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by
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University of Nebraska at Medical Center, 2018

Advisor: Kaustubh Datta, Ph.D.

Abstract

Bone metastasis is one of the major clinical concerns that causes skeletal related malignancies and increased mortality. Bone is one of the preferred sites for metastatic prostate cancer. The metastatic prostate cancer cells interact with bone cells (osteoblasts and osteoclasts) resulting in an imbalance in the bone homeostasis leading to increased activation of osteoblasts over osteoclasts. Our preliminary data indicated a non-tyrosine kinase receptor Neuropilin 2 (NRP2) is expressed in osteoclasts induced by metastatic prostate cancer cells and acts as a negative regulator of osteoclast differentiation and function. We hypothesize that prostate cancer -induced NRP2 expression in osteoclasts is necessary for low osteolytic activity and thus favors an osteoblastic lesion in prostate cancer bone metastasis.

Early experimentation discussed in my first section of my thesis demonstrated an increase in NRP2 expression in osteoclasts induced by RANKL and M-CSF and in PC3 and LNCaP C4-2B conditioned media (CM). TRAP staining and activity confirmed the differentiation of osteoclasts under these conditions. Interestingly, depletion of NRP2 and treatment with either in RANKL and M-CSF or LNCaP C4-2B CM exhibited a drastic increase in osteoclast differentiation and function.

An increase in expression of osteoclastic genes following NRP2 depletion in RANKL and M-CSF and LNCaP C4-2B CM was also detected. However, NRP2-depleted osteoclast precursors when treated with PC3 CM showed no change in osteoclastogenesis. It is important to note that LNCaP C4-2B promotes mixed bone lesions, which inclines more toward osteoblastic lesion, while PC3 promotes predominantly osteoclastic bone lesions. These findings therefore advocate a role of NRP2 in inhibiting osteoclastic activity in PCa bone metastasis with mixed lesions and that osteolytic PCa evades NRP2 inhibition.

In the second section of my dissertation, we elucidated the molecular mechanisms through which NRP2 regulates osteoclast differentiation and function in normal bone and in LNCaP C4-2B CM. Our studies suggest that NRP2 regulates the expression and translocation of NFATC1 which is a crucial osteoclastic transcription factor. Additionally, NRP2 controls NF-κB in the normal bone. These data imply that NRP2 restricts the translocation of critical transcription factors to regulate osteoclasts in prostate cancer bone metastasis.

The last part of my dissertation addressed how PC3 CM-induced OCs escapes the inhibition of NRP2. Presence of GM-CSF resulted in a delay in the differentiation and fusion of osteoclasts in RANKL and M-CSF and LNCaP C4-2B CM. It can be deduced that secretion of GM-CSF by PC3 CM may regulate the differentiation and fusion of osteoclasts and thereby escapes the regulation of NRP2.

Altogether, we report that NRP2 functions as a negative regulator of osteoclasts in prostate cancer bone metastasis but is rendered ineffective in osteolytic lesions. Hence, an insight into the regulation of NRP2 in osteoclasts can aid in the development of new and effective therapeutic strategies for the treatment of prostate cancer bone metastasis.

Acknowledgements

The completion of this dissertation would have been impossible without the invaluable contributions of so many people, scientifically, educationally, professionally and personally.

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Table of contents

This work is dedicated to my beloved grandmother and my parents

CHAPTER 1

INTRODUCTION

CHAPTER 1A: Introduction to prostate cancer

- **Progression to an advanced disease and related treatment strategies.**
- **Development of castration resistant prostate cancer and its clinical challenges.**

CHAPTER 1B: Prostate cancer bone metastasis

- **Metastatic advancement of castration resistant prostate cancer to bone.**
- **Bone cells and bone remodeling.**
- **Vicious cycle of bone metastasis.**
- **Treatment strategies for prostate cancer bone metastasis and their limitations.**

CHAPTER 1C: Molecular mechanisms in osteoclasts in bone metastasis

- **Recent evidence on molecular pathways in normal bone and metastatic bone.**
- **Introduction of Neuropilins (NRP1 and NRP2).**
- **Neuropilins in normal bone and prostate cancer bone metastasis.**

CHAPTER 1A: Introduction to prostate cancer

Prostate cancer is the most common disease occurring in American men following skin cancer. It affects 1 in every 7 men and 99% of the patients with prostate cancer are above the age of 50 years. Apart from age, factors such as family history, smoking, chemical exposure, obesity, geography and ethnicity increase the risk of prostate cancer. African American men are more prone to prostate cancer than the white American men[1,2]. As prostate cancer is mostly a slow-growing disease with exceptions of some cases where it is highly aggressive, no symptoms are observed until it reaches an advanced stage. In 2018, nearly 164, 690 new prostate cancer cases are estimated by American cancer society[3]. Most of the men diagnosed with prostate cancer do not die of the disease rather die of other causes with a majority of the cases still undetected.

In the early stages of prostate cancer when it is confined to the prostate gland, it can be treated with excellent chances for survival. Nearly 85% of American men are diagnosed with early-stage prostate cancer and with timely medical intervention, these patients can be treated with successful survival outcomes[4]. As prostate cancer is slow growing, many patients are safely followed by active surveillance or watchful waiting[5,6]. Active surveillance is a strategy used in which men with early stage prostate cancer undergo careful monitoring and serial prostate biopsies and the criteria for enrollment in AS include: clinical stage T1 or T2a; Gleason pathological score < 6 in the biopsy specimen with no Gleason pattern 4; 3 or fewer positive biopsies cores (minimum of 12 cores sampled), and no core with >50% involvement with cancer[1]. Other treatment strategies include a

combination of surgery, radiation, hormonal or chemotherapy[7]. In this context, understanding of Gleason grading is essential for the better judgement of the stage of prostate cancer and aid in deciding the treatment strategy. Based on the appearance of the cells under a microscope, a urologist grades the prostate cancer patient biopsies ranging from 1 to 5. Gleason grade 1 being well differentiated associated with favorable prognosis and 5 being the least differentiated which is correlated with poor prognosis. The final Gleason score is calculated by the sum of the grade of the primary tumor and the second number will be the pattern of the highest grade[8]. The Gleason score ranges from 2 to 10. Prostate cancer biopsy with highest score is more aggressive and have poor prognosis.

Prostate cancer is known to be advanced when it spreads beyond the prostate and its surrounding area into the nearby tissues, lymph nodes, bones, or other parts of the body. Nearly 10-20% of patients with newly diagnosed prostate cancer have advanced prostate cancer[7,9]. In patients diagnosed with localized prostate cancer, advanced prostate cancer will develop in nearly 40% men after they were initially treated for the localized disease[7]. Most prostate cancer–related deaths are due to the advanced stage of the disease, which results from a combination of lymphatic, hematogenous, or contiguous local spread[9,10]. Advanced-stage prostate cancer is detected more frequently in African Americans with a higher Gleason grade compared to white Americans[11]. Patients diagnosed with advanced or stage IV prostate cancer can be broadly divided into two groups. Patients who present with locally confined to the pelvis, but involving adjacent organs or lymph nodes have localized stage IV prostate cancer. Patients with

prostate cancer that has spread to distant organs, most commonly to the spine, ribs, pelvis and other bones have metastatic stage IV prostate cancer. In localized stage IV prostate cancer, patients are treated with radiation therapy and immediate hormonal therapy with successful 5 year-survival from the initiation of treatment without evidence of cancer progression or development of a distant metastatic disease[11,12].

Prostate cancer that has spread to distant organs and bones is treatable, but not curable with current standard therapies[13]. Androgen deprivation therapy (ADT) through either medical or surgical castration has been the standard treatment of metastatic prostate cancer for many years. As the growth of prostate epithelial cells requires physiological levels of androgen for its functioning, most of the patients with advanced prostate cancer usually can be controlled with ADT for a period of time[13-15]. Despite a good initial response to ADT, remissions last on average 2-3 years, eventually progressing into castration-resistant prostate cancer (CRPC) with no further benefit from ADT. Patients with CRPC have a worse prognosis and translate into a survival time of 16–18 months on an average from the beginning of progression[14,15]. These patients show a metastatic progression of the disease (Fig 1.1). Docetaxel or cabazitaxel in combination with prednisone is the first-line chemotherapy regimen for metastatic CRPC patients (mCRPC). However, chemotherapy is not well tolerated by all CRPC patients, who were often elderly men with limited bone marrow reserve and concurrent medical conditions[16]. Hence, the treatment strategy for mCRPC remains a significant clinical challenge.

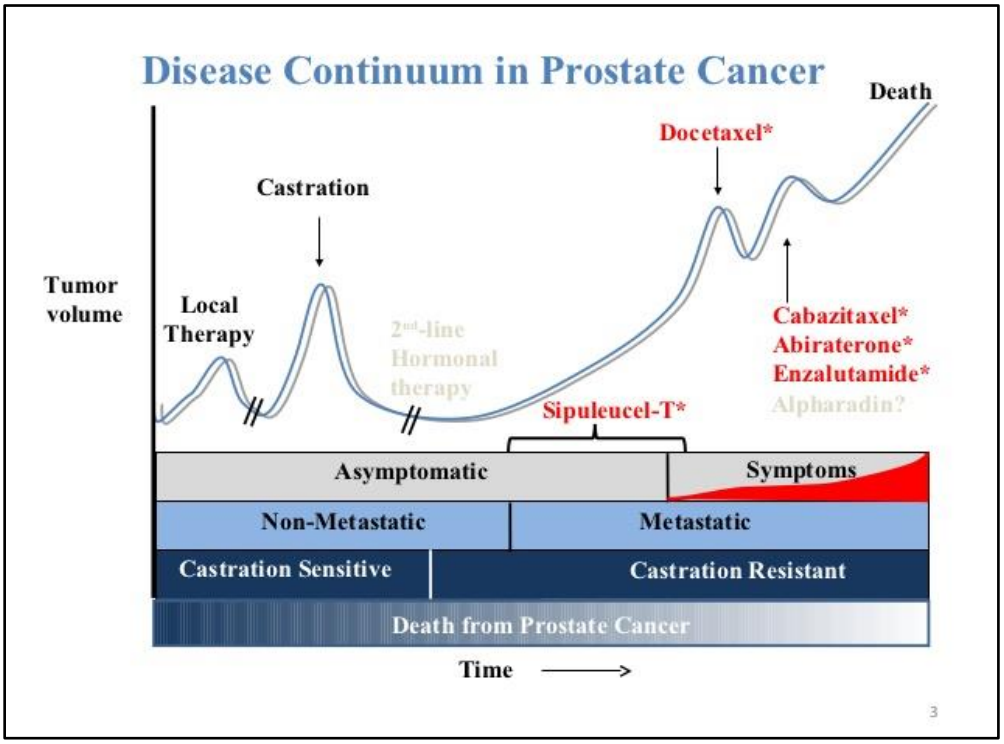


Figure **1.1**: Graphical illustration of Progression of Prostate cancer.
Obtained from Prostate cancer: Clinical update by James L. Gulley

CHAPTER 1B: Prostate cancer bone metastasis

In nearly 90% of mCRPC patients, the progression of metastatic lesions in bone is often the initial manifestation resulting in a significant increase in mortality and morbidity[6,14,16,17]. Metastatic prostate cancer progresses to the bone through a series of events. It starts with the dissemination of tumor cells from the primary site followed by invasion through the vasculature to reach circulation. Once in the circulation, they migrate across the sinusoidal wall in the bone marrow cavity and home to the bone. After localization in the bone, the tumor cells may remain dormant to overcome the harsh and challenging bone microenvironment, or under favorable conditions, they release growth factors to interact with the bone and deregulate bone microenvironment resulting in overt micrometastasis (Fig 1.2). These patients are symptomatic and are at high risk of developing skeletal-related events (SREs) which includes pathological fractures, spinal cord compression, bone pain, decreased hematopoiesis resulting in anemia and malignant hypercalcemia leading to reduced quality of life and detriment of overall survival.

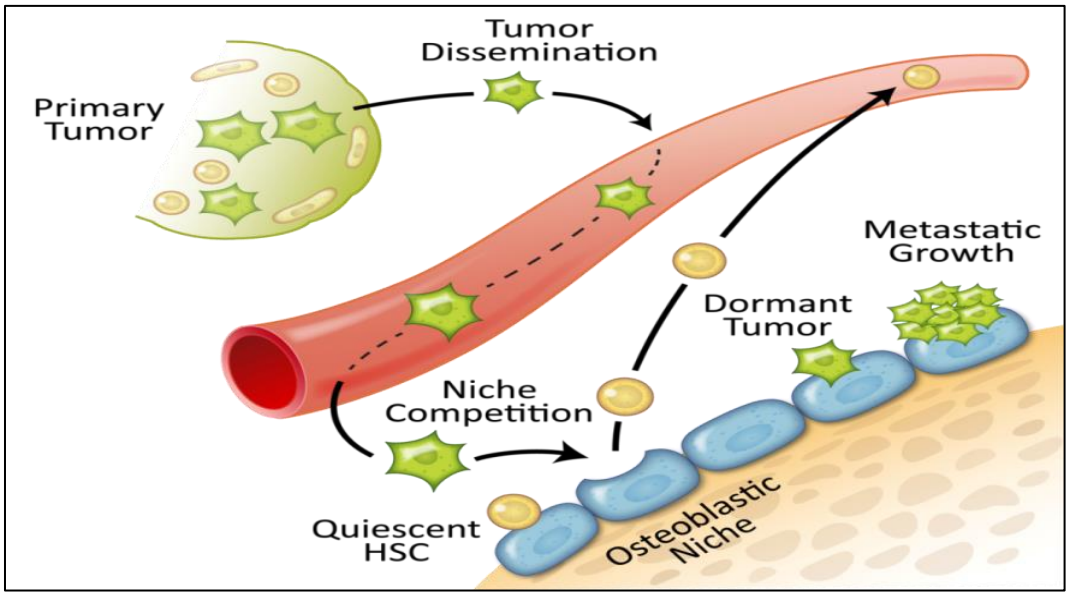


Figure **1.2**: Progression of Prostate cancer from primary tumor to the metastatic site, bone.
Obtained from https://prostatecancerinfolink.net/2011/03/23/12283/

Bone cells and Bone remodeling

Bone is a dynamic tissue that undergoes continuous remodeling to maintain its strength, stiffness, and integrity. In a healthy adult bone, bone remodeling is achieved by the functions of three types of bone cells: osteoblasts, osteoclasts, and osteocytes. Osteoblasts originate from mesenchymal stem cells present in the bone marrow. These cells differentiate and mature to mineralize the bone extracellular matrix by depositing calcium hydroxyapatite crystals[18-20]. This function of osteoblasts provides rigidity and strength to the bone. Osteoblasts are recruited to the site of resorption pits, injury or damage where they build and lay new bone to restore the integrity of the bone. Osteoclasts are bone-resorbing cells derived from hematopoietic stem cells. They release enzymes that can degrade the bone thereby forming a resorption pit. During bone remodeling, osteoclast removes the mineralized bone following which osteoblast lay down new bone matrix which subsequently becomes mineralized. This process occurs at discrete sites named basic multicellular units (BMUs). Molecularly, osteoblasts and cells such as stromal cells, chondrocytes, megakaryocytes, B cells, synoviocytes activated CD4+ and CD8+ T cells and macrophages in bone matrix release receptor activator of nuclear factor kb ligand (RANKL) which then binds to RANK receptor on osteoclastic precursors and activate osteoclast for bone resorption. Osteoclast activation is negatively regulated by the release of osteoblastic osteoprotegerin (OPG) which inhibits the binding of RANKL to RANK receptor[18-20]. The RANKL/RANK/OPG axis thereby orchestrates the bone remodeling and maintains bone homeostasis (Fig 1.3).

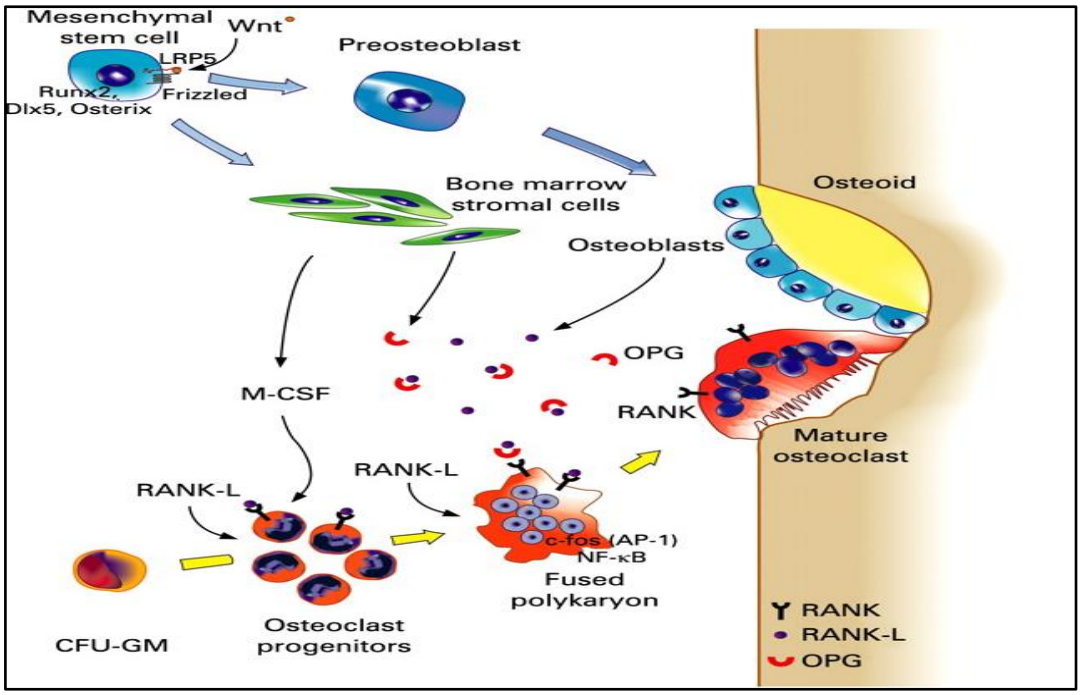


Figure **1.3**: Homeostasis in healthy bone.
Obtained from Tilg H et. al. ***Gut, inflammation and osteoporosis: basic and clinical concepts.*** *Gut. 2008 May;57(5):684-94.*

In addition to these cells, mature osteoblast cells become trapped in the newly formed bone matrix and become star-shaped osteocytes. These cells form network with each other for exchange of nutrients. Osteocytes are terminally differentiated osteoblasts that are no longer involved in active bone formation, and have become entrapped within the lacuna embedded deep in the bone matrix produced by them[20]. They are abundant in the bone matrix making up to 90% of all the bone cells and are long-lived than osteoblasts and osteoclasts. They are first cells to detect and respond to mechanical strain in the bone by communicating with surface osteoblasts and osteoclasts via a complex network of neuron-like processes referred to as canaliculi. Osteocytes play central role in bone remodeling by integrating both hormonal and mechanical signals to regulate bone mass. They undergo apoptosis to release apoptotic bodies containing RANKL to recruit osteoclasts to the site of resorption to either remodel or remove damaged bone area. Osteocytes also regulate osteoblast signaling by the release of sclerostin, which is an inhibitor of Wnt signaling in osteoblasts. In addition, osteocytes are critically important for bone mineralization, by regulating phosphate metabolism through release of FGF23 (Fig1.4)[21].

Apart from bone cells, the bone microenvironment includes myeloid and immune cells, platelets, bone marrow endothelial and hematopoietic cells and bone marrow-derived mesenchymal stem cells. Together, the interaction between osteoclasts, osteocytes, osteoblasts and the bone microenvironment maintains the physiology of healthy bone. However, this tightly regulated bone homeostasis is entirely skewed when metastatic prostate cancer cells invade bone. In bone me-

tastasis, the invading cancer cells aberrantly stimulate the activity of osteoblasts and osteoclasts leading to deregulation of bone homeostasis and disrupting the process of bone remodeling.

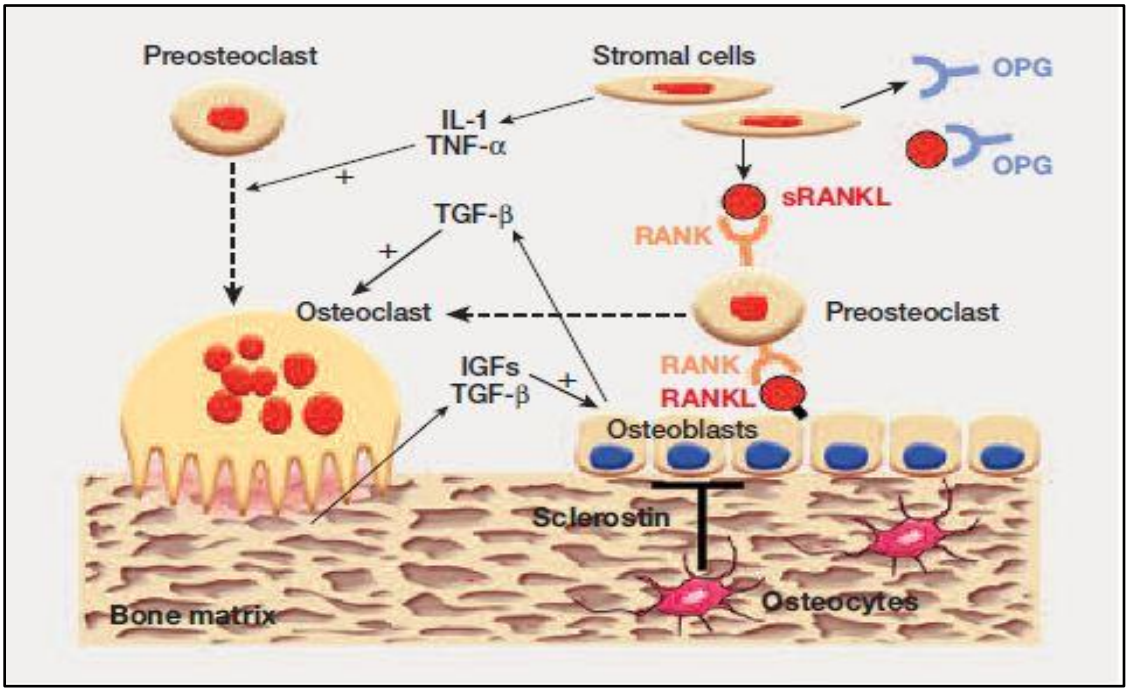


Figure **1.4**: Bone remodeling.
Obtained from Pierre J. MARIE. Bone remodeling: a social network of cells.

As this study focuses on osteoclasts, a detailed description of osteoclasts in general follows.

Osteoclasts

Origin: Osteoclasts are giant, multinucleated cells which remain to be one of the most complex and fascinating cells in vertebrates. These cells are short-lived and modest in numbers yet are uniquely endowed with the capability to degrade the complex organic and inorganic bone matrices. Osteoclasts originate from the hematopoietic stem cells present in bone marrow which also are common progenitors for lymphoid as well as myeloid lineage. Osteoclasts are derived from the myeloid lineage by the commitment of hematopoietic stem cells into common myeloid progenitors (Lin-c-kit^{+}sca1^{-}Fms^{+}) which then give rise to Macrophage/Dendritic cell precursors (MDP) which are identified as CX3CR1^{+}CD117^{+}Lin^{-}. These MDPs in the presence of M-CSF differentiate into monocytes (CD31^{-}Ly-6C^{-}cFMS^{+}Rank^{+}), myeloid blasts (CD31^{+}Ly-6C^{+}cFMS^{+}Rank^{+}) and common Dendritic cell precursor (Lin^{-}cKit^{lo} Flt3^{+}cFMS^{+}). Monocytes are known precursors of macrophages, dendritic cells, and osteoclasts. Further, macrophages in the presence of RANKL and M-CSF can form osteoclasts. Immature dendritic cells that arise from common Dendritic cell precursors can also trans-differentiate into osteoclasts in the presence of IL-1, TNF, and IL-10. Myeloid blasts are the early stage Ly-6C^{+} monocytes which on stimulation with M-CSF and RANKL become polykaryotic osteoclasts. However, these myeloid blasts can also retain the characteristics of Ly-6C^{-} monocytes[22-25] (Fig 1.5).

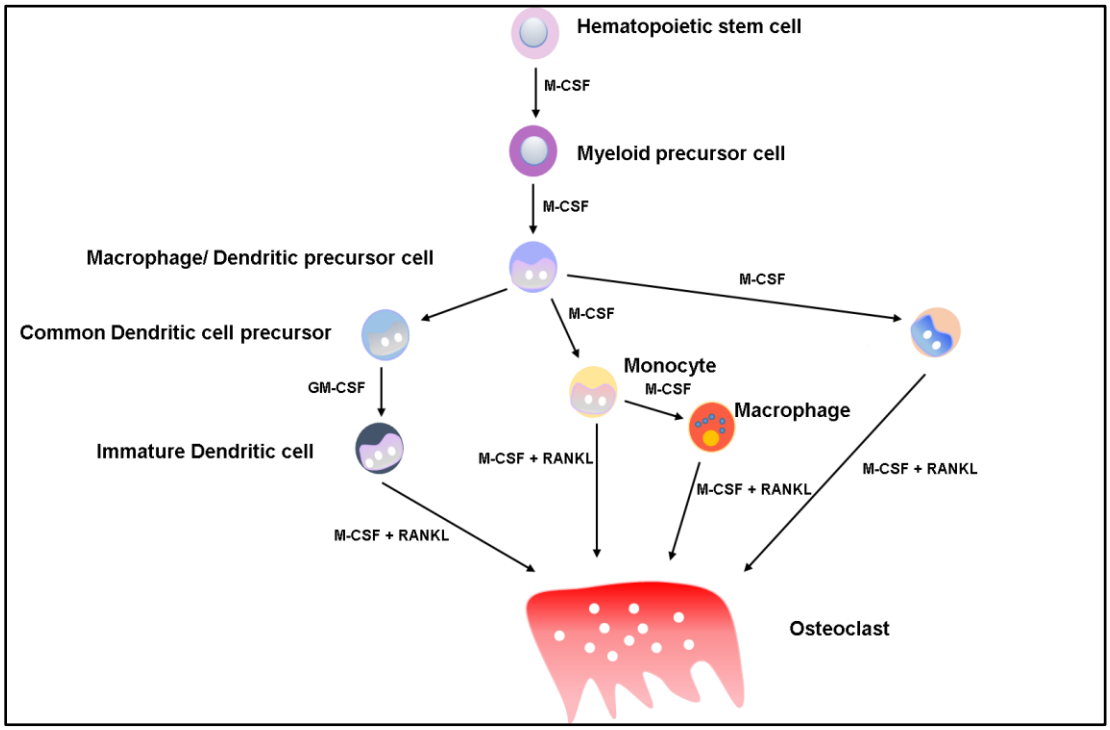


Figure **1.5**: Origin of osteoclasts.

***Commitment**:* The differentiation of hematopoietic stem cells into bone-resorbing osteoclasts involves a sequential activation of various signaling factors released by osteoblasts and surrounding cells in the bone environment. The first wave of signaling includes M-CSF (Macrophage- colony stimulation factor), CSF1r/ c-Fms (receptor for M-CSF) and transcription factor PU.1[26-28]. M-CSF is expressed mainly by endothelial cells followed by osteoblasts, stromal fibroblasts, activated T-lymphocytes and cells of the epithelium[26-28]. In response to M-CSF, the hematopoietic stem cells commit to myeloid lineage which then proceeds to become monocytes or macrophage colony-forming units (CFU-M). Monocytes are the common precursors of macrophages and osteoclasts. PU.1 is the first signaling molecule known to influence the commitment and differentiation of myeloid precursors to osteoclast lineage[26-28]. During the stage of commitment, PU.1 binds to the promoter site of Csf1r as well as ITGAM and promote their transcription which is intrinsic to osteoclast biology. PU.1 deficient mice die at birth and show signs of osteopetrosis. The absence of PU.1 causes an arrest in the normal differentiation of myeloid precursors to osteoclasts and macrophages suggesting that PU.1 is a central player in the commitment and differentiation of osteoclasts[26-29] (Fig 1.6).

Activation of M-CSF is vital for the proliferation and survival of the monocytes. In fact, CSF1-deficient osteopetrotic mice (Csf1op/Csf1op) showed severe osteopetrosis and a range of hematologic abnormalities in the peripheral blood, bone marrow, and spleen along with growth retardation, reduced macrophage densities, defect in male and female reproductive functions and failure of tooth erup-

tion. Administration of M-CSF corrected several developmental disorders entirely in CSF1-deficient osteopetrotic mice while partial restoration of macrophage densities in liver, adrenal, spleen, and large intestine was observed. However, presence of residual osteopetrosis caused a tremendous delay in the resorption of trabecular bone in the sub epiphyseal region of the long bone along with partial normalization of hematological abnormalities in the bone marrow, peripheral blood and spleen suggesting the effects of M-CSF on osteoblasts and osteoclasts in these mice[26,27,29].

During the early myeloid differentiation, M-CSF along with other growth factors such as IL-1 and IL-3 produce mononuclear progenitors. Following the initial myeloid commitment and differentiation into monocytes, M-CSF signaling regulates proliferation, survival, and differentiation of monocytes to macrophages and osteoclasts. Molecularly, binding of M-CSF with c-Fms/ CSF-1R (Colony Stimulating Factor 1 Receptor) initiates a signaling cascade which stimulates PI3K/Akt pathway which in turn activates GSK3 and the FOXO family of transcription factors. Once active, GSK3 promotes the proteasomal degradation of cyclin D1 causing cell cycle arrest in G1 phase. FOXO proteins inhibit the transcription of cyclin D1 while increasing the cell cycle inhibitors, p27 and p130[26,27]. Further, M-CSF induces the expression of MAPK phosphate-1 (MKP-1) which then dephosphorylates ERK leading to its inactivation. This signaling results in the decrease in monocyte proliferation to enable the differentiation of monocytes into osteoclasts. M-CSF also regulates the proliferation of pre-osteoclasts via the ubiquitination, endocytosis and subsequent degradation of c-Fms, c-Src, and c-

Cbl[26]. Microphthalmia-associated transcription factor (MITF) is activated as result of M-CSF signaling stimulation which in turn binds to Bcl-2 promoter to regulate its protein expression in monocytes. Contrary to the effect of M-CSF signaling on proliferation of monocytes, reports by Zhou et al. suggest that PI3K mediates the suppression of p27 to promote proliferation of M-CSF-induced monocytes. M-CSF also induces the expression of RANK receptor which is one of the key players involved in the differentiation of monocytes to osteoclasts (Fig 1.6) [26,27,29].

Interactions between PU.1 and MITF are critical for the osteolysis biology. MITF mi/mi mice have severe osteopetrosis and inactivate essential osteoclastic factors such as Tartrate-resistant acid phosphatase (TRAP) and carbonic anhydrase 2 (CA-II). These mice also contain abundant macrophages, which imply that PU.1 controls the osteoclast formation via downstream regulation of MITF[30].

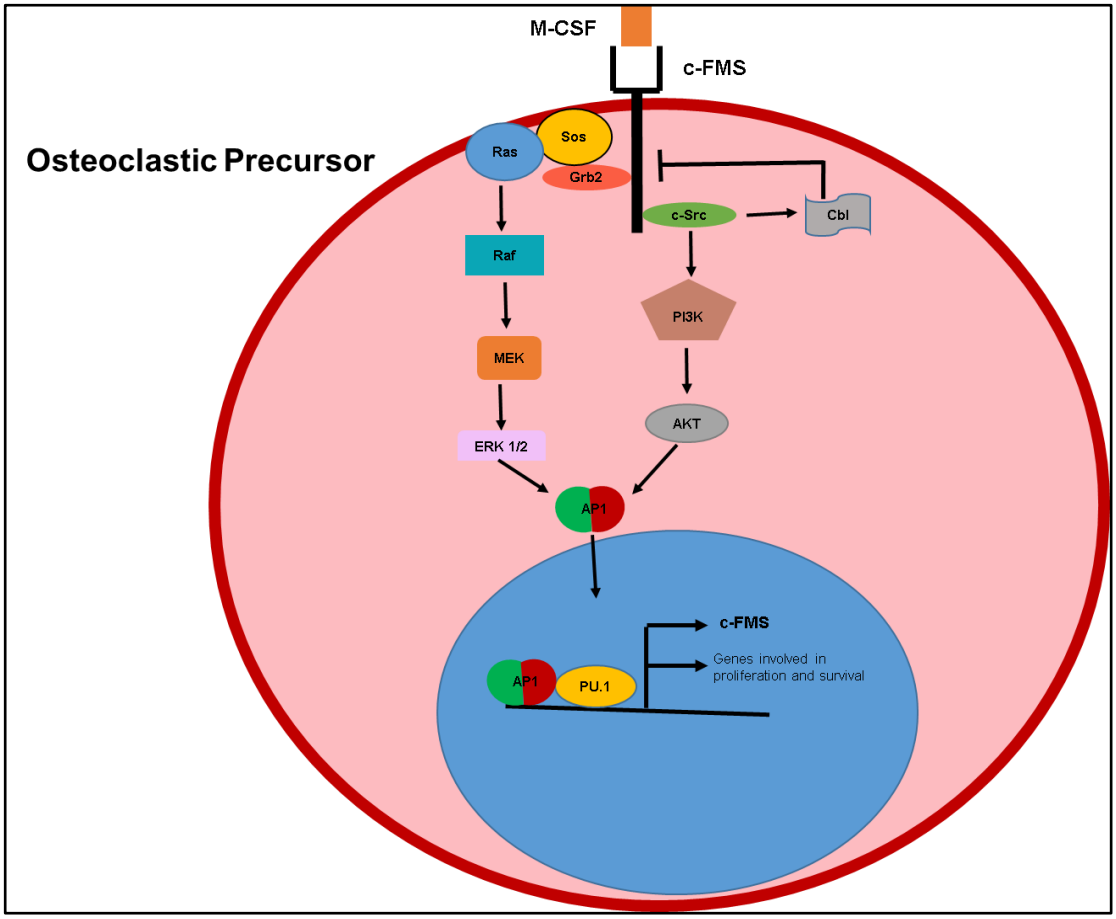


Figure **1.6**: M-CSF signaling in Osteoclastic precursors.

***Migration and Targeting**:* The recruitment of osteoclast precursors to the bone surface lined with RANKL releasing osteoblasts is a crucial step for osteoclast differentiation. Osteoclast precursors are recruited to the site of resorption by chemotaxis and chemoattractants that are responsible for this function are released by mature osteoblasts as well as the bone matrix itself. Many chemokines govern the chemotaxis of the monocytic osteoclast precursors. One of the well-characterized chemokines in the regulation of osteoclast precursors' migration is CXCL12/ SDF-1 (stromal cell-derived factor-1)[31,32]. CXCL12 is expressed by stromal cells in the perivascular regions in the bone marrow cavity. The chemokine receptor CXCR4 is widely expressed on nearly all of the hematopoietic cells including osteoclast precursors. CXCL12 promotes chemotactic recruitment, development, and survival of osteoclast precursors that express CXCR4[31,32]. Secondary to this, another chemokine belonging to the same family as CXCL12, CX3CL1 is expressed by osteoblasts, and its cognate receptor CX3CR1 is preferentially expressed on osteoclast precursors. CX3CL1 was indicated to play a crucial role in the recruitment of osteoclast precursors into the bone marrow cavity and in the firm adhesion on the bone surface[32]. Discussion leading to a hypothesis by Noble *et al.* and Heino *et al*. suggested that the dying osteocytes embedded in the bone matrix release RANKL in the form of apoptotic bodies to attract the osteoclast precursors to their location for degradation and healthy osteocytes downregulate the resorption (Fig 1.7).

Sphingosine-1-phosphate (S1P) is lipid mediator present in all the cell types in the e bone and enriched in blood and is known a critical regulator of osteoclast

precursor migration[31]. Osteoclast precursors express S1PR1 and S1PR2 on its surface, and the migration and targeting of these cells are dynamically regulated by S1P. S1PR1 and S1PR2 function in opposing directions in the migration of osteoclast precursors. S1PR1 promotes chemotaxis of pre-osteoclasts into blood circulation where S1P levels are high while S1PR2 induces chemorepulsion. S1PR2 require high concentrations of S1P to conduct chemo repulsion. Once activated, it negatively regulates S1PR1 function and induces chemotaxis in the opposite direction of S1P gradient where S1P levels are low which is key to direct osteoclast precursors into the bone marrow cavity (from the blood vessel where S1P levels are high) and to the site of resorption. After the entry into bone marrow, S1PR1 is reactivated, and the osteoclast precursors required for resorption remain while the rest are returned to the blood vessels by S1P gradient (Fig 1.7)[31]. This mechanism of chemoattraction and chemorepulsion is a checkpoint for controlling osteoclastogenesis as it determines the number of osteoclasts precursors that are necessary for the degradation of bone matrix.

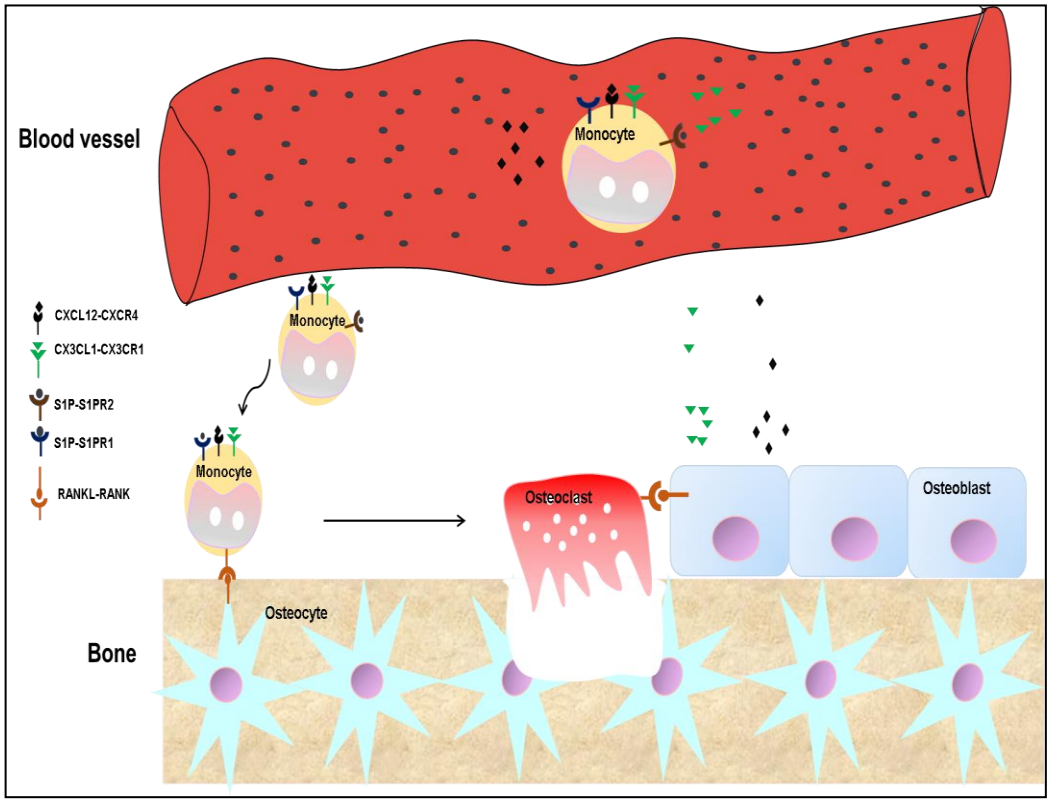


Figure **1.7**: Mechanism of Osteoclastic Precursors Migration and Targeting.

***Differentiation**:* The function of RANKL-RANK-OPG axis tightly regulates the differentiation of myeloid precursors into polykaryon, bone-resorbing osteoclasts. RANKL (receptor activator of nuclear factor-κB transcription factor ligand) is the most potent osteoclastogenic cytokine expressed widely on the surface of osteoblasts, stromal cells, chondrocytes, megakaryocytes, B cells, synoviocytes activated CD4+ and CD8+ T cells and macrophages. RANKL binds to its receptor, RANK present on the surface of monocytes and immature dendritic cells to initiate signaling cascade leading to osteoclast differentiation and activation[22,33,34]. Downstream of RANK, TNF receptor-associated factor (TRAF) proteins such as TRAF2, TRAF3, TRAF5, and TRAF6 are activated. Of all the TRAFs, mice lacking TRAF6 has a defect in osteoclast differentiation and function which implies that TRAF6 is a major mediator of osteoclast formation. Activation of RANK by RANKL leads to recruitment of TRAF2 AND TRAF5 to the RANK-RANKL complex, and the subsequent binding of TRAF6 leads to the phosphorylation of TAK1, a member of MAP3K via TAB2. TAK1, in turn, phosphorylates NIK (NF-κB-inducing kinase) to activate IKK (IB kinase) complex in a non-canonical NF-κB manner[22,33,34]. Activated IKK phosphorylates IB to destabilize the IB-NF-κB complex thereby liberating NF-κB which subsequently translocates into the nucleus for the transcription of osteoclastic genes which includes one of the key transcription factor, NFATc1.[35] The phosphorylated IB is ubiquitinated and degraded by the proteasome machinery. In a canonical NF-κB pathway, activation of TRAF6 and p62 can also phosphorylate IKK via PKC which binds to p62 through its N-terminal PB1 domain. PKC initiates the activation of NF-κB via the

IKK phosphorylation and release of NF-κB P65 and P50 and their translocation into the nucleus.[35] Both the canonical and non-canonical NF-κB pathways have been reported to be involved in the osteoclast differentiation and survival. In the case that one of the NF-κB pathways is blocked or downregulated, the other pathway compensates for the functioning of osteoclasts (Fig 1.8) [22,33-36].

Although RANKL –RANK signaling is the key pathway for osteoclast differentiation, it is not sufficient to drive osteoclastogenesis without the co-stimulation by several transmembrane adaptor proteins such as DAP12, FcR, and DAP10. The extracellular domains of these adaptor proteins are small and cannot interact directly with the ligands. They associate with cell surface receptors via paired charged residues in the transmembrane regions of these adaptors known as immunoreceptor tyrosine-based activation motifs (ITAM). During the early stages of osteoclast differentiation, DAP12 co-stimulates RANK signaling by recruitment and activation of Syk, which sequentially induces the activation of BTK and Tec kinases as well as PI3K leading to subsequent activation of PLC and thereby driving Ca2+ oscillations. Ca2+ stimulates calcineurin to dephosphorylate NFATC1 to enable its translocation into the nucleus for transcription of osteoclast genes[22]. Ca2+-mediated activation of NFATc1 also triggers the autoamplification loop of NFATc1 and ensures a sustained NFATc1-dependent transcriptional program in which osteoclast-specific genes are activated by a transcriptional complex that involves NFATc1, AP-1, and other cooperators (Fig 1.8)[22,33,34,36,37].

The induction of NFATc1 is a distinctive characteristic of pre-osteoclasts. It is one of the key target genes of NF-κB as well as Ca2+-calcineurin pathway during the early stage of osteoclastogenesis[37]. Reports on NFATc1- deficient mice indicate that deletion of NFATc1 causes embryonic lethality and that ectopic expression of NFATc1 caused bone marrow-derived precursor cells to differentiate into osteoclasts even in the absence of RANKL[22]. Interestingly, another molecule which is a member of NFAT family, NFATc2 preexists even before RANKL stimulation and is also activated and recruited to the promoter site of NFATc1 along with NF-κB to activate NFATc1 cooperatively[37,38]. This step is crucial for the robust induction of NFATc1 during osteoclast differentiation within a short time of RANKL induction (Fig 1.8)[22,33,34,36].

c-Fos is also identified as a key determinant in the differentiation of myeloid precursors to osteoclasts and macrophages.[39] As a result of RANKL-RANK signaling, c-Fos is activated which in turn complexes with c-Jun to form the Activator protein-1 (AP1) transcription factor. It functions as a switch between osteoclast and macrophage differentiation from myeloid precursors. In the absence of c-Fos, the myeloid precursors differentiate into macrophages but not osteoclasts causing severe osteopetrosis as exhibited in c-Fos-deficient mice.[35,39] This phenotype can be rescued by bone marrow transplantation or ectopic overexpression of c-Fos. Also, DAP12-induced Ca^{2+} prompts CaMKIV (calcium/calmodulin-dependent protein kinase type IV) to activate c-Fos in RANK-dependent manner which translocate into the nucleus to form AP-1 dimers and work in coordination with NFATc1, NF-κB, MITF, PU.1 and other transcription factors to promote the

transcription of osteoclastic genes.[35,38] Accumulating evidence indicates that the cooperation of NFAT and AP1 is responsible for the specific activation of NFATc1 promoter and its auto-amplification (Fig 1.8)[33,34,36,39].

***Maturation**:* The initiation of maturation phase is characterized by the fusion of mononucleated osteoclasts to form multinucleated polykaryons followed by their recruitment to site of resorption. The multinucleation of osteoclasts is the most striking morphological feature that distinguishes osteoclasts from their precursors.[38] The osteoclast cell-cell fusion is highly regulated since their functions of bone resorption, and secretion of digested bone are so dependent upon extremely active membrane dynamics, including formation of the ruffled border and high rates of endocytosis, vesicle fusion, and transcytosis.[34] The function of syncytin1, CD47, dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast-stimulatory transmembrane protein (OC-STAMP) in early fusion events, and of connexin 43 at a later stage enables the engulfment of mononuclear cells by more mature, multinucleated osteoclasts.[34,38] DC-STAMP and OC-STAMP are two related transmembrane proteins which have been reported to be essential for mononucleated osteoclast fusion and are strongly induced by RANKL during the osteoclastic differentiation.[34,40] Recent work by Yagi *et al*., Miyamoto *et al*. and Hanna *et al*. showed that mice with knockout of DC-STAMP and OC-STAMP had defect in the fusion of mononuclear osteoclasts as characterized by the block in the formation of fusion bridges which are necessary for cell-cell junction.[34,41-43] Also, the mononucleated osteoclasts from these mice demonstrated decreased pit resorption capacity.[42,43] Another study by Noam Levaot *et.al*. attempted to un-

derstand the cellular mechanisms underlying the fusion biology of osteoclasts and suggested that the process of fusion is initiated by a small subset of mature osteoclast precursors (~2.4%) stimulated by M-CSF and RANKL are known as fusion-founder cells. These cells can fuse with less mature osteoclast precursors to generate multinucleated osteoclasts. Diffusible cytoplasmic molecules are exchanged between fusion-founder and fusion-competent cells by the formation of protrusions interconnecting the fusing cells.[44] However, it is still unclear from this study whether fusion occurs precedes osteoclast differentiation or vice versa or they occur simultaneously (Fig 1.8)[38].

During maturation, several osteoclast-specific genes such as Cathepsin K, matrix metalloprotease 9 (MMP9), c-Src, osteoclast-associated receptor (OSCAR), Tartarate- resistant acid phosphatase (TRAP), chloride channel protein 7 (CIC7), β3 integrin, αv integrin and Latent transforming growth factor binding protein 3 (LTBP3) are activated by a transcriptional complex that contains NFATc1 along with other transcription factors such as AP1, PU.1 and MITF.[45,46] NFATc1 - AP-1 complex is essential for the induction of the TRAP and calcitonin receptor genes.[34] The transcriptional complex with NFATc1, PU.1, and MITF is necessary for the expression of cathepsin K and OSCAR. Importantly, the components of the NFATc1 complex are not always the same, i.e., for instance, the cooperation between NFATc1 and PU.1/MITF was not observed on the calcitonin receptor promoter, suggesting that the differential composition of the transcriptional complex may contribute to the spatiotemporal expression of each gene during osteoclastogenesis (Fig 1.8).[38,45]

NFATc1 plays a vital role in the process of osteoclast fusion via up-regulation of the DC-STAMP and the d2 isoform of vacuolar ATPase V0 domain (Atp6v0d2). Recently, NFATc1 has been shown to activate Atp6v0d2 via co-activation with MEF2 and MITF.[34,38]

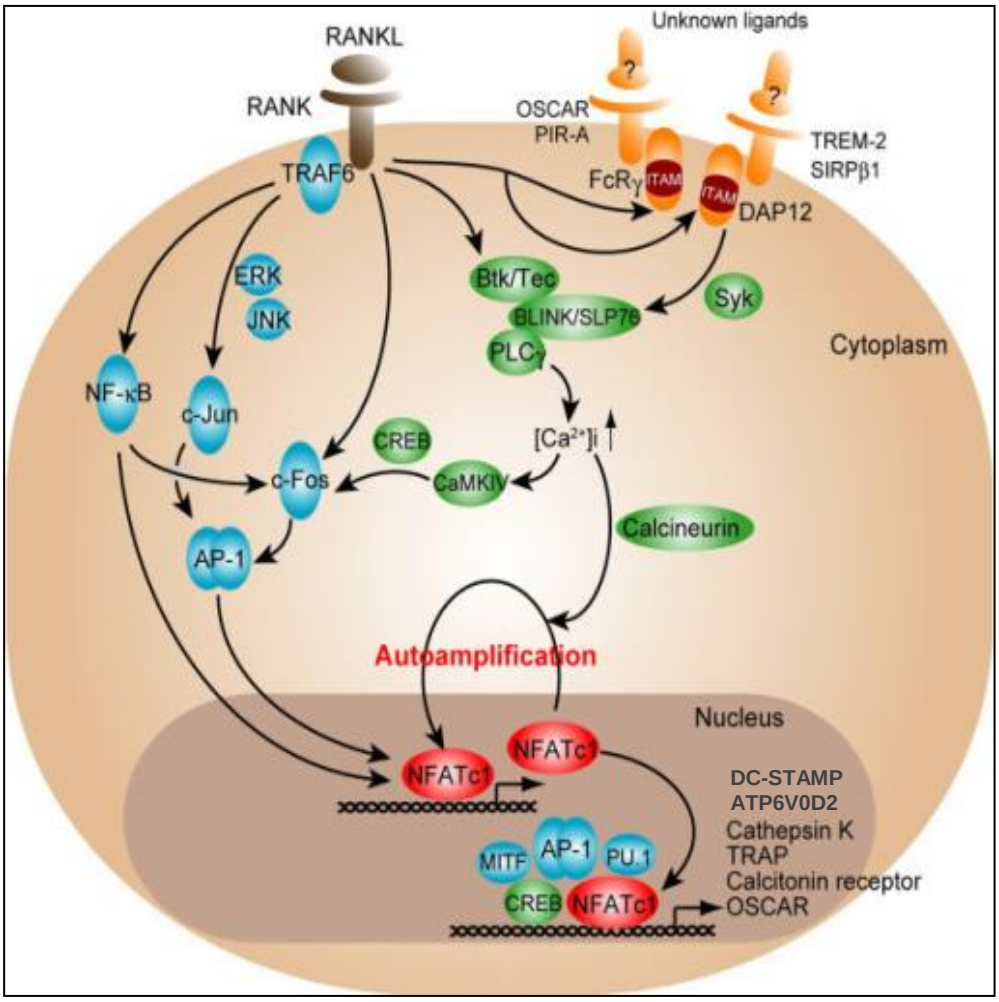


Figure **1.8:** Signaling cascade involved in osteoclast differentiation and maturation.

Modified from Kazuo *Okamoto and Hiroshi Takayanagi. Regulation of bone by the adaptive immune system in arthritis. Arthritis Res Ther. 2011; 13(3): 219.*

***Activation**:* Osteoclast activation is a multi-step process which follows multinucleation of osteoclasts. The process of osteoclast activation is divided into four steps which are: (1) Polarization of the osteoclast to the mineralized bone matrix and reorganization of actin cytoskeleton. (2) Formation of the ruffled border. -3) The release of bone matrix-degrading enzymes and acid within the ruffled border and sealing zone to degrade the organic matrix and minerals. (4) Removal of resorbed products from the osteoclast (Fig. 1.9)[47].

Osteoclast polarization: During bone resorption, osteoclasts are polarized with an apical membrane at the contact with bone and a basolateral membrane at its opposite. Osteoclasts recognize proteins containing Arg-Gly-Asp sequence motif on the bone matrix and attach through vitronectin is the first step in the polarization of osteoclasts.[48] Physical properties of bone such as hardness and roughness contribute to the induction of osteoclast polarity. Presence of hydroxyapatite – collagen complex also enables the polarization of osteoclasts. The release of protons from osteoclasts onto the bone matrix aids in recognition of bone by the osteoclasts. Further, attachment to the bone surface occurs through the function of integrin specifically, αvβ3 integrin, a vitronectin receptor. The αvβ3 integrin forms a thin actin complex known as podosome which recognizes the proteins with RGD-motif such as osteopontin, vitronectin and bone sialoprotein in the bone matrix.[47,49] Individual podosomes form part of the subcellular structures that continuously self-organizes during osteoclast attachment and polarization.[48,49] Podosome clusters and evolves into dynamic rings at intermediate stages and end up forming peripheral podosome belts in mature cells. These

podosome belts fuse to form the sealing zone.[49] During polarization, osteoclasts become apical-basal polarized with an apical membrane at the contact with bone and a basolateral membrane at its opposite along with specific submembrane domains to form the sealing zone.[49] In actively resorbing osteoclasts, the sealing zone is characterized by a broad circular band of actin surrounded by a double ring of vinculin.[47,48]

At molecular level, podosomes contain F-actin core and actin regulatory proteins including cortactin, Wiskott–Aldrich Syndrome Protein (WASP) and Arp2/3.[49] During osteoclast polarization, cytoskeleton rearrangement occurs, and M-CSF plays a crucial role in controlling the v3 integrin signaling via c-Src, Pyk2, Syk and c-Cbl.[48] In fact, DAP12/Syk association is the point of convergence between M-CSF and αvβ3 integrin signaling during cytoskeleton rearrangement. M-CSF binding to c-Fms promotes the stable interaction with αvβ3 integrin via phosphorylated sites on c-Fms. Integrin αvβ3 mediates cytoskeleton organization through the cytoplasmic domain of β3 subunit. Binding of integrin αvβ3 to its ligand leads to phosphorylation and activation of c-Src, which is constitutively associated with the cytoplasmic domain of β3 subunit. Activated c -Src then phosphorylates Syk, whose SH2 domains recognize and bind to the phosphotyrosine residues of the ITAM protein and DAP12 to activate their coupling to c-Fms.[48,49] The αvβ3 –c-Src-Syk complex then recruits Slp76, an adaptor for Vav3. Vav3, a guanine nucleotide exchange factor is activated via Slp76 which converts small GTPases of the Rho family from the inactive GDP form to the active GTP-bound form.[47] Rac is a Rho family GTPase expressed in osteoclasts and is involved in αvβ3 -

mediated reorganization of the osteoclast cytoskeleton.[48,50] Rac1 and Rac2 are two key effector molecules of the αvβ3 integrin signaling and their deletion results in a severe dysregulation in the osteoclast cytoskeleton and osteopetrosis.[48,50] Cdc42 is another Rho GTPase, which promotes cytoskeletal effect through PKC. Integrin αvβ3-mediated cytoskeleton organization leads to the formation of the dense actin belt-like structures called the F-actin ring, which isolates the resorptive space from its surroundings therefore forming a sealing zone. The resorptive bone lacuna is separated by the F-actin ring to limit the flow of protons and bone-degrading enzymes thereby maintaining low pH for effective degradation of the bone matrix.[48,50]

Formation of ruffled borders: During resorption, the plasma membrane enclosed by the F-actin ring expands and rearranges to attain highly convoluted structures upon attachment to the bone surface called the ruffled borders. These borders are not only a morphological characteristic of the osteoclast but also the cell's resorptive organelle as they serve as exit for protons and lysosomal proteases onto the resorbing surface.[51] The ruffled border membrane is formed by the insertion of the proton pump, H-ATPase, and cathepsin K-bearing vesicles into the plasma membrane attached to the bone.[52] These borders vary in size based on the activity of the cell and dependent on αvβ3 integrin signaling thereby providing a large surface area for the release of protons and osteolytic enzymes. In unbound osteoclasts, acidifying vesicles containing the proton pumps are distributed diffusely throughout the cytoplasm and upon attachment of osteoclasts to bone, matrix-derived signals such as αvβ3 integrin prompt these vesicles to lo-

calize and integrate into the plasma membrane facing the bone.[51,52] Acidic vesicles fuse with the plasma membrane in the ruffled borders which is mediated by SNARE proteins. Fusion of exocytotic vesicles with the osteoclast plasma membrane is mediated by SNARE proteins (v-SNARE and t-SNARE). The function of SNARE function is regulated by synaptotagmins (Syt), a family of vesicular trafficking proteins.[47,51,52]

Studies on Ca^{2+} flux and ruffled borders in resorbing osteoclasts suggested that calcium regulates the secretion of intracellular granules at the time of resorption. The osteoclasts that are actively resorbing are exposed to high calcium concentrations in the ruffled borders but the internal concentration of calcium is maintained at ~100Nm. However, αvβ3 integrin signaling induces a robust increase in the intracellular calcium to 0.5-0.8 mM along with formation of calcium puffs.[47,52] Hence, it was postulated that these drastic increases in calcium levels may correlate with the pronounced increase in the release of intracellular vacuoles but no evidence can corroborate this hypothesis.

Release of bone matrix-degrading enzymes and acid to degrade the organic matrix and minerals: During bone resorption, osteoclasts activate a variety of intracellular pathways. The ruffled border of osteoclast has a vacuolar-type proton ATPase (V-ATPase) inserted into it which enables the release of large amounts of acid to degrade bone matrix. V-ATPase is placed in the ruffled borders via its release from lysosome.[47,51,52] The source of the cytoplasmic protons is carbonic acid, which is generated by carbonic anhydrase II from carbon dioxide and water. Carbonic anhydrase II is localized close to the ruffled border. To maintain elec-

troneutrality, Cl^- are also transported into the resorption compartment via chloride channels which are charge-coupled to the V-ATPase and also present in the ruffled border membrane.[51,52] The secretion of protons across the ruffled border membrane into the extracellular resorbing compartment leaves the conjugate base, bicarbonate (HCO 3-) inside the osteoclast and to maintain electroneutrality, bicarbonate must be removed from the cell. Further, the osteoclast also must maintain efflux of Cl^- ions to remove excess bicarbonate as well as supply for chloride channels. For this, a passive chloride-bicarbonate exchanger is present in the basolateral position of the plasma membrane of osteoclasts. The coordinated function of V-ATPases and chloride channels leads to a net release of hydrochloric acid into the resorption lacuna (Fig. 1.9).[47,51,52]

TRAP is an acid phosphotase that cleaves phosphomonoesters and is the only known enzyme to be expressed in the osteoclast precursors. Osteoclasts generate large quantities of TRAP which localize to the intracellular vesicles and vacuoles near the ruffled borders. TRAP is released in the resorption lacuna to partially dephosphorylate osteopontin, bone sialoprotein and bone matrix phosphoproteins. This allows the osteoclast migration and further resorption to occur. In knockout studies, $TRAP^{-/-}$ mice exhibit a mild osteopetrosis and osteoclasts derived from these mice are unable to dephosphorylate osteopontin leading to an accumulation of the protein in the resorption zone and inside the cells in electron dense vacuoles (Fig. 1.9).[47,52]

The degradation of the organic component of bone matrix is accomplished by the lysosomal protease, cathepsin K. High expression levels of cathepsin K are pre-

sent at the ruffled border and released into the resorption lacuna via lysosomes to digest native collagen fibers at acidic pH.[53,54] These information indicates that lysosomal secretion is a major pathway for resorbing enzymes via the localization of LAMP1 and LAMP2 to the surface of lysosomes.[53] Lysosomes are of two types which have been reported to be active in resorbing osteoclasts. One is secretory lysosomes and the other is conventional lysosomes.[54] Cathepsin K transport occurs through secretory lysosomes.[53] Cathepsin K contains a mannose-6-phosphate (M-6-P) moiety which binds to Mannose-6-phosphate receptors (MPRs) to assist in the transport of soluble lysosomal enzymes to the endosome-lysosome compartments.[53,54] MPRs are highly expressed in osteoclasts to mediate the sorting of cathepsin K from the trans-golgi network to the secretory lysosomes. Lysosomal enzymes and MPRs are present in the endoplasmic reticulum, golgi apparatus and in transport vesicles which fuse with the ruffled border in osteoclasts. The inhibition of M6P targeting pathway as a result of deletion of GNPTAB (GlcNAc-1-phosphotransferase α, β-subunits) dysregulates the formation of secretory lysosomes.[54] Due to a hindrance in the secretory lysosomal pathway, cathepsin K and TRAP are sorted into small post trans-golgi network vesicles leading to their increased secretion in the ruffled borders of these osteoclasts in comparison to wild-type osteoclasts (Fig. 1.9).[47,53]

Several MMPs have been identified to solubilize collagen in the bone areas during resorption. Osteoclasts express MMP1, MMP9, and MMP14 which localizes to the ruffled borders to resorb bone matrix.[55] Deletion of these MMPs did not show any evident difference in the osteoclast resorption but had slower osteo-

clast migration. However, calvariae of MMP13 knockout mice showed slower resorption compared to their wild-type counterparts.[55,56] These evidences suggest that none of the MMPs of osteoclastic origin are limiting for resorption but MMP13 which originates from osteocytes has proved to be limiting for resorption.[55]

In addition to the matrix-degrading enzymes, many different families of proteins are known to play a crucial role in promoting an effective resorption. Rabs are small GTPases localized in the ruffled border of the resorbing osteoclast to regulate late endosomal / lysosomal vesicular trafficking. Rab 7 governs osteoclast lysosome biogenesis and its absence impairs osteoclast ruffled border formation and secretion of enzymes.[53,57] Specifically, Rab 7 regulates the movements of lysosomes and actin cytoskeleton rearrangement through its interaction with Rac1. Plekm1 whose mutations cause osteopetrosis in ia/ia (incisors absent) rats and in a small set of patients with intermediate osteopetrosis, is reported to interact with Rab 7 to function in osteoclast lysosomal trafficking. Osteoclasts in the ia rats and human patients have normal phenotype but with reduced resorptive function.[57] Rab 3D is another Rab family protein that plays a crucial role in osteoclast activation and resorption. It governs the small post-trans golgi network vesicles which are involved in ruffled border formation and bone resorption.[58] Recent studies on Syt VII in osteoclasts suggested that it is a lysosome-associated protein that co-localizes with cathepsin K and LAMP2 and promotes the fusion of lysosomes containing osteoclast enzymes, proton pumps and chloride channels into the ruffled border.[59] It interacts with lysosome-specific SNARE/ TI-VAMP,

which in turn complexes with syntaxin 4 to localize lysosomes containing resorptive proteins to the ruffled borders.[47,53]

Removal of resorbed products from the osteoclast: Osteoclast function in bone resorption requires both the dissolution of crystalline apatite and the enzymatic degradation of the organic bone matrix. This results in the release of large amounts of calcium, phosphate and collagen fragments, which need to be removed from the resorption lacuna to prevent their accumulation to levels that can be toxic to osteoclasts. The removal of these products is accomplished by transcytosis via a vesicular process. The transcytosis is a sequential multistep process. First, degraded products are endocytosed, then transported along a transcytotic vesicular pathway toward the anti-resorptive side of the cell, and finally released out of the cell by exocytosis at the top of the osteoclast.[60] Rab 7 and Rab 3D has been reported to be involved in the regulation of transcytosis and exocytosis respectively.[58] Rab 7 promotes the processing and degradation of collagen fibers via transcytosis.[57] Clathrin, its adaptor protein AP-2, and the large GTPase dynamin are localized at the central area of the ruffled border to enable clathrin-mediated budding necessary for the initial formation of the transcytotic vesicles.[54] Diverse sizes of the transcytotic vesicles are observed suggesting that in addition to endocytosis, micropinocytosis occur to give rise to large endocytic vacuoles called macropinosomes.[60,61] However, it is still to be elucidated how micropinocytosis occurs in osteoclasts and what proteins are involved (Fig. 1.9).[38,47]

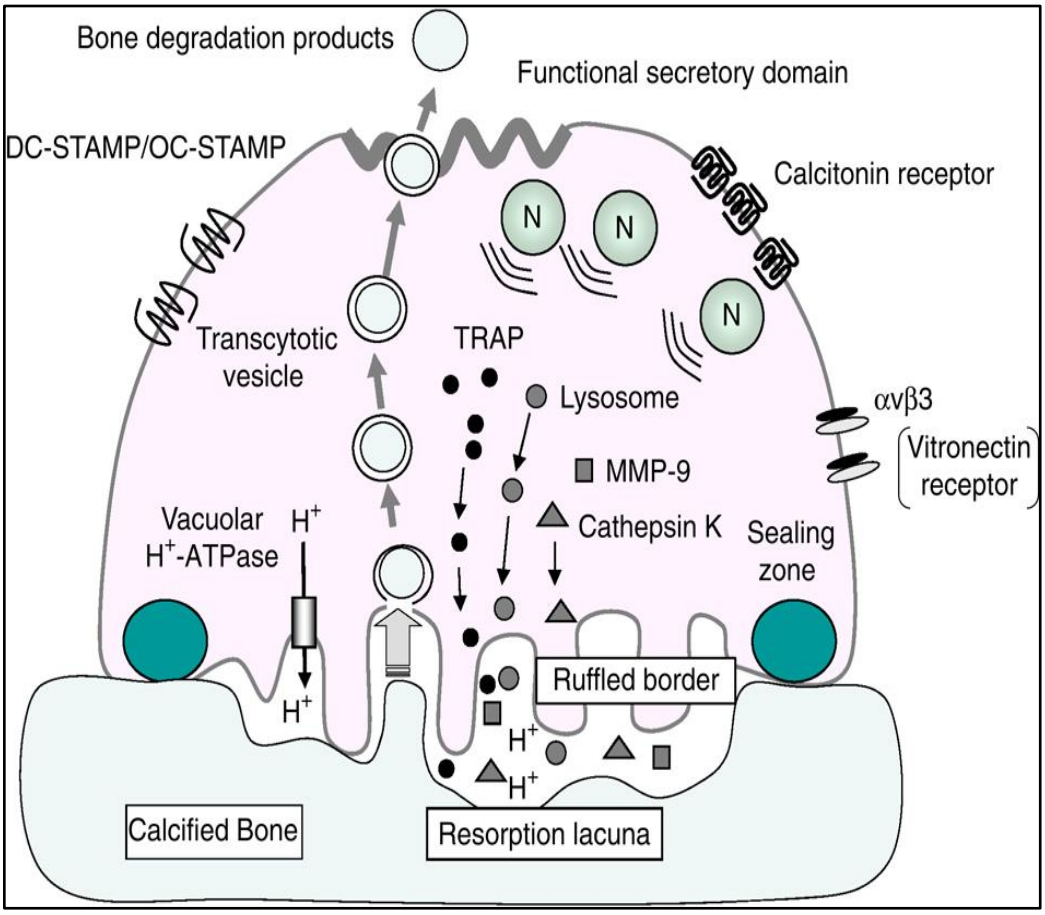


Figure **1.9:** Mechanism of osteoclast activation and bone resorption.

Obtained from Naoyuki Takahashi, Nobuyuki Udagawa & Tatsuo Suda . Vitamin D endocrine system and osteoclasts. BoneKEy Reports (2014) 3, 495.

***Apoptosis**:* osteoclast apoptosis is recognized as a critical regulatory mechanism to control osteoclast differentiation and function and alteration in it can lead to pathological condition. One of the most potent mechanism for osteoclast apoptosis in mature osteoclasts is the Fas/ FasL signaling.[62] Fas is a member of the death receptor family and is expressed in mature osteoclasts as well as in unattached TRAP positive osteoclastic precursors. Its expression increases during differentiation suggesting that Fas is important for the survival of osteoclasts. FasL binds to Fas and recruits FADD which initiates downstream caspase activation cascade and amplifies the death signal to the downstream targets such as BID. Truncated BID translocates to the mitochondria from the cytosol to activate Bax/Bak for the release of cytochrome c from the mitochondria leading to activation of caspase 3 and caspase 9 thereby causing cell death. Binding of RANKL to RANK receptor initiates the recruitment of c-Src, PI3K and AKT to the TRAF6 complex. Activated Akt phosphorylates Bad and caspase 9 thus preventing the activation of apoptotic machinery.[63]

Among BCL-2 family members, Bcl-xL is highly expressed in osteoclast for their survival. M-CSF, RANKL and TNF signaling promotes the expression of Bcl-xL in mature osteoclasts to prolong their life span and prevent apoptosis.[64] Another anti-apoptotic protein, Bim is expressed by osteoclasts to increase osteoclast survival. In the presence of M-CSF, Bim expression is downregulated and the cells undergo apoptosis. $Bim^{-/-}$ osteoclasts are increased in number because of their increased survivability but have decreased resorptive function which indicates that Bim might regulate osteoclast activation.[65,66]

Vicious cycle of bone metastasis

Conventionally, bone metastases are characterized as either osteolytic, osteosclerotic or mixed based on the radiographic and pathological appearance of the lesions.[67,68] Osteolytic bone lesions are the most common feature of the breast and multiple myeloma cancer. Osteolysis is caused by the release of osteoclast activating factors by the tumor cells in the bone microenvironment to stimulate the differentiation and activation of osteoclasts which results in bone resorption at a highly elevated rate. The function of osteoclasts provides an environment that is favorable to the metastasized tumor cells and allows them to survive, proliferate and establish as a bone metastasis at the interface of bone and bone marrow.[68] Although osteolysis is the dominant type of lesion occurring in bone metastases in breast and myeloma cancer, there is always a basal level of bone formation that is associated with it.[67] The basal activity of osteoblasts represents a physiological attempt to repair damaged bone. However, as the rate of bone resorption is higher than the rate of bone formation, the predominant effect of bone metastases in breast and myeloma cancer remains to be a significant bone loss. In contrast to this, bone metastases occurring in melanoma cancer and to an extent in prostate cancer is predominantly osteoblastic in nature.[69] Osteoblastic metastases are a result of the release of factors by tumor cells that stimulate the initiation, proliferation, differentiation and subsequent activation of osteoblasts leading to an uncontrolled bone formation.[67] The complex interactions between the tumor cells, bone and bone matrix constitute a vicious cyclic feedback loop of osteoblast-mediated bone metastases. Moreover, newly formed bone

provides a fertile soil for the tumor cells to survive, proliferate and in turn activate osteoblasts to promote bone metastasis. Interestingly, the rate of bone metastases occurrence is slower in osteoblastic lesions than in osteolytic disease. This phenomenon can be due to an initial increase in bone as a result of osteoblastic activity and may limit the space available for the tumor cells thereby confining the tumor cells.[68,69] Release of tumor-derived factors and RANKL-secreting osteoblasts can both activate osteoclasts leading to a nominal level of bone resorption, which subsequently creates more space for more osteoblastic lesions. As a result of bone resorption, cytokines are released from the bone matrix which in turn can enhance this vicious cycle by facilitating the continued proliferation of tumor cells and osteoblasts causing bone metastasis. Osteolytic and osteoblastic bone lesions are two extremes of the extent of activity of bone cells to drive tumor-mediated bone destruction (Fig. 1.10).[67,68]

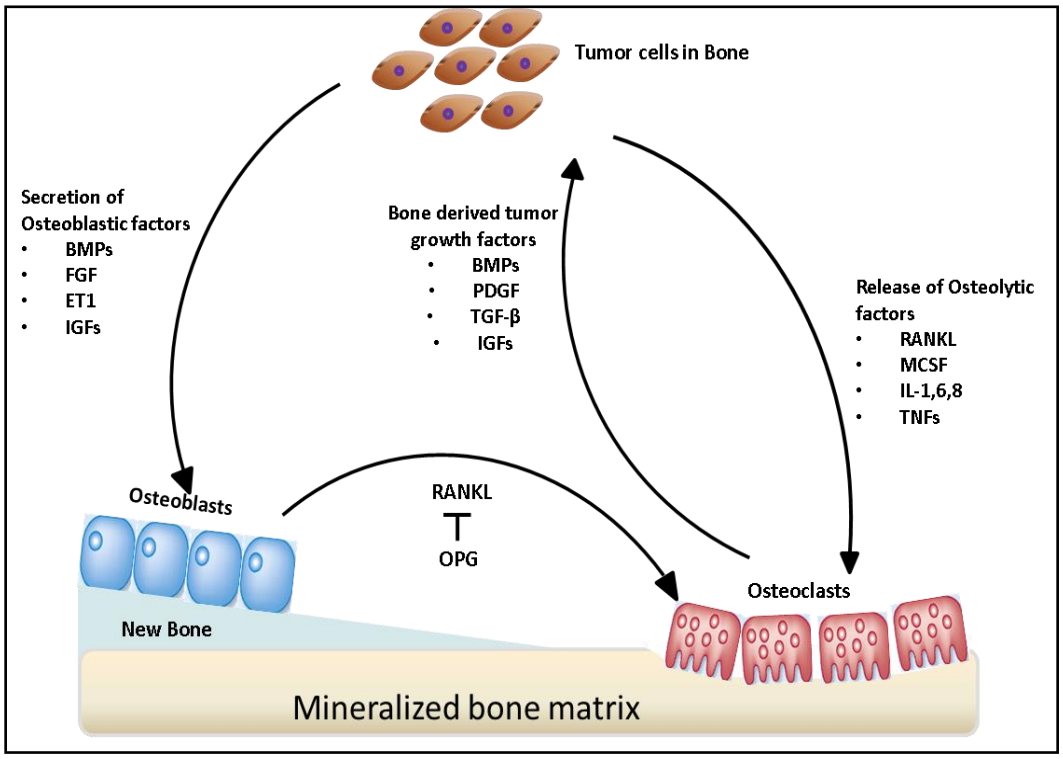


Figure **1.10:** Vicious cycle of bone metastasis

Based on the information from case studies of prostate cancer patients with bone metastasis, mixed osteoblastic/ osteolytic lesions are observed in these patients with the balance slightly shifting towards more osteoblastic lesions with a basal level of osteolysis.[69] Recent reports by Russell Taichman group suggested that tumor cells that metastasize to bone are initially homed in the hematopoietic niche which is present as discrete areas near the interface of bone and bone marrow.[70,71] In the niche, these tumor cells remain dormant release growth factors that can direct the differentiation of hematopoietic stems cells into osteoclasts. The active osteoclasts resorb bone and release growth factors that enable the survival of tumor cells in the bone microenvironment. Once the tumors grow and proliferate, they release osteogenic factors to induce osteoblast differentiation and maturation leading to increased osteogenesis (Fig1.11).[70,71] The osteoblasts induced by prostate cancer cells lay the bone in an irregular manner leading to an uneven bone formation. As a result of this, the bone that is formed is weak and more prone to fracture as it loses its strength and integrity. Furthermore, the bone homeostasis is dysregulated because of increased osteogenesis with a nominal level of osteolytic function causing SREs in patients with bone metastatic prostate cancer.

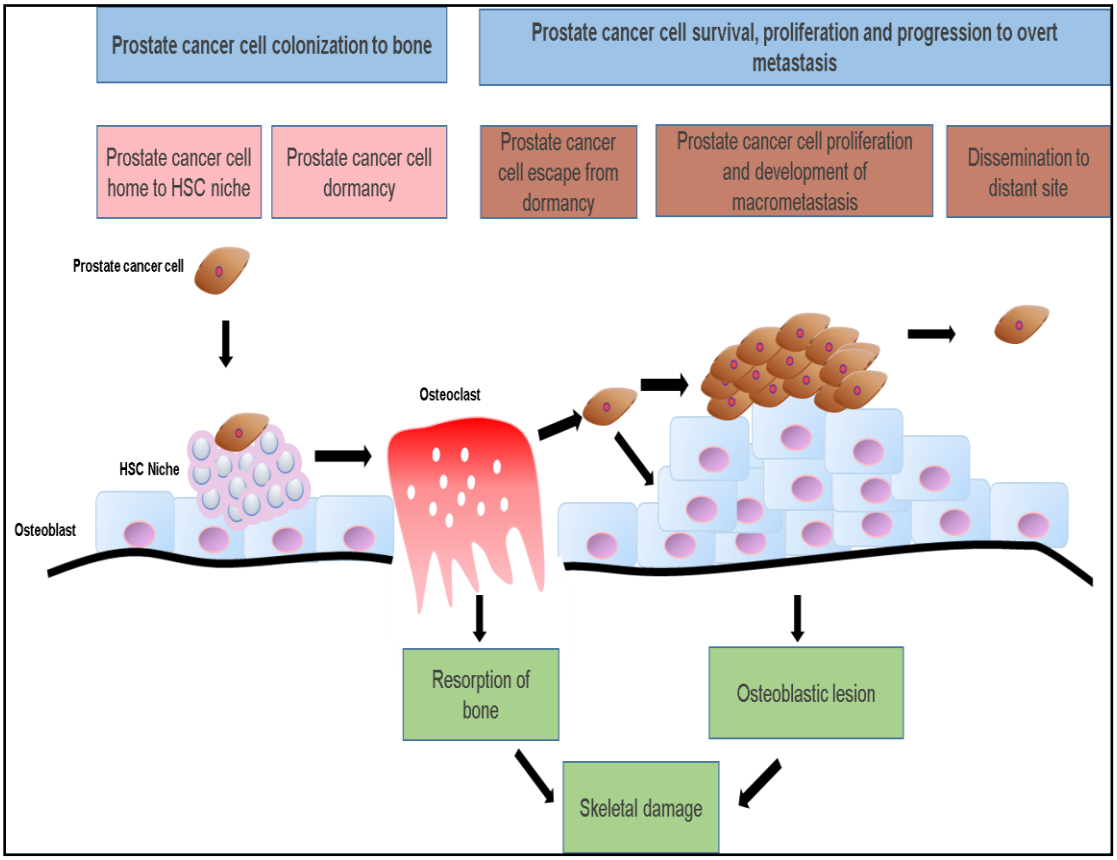


Figure **1.11:** Schematic illustration of prostate cancer cells homing to the Hematopoietic Stem Cell Niche within the Bone Microenvironment and development of overt metastasis.

Treatment strategies for prostate cancer bone metastasis

In mCRPC patients with bone metastasis, the current standard treatment regime includes systemic therapy with taxanes such as Docetaxel who in randomized trials showed to increase the overall survival of the patients with least palliation of bone pain. Furthermore, not all can tolerate the treatment as many patients with mCRPC might not be healthy enough to receive docetaxel owing to poor performance status and comorbidities.[72] Other systemic disease-modifying agents include cabazitaxel, novel hormone-based therapies (abiraterone and enzalutamide) and immunotherapy (sipuleucel-T). Currently, Cabazitaxel is administered to patients progressing on or after docetaxel treatment. Hormone-based therapies with abiraterone and enzalutamide are approved for use in patients with mCRPC either before or following chemotherapy. These hormone therapies have shown to improve survival and skeletal outcome at least time to the first skeletal related events (SRE) in this setting.[73] Treatment with an immunotherapeutic vaccine, Sipuleucel-T has significantly improved the overall survival of men with mCRPC. Sipuleucel-T is preferred in chemotherapy-naive mCRPC patients, which limits its use in combination therapy. Further, administration of Sipuleucel-T in mCRPC patients has severe side effects with no change in the status of SREs.[74]

Use of osteoclast-targeting agents such as zolendronic acid and denosumab is widely accepted for the treatment of bone metastatic prostate cancer. Preclinical studies revealed that nitrogen-containing bisphosphonates such as zolendronic acid is highly potent in inhibiting the osteoclast-mediated bone resorption.[75] From the studies conducted separately by Alliance (CALGB 90202) and ZEUS groups,

it can be concluded that zolendronic acid is specifically effective in treating SREs in patients with early stages of mCRPC and bone metastasis and ineffective for the prevention of bone metastasis in high risk localized and castration-sensitive prostate cancer patients.[75] Denosumab is a human monoclonal antibody to RANKL that blocks the binding of RANKL to RANK receptor on osteoclasts to prevent their differentiation and function leading to decreased bone loss. Information from two independent phase II trials indicates that denosumab reduced the bone resorption in patients independent of prior bisphosphonate treatment. Comparing the potency of denosumab and zolendronic acid in patients with mCRPC and bone metastasis, a randomized, double-sided phase III trial showed that denosumab was able to prolong the time to the first occurrence of SREs and decrease bone marker turnover (BALP and uNTx) than zolendronic acid. However, administration of osteoclast targeting agents on patients has adverse complications which include hypocalcemia, nephrotoxicity (only with use of zolendronic acid), osteonecrosis of the jaw and immune dysfunction.[76] The limitations of the current treatment strategies advocate the necessity of the understanding of the molecular mechanisms that can be extrapolated to develop an effective therapy.

CHAPTER 1C: Molecular mechanisms in osteoclasts in bone metastasis

Recent evidence on molecular pathways in healthy and metastatic bone

In healthy bone, M-CSF and RANKL are the critical inducers which work in concert to enable the commitment, survival, differentiation, maturation, and activation of osteoclasts. Osteoblasts remain the primary source of these cytokines along

with fibroblasts and T regulatory cells contributing to the overall pool. OPG released by osteoblasts inhibit the RANK-RANKL pathway thereby controlling the osteoclast differentiation and function. Growth factors such as TNF-, PTHrP, VEGF, and interleukins such as IL-1, IL-6, IL-8, IL-11, IL-15, IL-17 and IL-32 are reported to work along with M-CSF and RANKL to promote osteoclastogenesis.[77,78] Current reports on prostate cancer bone metastasis suggest that metastatic prostate cancer cells once in the hematopoietic niche release growth factors such as EGF, TNF-, PTHrP, IL-1, IL-6, IL-8 and VEGF to manipulate the hematopoietic stem cells to commit to osteoclast lineage.[78,79] These tumor-induced osteoclasts degrade bone to release factors such as TGF-, IGF-1, BMP6, FGF-8, and PDGF to enable the survival of metastatic prostate cancer cells in bone and advocate their interaction with osteoblasts to develop into overt osteoblastic metastasis.[80] Although the identification of factors has provided insight into their role in prostate cancer bone metastasis, use of inhibitors or osteoclast targeting agents could not rescue the overall architecture of the bone causing severe bone pain and can result in decreased mortality in prostate cancer patients with bone metastasis. There is growing evidence that osteoclasts are the potential proponents for the prostate cancer cells to survive in strenuous bone microenvironment during the early stages of homing and survival thereby making osteoclasts a potential target for therapy. However, there exist gaps in the knowledge at molecular level that needs addressing to develop a successful treatment.

Introduction to Neuropilin family

Neuropilins are non-tyrosine kinase glycoproteins expressed on the surface of the cell to function mainly as co-receptors for vascular endothelial growth factor (VEGF) and the class III Semaphorin family of molecules by interacting with VEGF receptors and Plexins, respectively.[81] They were initially reported to be involved in the chemorepulsion function of Semaphorins in axonal guidance during neural development. They are extensively known for their functions in physiological processes such as angiogenesis, lymphangiogenesis, cardiovascular development, cell survival, migration and in pathological disorders such as cancer. Neuropilins are type I transmembrane proteins with molecular mass of 120 kDa and are reported to be expressed in neuronal cells, endothelial cells, epithelial cells of endodermal origin and various immune cells such as macrophages, dendritic cells (DCs), T cells, B cells, and mast cells.[81]

Neuropilins exist as two isoforms as a result of gene duplication namely Neuropilin 1 (NRP1) and Neuropilin 2 (NRP2). Structurally, NRP1 and NRP2 share similar structural layout consisting of an N-terminal extracellular domain followed by a transmembrane region and a short cytosolic tail of 43–44 amino acids. Neuropilins comprise of four different domains, two repeats of CUB [complement binding factors C1s/C1r, Uegf, BMP1 (bone morphogenetic protein 1)] (a1/a2) domains, two repeats of Factor V/VIII homology (b1/b2) domain, a b-c linker followed by a MAM (meprin, A5 antigen, receptor tyrosine phosphatase μ) (c) domain, a single transmembrane domain and relatively short cytoplasmic domains (44 amino acids for NRP1 and 43 for NRP2). The CUB (a1/a2) domains (approx.

110 amino acid residues each) is essential for the binding of class 3 Semaphorin group of ligands. The b1/b2 domain (approx. 150 amino acid residues each) with its ability to bind cell surface-specific anionic phospholipids, is necessary for cell-cell adhesion and ligand binding for VEGF and Sema3A. The MAM domain is a 170 amino acid residue region which is thought to mediate homodimerization or oligomerization of hemophilic proteins to maintain protein stability (Fig. 1.12).[81]

NRP1 and NRP2 have several isoforms as a result of alternate splicing. NRP1 has been reported to exist as membrane-bound truncated NRP1 (Δexon16) (lacks 51 nucleotides corresponding to exon 16) and soluble NRP1 (sNRP1), i.e., s12NRP1 (644 amino acids), sIIINRP1 (551 amino acids) and sIVNRP1 (609 amino acids). SNRP1 has the a1/a2 and b1/b2 domains but lacks the MAM (c), transmembrane and cytoplasmic domains which enable it to become a decoy receptor that can competitively bind and sequester ligands such as VEGF165 and Sema3A to inhibit their signaling process. NRP2 also exists as membrane-bound NRP2a and NRP2b and soluble sNRP2 isoforms. NRP2a share 44% homology with NRP1 at amino acid level. The C-terminal three amino acids, SEA, present in NRP1 and the NRP2a isoform, form a consensus PDZ domain-binding motif, which mediates association with PDZ domain proteins such as NIP1 (neuropilin-interacting protein-1), synectin and GIPC (RGS-GAIP-interacting protein). NRP2b is identical to NRP2a in its extracellular domain but has 11% homology at transmembrane and cytoplasmic domains. The difference in the cytoplasmic domains of NRP2a and NRP2b may suggest their diverse physiological and pathological functions. Expression of NRP2a and NRP2b is high in brain and NRP2a is specif-

ically expressed in the liver, lung, small intestine, kidney, and heart but NRP2b is restricted to heart and skeletal muscle. NRP2a exists as splice variants such as NRP2a (17), and NRP2a (22) in humans, and NRP2a (0), NRP2a (5), NRP2a (17), and NRP2a (22) in mice. These splice variants are a result of insertion of 0, 5 and 17 amino acids after 809 residues located between the MAM and transmembrane domains. NRP2a (22) is a derivative of NRP2a (17) with five additional amino acids. NRP2b as a result of alternate splicing has insertion of 0 and 5 amino acid residues after residue 808 (NRP2b (0) & NRP2b (5)). Along with these membrane-bound splice variants, there exists s9NRP2 (1785 bp, 555 amino acids, 62.5 kDa) consisting of the two a1/a2 domains the b1 domain and a truncated b2 domain followed by the nine amino acids VGCSWRLPL encoded by intron 9 (Fig. 1.12).[81]

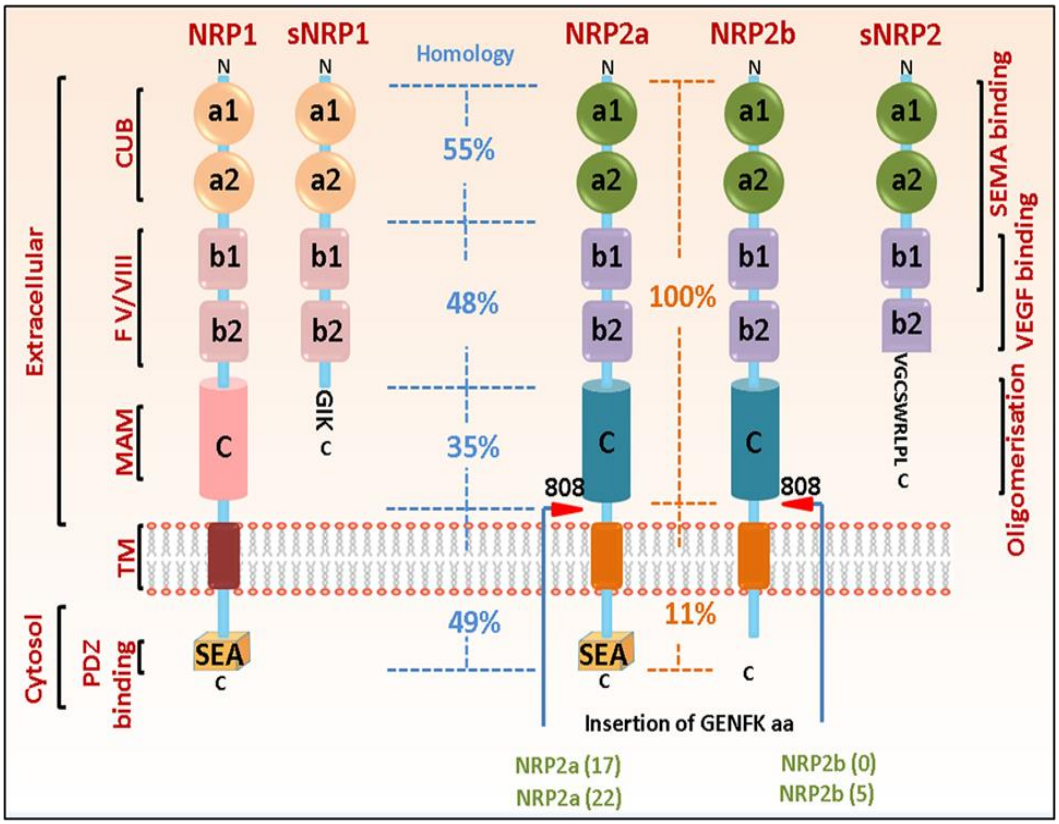


Figure **1.12:** NRP basic structure and splice variants.
Obtained from Sohini Roy et. al. Multifaceted Role of Neuropilins in the Immune System: Potential Targets for Immunotherapy. Front Immunol. 2017 Oct 10;8:1228.

Neuropilins transduce signals for all five VEGFs along with three VEGFR members of VEGF family. Knockout of NRP1 in mice demonstrates significant cardiovascular and neuronal dysfunction along with defect in angiogenesis causing embryonic lethality. In addition, overexpression of NRP1 also causes embryonic lethality due to hypervascularization. The vascular phenotype of NRP1 depletion in mice coincides to that of VEGF-A heterozygous mice and knockout of VEGR-2. NRP1 works in cooperation with VEGF-A/ VEGF-R2 to promote angiogenesis and NRP1 localizes to the endothelial tip cells for their fate determination. It functions downstream of Notch and limits BMP9 and TGF-β signaling in tip cells. Specifically, isoforms of VEGF i.e. VEGF165 binds VEGFR2 induces cell proliferation and migration and these functions are enhanced by the complexing with NRP1. NRP1 also interacts with VEGF-B and PIGF-2 via VEGF-R1 and VEGF-E through VEGF-R2 to promote cell migration and mitogenic activity. NRP1 has been reported to function as a receptor for extracellular microRNAs (miRNAs) commonly found in biological fluids and circulate either in encapsulated form or bound to protein argonaute-2 (AGO2). NRP1 binds AGO2/miRNA complexes to facilitate their cellular internalization, which may have consequences under normal and pathophysiology. NRP2 is reported to bind to VEGF-A isomers, VEGF165 and VEGF145 to promote vascular development. NRP2 also associates with VEGF-C and VEGF-D to a lesser extent along with VEGF-R2 to induce angiogenesis and with VEGF-R3 to promote lymphangiogenesis (Fig. 1.13).[82]

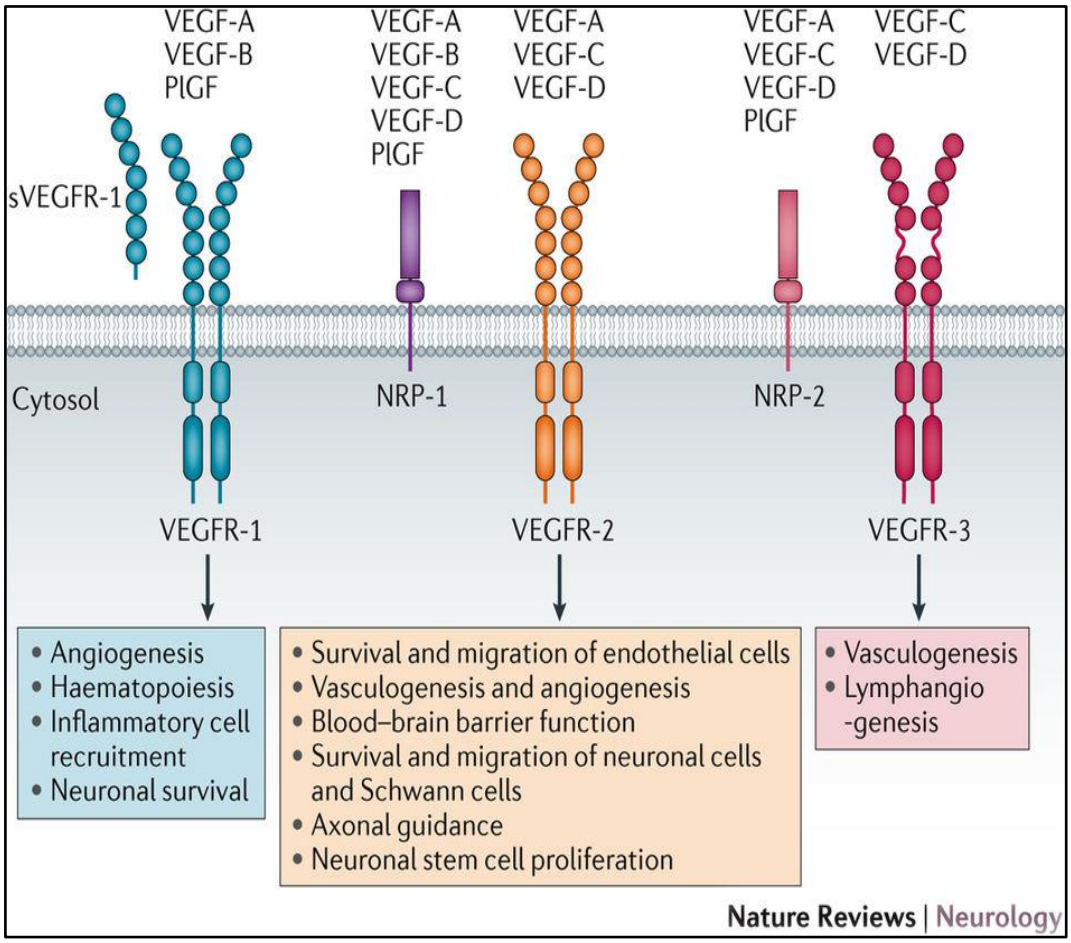


Figure **1.13:** NRPs and their binding partners-VEGF family growth factors.

Obtained from Christian Lange et. al. Vascular endothelial growth factor: a neurovascular target in neurological diseases. Nature Reviews Neurology vol12, pages439–454 (2016).

NRPs are known for their role in neuronal guidance. For this, they act as modulates with dual binding sites, one for semaphorin (ligand) and the second for plexin (receptor). Class III semaphorins are reported to bind to the a1/a2/b1 domain of NRP1 or NRP2 with different affinities and specificities and form a holoreceptor complex with NRPs and PlexinA1 or PlexinA2. A unique feature of class III semaphorins is that they bind to NRPs but not to plexins and thus NRPs act as mediators of semaphorin - plexin signaling. NRP1 binds all class III semaphorins with high affinity for Sema3A to modulate growth cone collapse activity. NRP2 mainly binds to Sema3F, but not 3A and Sema3C with low affinity and plexinA3 to facilitate selective axonal guidance of neurons. Recent report on NRP2 concluded that Sema3F-NRP-2/PlexA3 signaling controls both synapse development and synaptic plasticity (Fig. 1.14)[82]

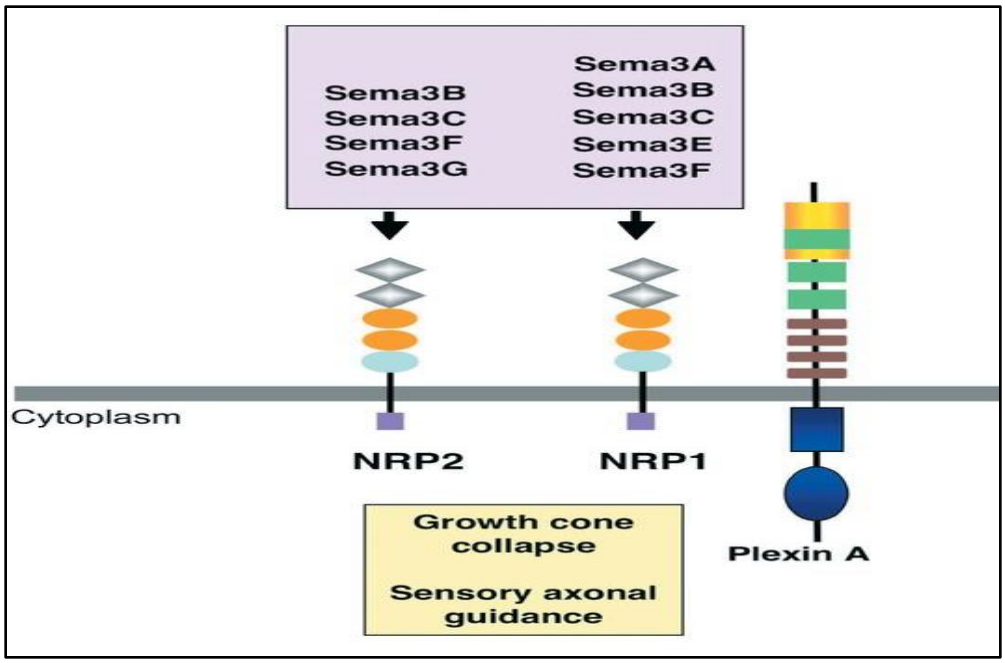


Figure **1.14:** NRPs and Semaphorin-Plexin signaling.
Obtained from Caroline Pellet-Many et. al. Neuropilins: structure, function and role in disease. Biochem J. 2008 Apr 15;411(2):211-26.

Neuropilins in normal bone and prostate cancer bone metastasis.

In the bone, Neuropilins are expressed by both osteoblasts and osteoclasts. Reports by Hayashi *et al*. suggested that NRP1 binds Sema 3A released by osteoblasts impairs immunoreceptor tyrosine-based activation motif (ITAM) and RhoA signaling to inhibit the RANKL-induced osteoclastogenesis. Interestingly, they also reported that Sema3a−/− or Nrp1Sema– mice showed severe dysfunction of osteogenesis with increase osteoclast and adipocyte number and that Sema 3A works via canonical Wnt signaling in part through FARP2-mediated Rac1 activation during osteogenesis.[83] Further, binding of NRP1 with PlexinA1 via discoidin domain receptor 2 resulted in the inhibition of osteoclast differentiation and function. In human peri-prosthetic osteolysis (PPO) samples, high NRP1 expression in osteoclasts was observed suggesting its role in the disease.[84]

Osteoblasts, as well as osteoclasts, also express NRP2. Evidence from the comparison of the bones from $Nrp2^{+/+}$ and $Nrp2^{-/-}$ mice showed a reduction in trabecular bone mass characterized by decreased osteogenesis but increased osteoclast number in NRP2 deficient mice. However, no study has been conducted to understand the exact molecular mechanism/s through which NRP2 functions to regulate bone homeostasis. In pathological conditions such as osteosarcoma, RNA levels of NRP2 were highly elevated and correlates with hypervascularity and poor prognosis.[85] Cumulatively, NRP2 is crucial for the functioning of bone cells (both osteoblasts and osteoclasts), but the exact mechanism of action is still unknown.

Multiple studies on NRP2 has shown that NRP2 provides chemoresistance to prostate cancer cells during therapy and that removal of NRP2 from the prostate cancer cells sensitizes them to chemotherapeutic drugs. Evidence from the studies by HL Goel *et al*. indicated that NRP2 is expressed by prostate cancer stem cells and when bound to VEGF causes the activation of PREX-RAC1-ERK signaling leading to ineffectiveness of anti-VEGF therapy (bevacizumab) on prostate cancer stem cells.[86] Further, these chemoresistant prostate cancer cells can progress and metastasize to different organs specifically to bone. In the bone, prostate cancer cells home to hematopoietic niche where they induce osteoclast differentiation and activation to release growth factors trapped in the bone matrix necessary for their survival and advancement into osteoblastic metastasis. As osteoclast activation precedes the osteogenesis in prostate cancer bone metastasis, targeting osteoclast function can prove to be beneficial during therapy. Further, NRP2 is expressed by osteoclasts suggesting its role in bone homeostasis. Since, metastatic prostate cancer cells dysregulate bone homeostasis, it would be essential to understand the role of NRP2 in osteoclasts in promoting prostate cancer bone metastasis.

General objectives and Hypothesis

Over the past few decades, multiple studies have established vital roles of NRP2 in malignant diseases such as prostate cancer, breast cancer, osteosarcoma and colon cancer.[87-89] The expression of NRP2 is significantly altered during the progression, metastasis, and chemoresistance of prostate cancer. This propagated a question as to what role does NRP2 have in the bone which is the most common site for mCRPC to metastasize. Specifically, little is known about the function of NRP2 in the tumor-bearing bone microenvironment, i.e., the NRP2 expressing- bone cells which interact with cancer cells during colonization and development of overt bone metastasis. Previous reports on NRP2 suggested its role in the regulation of bone homeostasis in a healthy bone by explicitly controlling osteoclast differentiation and activation while promoting osteogenesis.[85] Although, mCRPC promotes hyperactivation of osteoblasts, osteoclasts are the first cells which are activated by mCRPC in the hematopoietic niche and NRP2 may have a crucial role in the regulation of osteoclast function to promote osteoblastic bone metastasis.[71] Hence, a comprehensive understanding of the role of NRP2 in osteoclasts in promoting prostate cancer bone metastasis can aid in developing a successful treatment strategy. The focus of my study is to understand the function of NRP2 in osteoclasts in prostate cancer bone metastasis. I hypothesized that mCRPC induces NRP2 expression in OC and is necessary for promoting prostate cancer bone metastasis.

Our study therefore can address whether NRP2 on osteoclasts can be targeted for therapy in prostate cancer patients with bone metastasis which is incurable.

Altogether, the objectives of my dissertation research are:

1. To investigate the role of NRP2 in osteoclasts in promoting prostate cancer bone metastasis.

2. To elucidate the molecular mechanisms of NRP2 in regulating the osteoclastic differentiation and activation.

CHAPTER 2

Materials and methods

CHAPTER 2A: Materials

- **Prostate cancer cell lines**
- **Mouse models**
- **Reagents**

CHAPTER 2B: Methods

- **Cell culture**
  - **Prostate cancer cell lines**
  - **Preparation of conditioned media**
  - **Isolation of mouse bone marrow-derived osteoclasts**
- **Depletion of NRP2 using RNA interference and knockout model**
- **Tartarate-resistant acid phosphatase staining**
- **Pit formation assay**
- **Immunoblotting**
  - **Separation of nuclear and post nuclear fractions**
- **RNA isolation and cDNA preparation**
- **Real-time quantitative PCR**
- **Enzyme-linked immunosorbent assay**
- **Immunofluorescence**
- **Statistical significance**

CHAPTER 2A: Materials

Prostate cancer cell lines

Bone metastatic prostate cancer cell line, LNCaP C4-2B was kindly gifted by Dr. Donald Tindall, and PC3 bought from ATCC. LNCaP C4-2B is a selected derivate of a human prostate cancer cell line, LNCaP. These cells were isolated from metastatic prostate cancer lesions found in the lumbar spine of an athymic mouse. The mouse implanted with LNCaP C4-2 and by selective mechanism caused bone metastasis. LNCaP C4-2B has faster growth rate when compared to its parental cell line and is osteoblastic with low osteolytic function. Hence, LNCaP C4-2B provides a model to explore the mechanisms of osteoblastic lesions in prostate cancer.

PC3 (purchased from American type culture collection) is one of the most commonly used human prostate cancer cell line used in studying bone metastasis. It was derived from human bone metastasis and produce osteolysis and metastasize to femur, tibia, rib, pelvis, mandibles and lymph nodes in NOD/SCID mice after orthotopic or intracardiac injection. As PC3 is purely osteolytic in nature, it can be an ideal model for studying the function of osteolysis in prostate cancer bone metastasis.

Mouse models

All Mice used in this study were maintained under specific pathogen-free conditions. All procedures performed were in accordance with institutional guidelines

and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). Our study used two different mouse models, C57BL/6 and Transgenic CSF1R-cre; NRP2 Flox/Flox mice.

C57BL/6: These mice were purchased from Charles river at the age of 6-10 weeks for isolation of bone marrow.

Generation of transgenic CSF1R-cre; NRP2 Flox/Flox mice: The $NRP2^{flox/flox}$ mouse was developed Max Planck Research Unit for Neurogenetics by Dr. Peter Mombaerts. These mice were initially generated by insertion of Nrp2 targeted mutation in 129P2/OlaHsd derived E14 ES cells and had a loxP-tauGFP-pA+-FNF cassette inserted into the first intron and a corresponding loxP site inserted upstream of the start codon. The generated mice were bred with Tg(ACTB-flp)4917Dym to remove FRT-flanked neomycin resistance sequence (FNF) which yielded Nrp2tm1.1Mom targeted mutation in a mixed background that is predominantly 129P2 and C57BL/6. Our research collaborator in University Hospital Bonn, Germany, Dr. Michael Muders bred these mice with C57BL/6 to get pure background mice and gave them as a kind gift to us. The transgenic FVB-Tg(Csf1r-Mer-iCre-Mer)1Jwp/J mice were purchased from Jackson Laboratories. These mice were initially developed by Dr. Jeffrey W Pollard, Albert Einstein College of Medicine, NY. A transgenic construct containing tandem copies of icre/Esr1*, or Mer-icre-Mer, under the control of the mouse Csf1r, colony stimulating factor 1 receptor, promoter, was injected into fertilized FVB/N mouse eggs. The founder line 1 was established and submitted to the Jackson Laboratory where they were crossed with FVB/NJ to establish the colony. These mice ex-

press a Cre recombinase/mutant murine estrogen receptor double-fusion protein under the control of the Csf1r (colony stimulating factor 1 receptor) promoter. The MerCreMer double fusion protein consists of Cre recombinase flanked on each end with a mutated murine estrogen receptor (mer) ligand binding domain (amino acids 281-599, G525R); which does not recognize its natural ligand (17'-estradiol) at physiological concentrations but will bind with higher affinity to the synthetic estrogen receptor ligands 4-hydroxytamoxifen (OHT or tamoxifen) and, with lesser sensitivity, ICI 182780. Restricted to the cytoplasm, MerCreMer can translocate from cytoplasm to the nuclear compartment only after exposure to tamoxifen. When these Tg(Csf1r-Mer-iCre-Mer)1Jwp mice are bred with mice containing a loxP-flanked sequence of interest, tamoxifen-inducible, Cre-mediated recombination will result in deletion of the flanked sequences in Csf1r-expressing cells specifically myeloid lineage cells.

For our research, the CSF1R-cre; NRP2 Flox/Flox mice in a pure background were generated by a three-step breeding process. The first step involved back-crossing of FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J (CSF1R- Cre) mice with C57BL/6 pure background mice. Backcrossing was done for at least seven generations. This step enables the generation of 80% pure background CSF1R-Cre mice. The second step involves breeding of CSF1R- Cre with Nrp2tm1.1Mom/MomJ (NRP2flox/flox) to generate transgenic mice with CSF1R-Cre; $NRP2^{flox/+}$ geno-type. These mice were then bred with $NRP2^{flox/flox}$ mice to obtain CSF1R-Cre; $NRP2^{flox/flox}$ mice. The generated transgenic mouse (CSF1R-Cre; $NRP2^{flox/flox}$) expresses a tamoxifen-inducible Mer-iCre fusion protein driven by the Csf1r pro-

moter which upon administration with either tamoxifen intraperitoneally (75 mg/Kg body weight) in mice or (Z)-4-Hydroxytamoxifen in-vitro leads to activation of Cre recombinase. The Cre recombinase targets the deletion of NRP2 in the loxP-flanked regions. The ablation of NRP2 occurs in myeloid cells as they specifically express CSF1R. These transgenic mice are ideal for studying the osteoclast-related functional as well as molecular mechanisms in physiological and pathological conditions (Fig. 2.1)

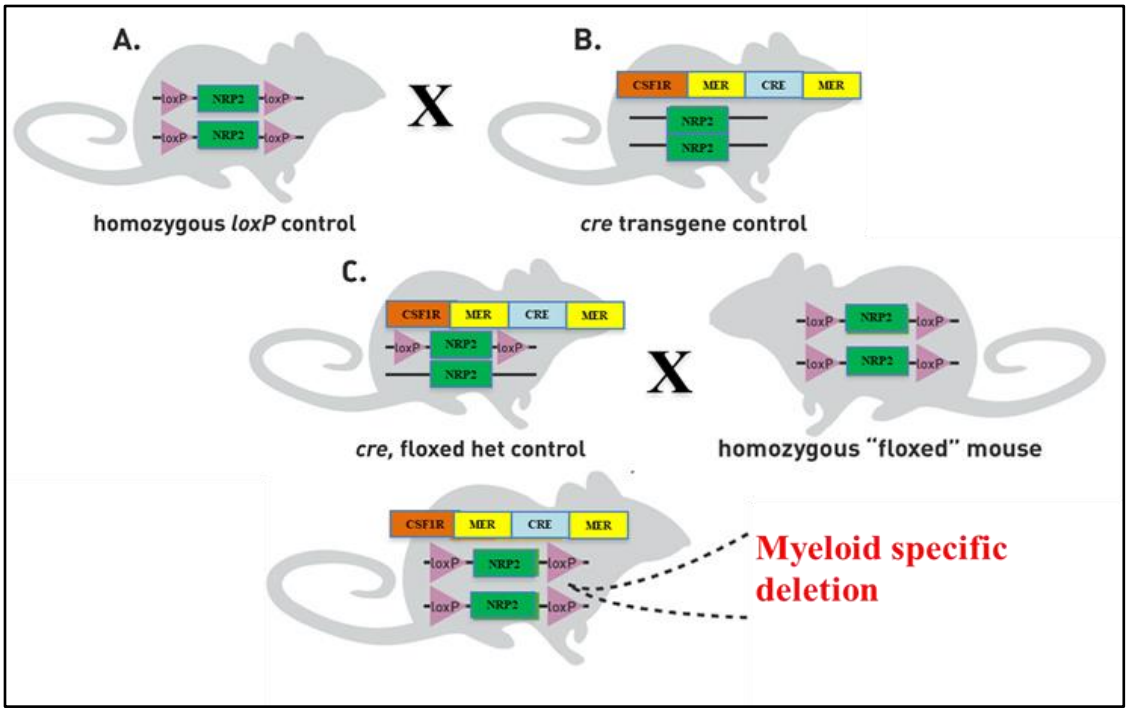


Figure **2.1:** Illustration of sequential breeding for Generation of experimental transgenic mice, NRP2$^{Flox/Flox}$; Csf1r-cre.
Obtained from Dr. Sohini Roy.

Reagents

RPMI 1640 medium, DPBS, 0.25% (w/v) Trypsin, MEM Non-Essential Amino Acid solution (100X), sodium pyruvate (100 mM), HEPES (1 M) and Penicillin-Streptomycin (5,000 U/ml) were procured from ThermoFisher Scientific. Minimum Essential Medium (MEM) Alpha Medium (10-022-CV) was purchased from Corning. Fetal bovine serum and goat serum were obtained from GIBCO.

Antibodies such as NRP2 (D39A5), NF-KB (D14E12), -Actin (D6D8), Histone H2A (D603A) were purchased from Cell signaling technology, NFATc1 (7A6) from ThermoFisher Scientific, HDAC1(ab7028) from Abcam, Rho-GDI (C2, sc-374579) and HSC70 (B-6, sc-7298) from Santa Cruz Biotechnology. Secondary antibodies were ordered, i.e., goat anti-rabbit IgG-HRP (sc-2004) from Santa Cruz Biotechnology and sheep anti-mouse IgG-HRP (AC111P) from EMD Millipore. Secondary antibodies conjugated with Alexa fluor such donkey anti-rabbit Alexa Fluor 546 for NF-B (cat.no.- A10040) and goat anti-mouse Alexa Fluor 660 for NFATc1 (cat.no. – A21054) were obtained from ThermoFisher Scientific. siRNA against mouse NRP2 and non-targeting control (ON-TARGET plus, smart pool) was bought from Dharmacon. Recombinant murine M-CSF (315-02) and RANK Ligand (315-11) were acquired from Peprotech. TRAP staining kit was purchased from Cosmo bio (PMC-AK04F-COS) and Osteo assay surface microplates (24-well, #3987) from Corning. Reagents such as (Z)-4-Hydroxytamoxifen (H7904), HEPES, KCl, DTT, NP-40, Glycerol, MgCl2, EDTA, PMSF, cyclosporine A, protease inhibitors such as aprotinin, and leupeptin were purchased from Sigma-Aldrich. Halt, Trizol and Powerup SYBR Green master mix were bought

from ThermoFisher Scientific. cDNA kit was obtained from Roche and primers from IDT. The primer sequences are listed in Table.

CHAPTER 2B: Methods

Cell culture

Prostate cancer cell lines

PC3 and LNCaP C4-2B were cultured in RPMI complete medium supplemented with 10% fetal bovine serum and antibiotics (Penicillin-streptomycin). Upon confluency, these cells were washed with DPBS and either brief rinse (LNCaP C4-2B) or treated with 0.25% (w/v) Trypsin-EDTA (PC3) to detach the cells from the plate. The cells were collected in equal volumes of complete medium to neutralize the effect of trypsin. The cells were pelleted by centrifugation at 1000 g for 5 mins. The cells are then suspended in fresh complete media and plated in a T-175 flask and cultured in a tissue culture incubator maintained at 37 0C and 5% CO2.

Collection of conditioned medium

To collect conditioned medium from PC3 and LNCaP C4-2B, the 1 x 10^6 cells were plated in a T-75 culture flask and allowed to grow until 70% confluency. Once 70% confluent, the cells were washed with DPBS to remove any traces of exhausted medium and 5 ml of fresh serum-free RPMI complete medium is added to the flask. The cells were then incubated for another 24hrs to collect conditioned medium. After obtaining the conditioned medium, it was centrifuged at

1000 g for five mins to remove cells and cell debris from the medium and filtered using 0.45 m filter to remove any residual debris remaining after centrifugation. The conditioned medium was either used fresh for experiments or aliquoted in small volumes based on usage and stored at -80 ^{0}C for future use.

Isolation of mouse bone marrow-derived osteoclasts

Mouse osteoclastic precursors were isolated from 6-10 weeks old C57BL/6 male mice and transgenic mouse model (CSF1R-Cre; NRP2flox/flox) after euthanizing and cervical dislocation. Long bones from the mice were harvested, and the bone marrow was collected in centrifuge tubes by flushing the bones with a 30 ml syringe with needle containing DPBS. The remaining processing was done in sterile conditions. The bone marrow was subjected to centrifugation at 2000 g for ten mins. The pellet obtained was suspended in MEM alpha medium containing 10% fetal bovine serum, 1% antibiotic, 1% sodium pyruvate, 1% MEM Non-Essential Amino Acid and HEPES (pH 7.4). The cells are then filtered through a cell strainer of 70 µM pore size to remove fibrous cellular debris and make a single cell suspension. The cell suspension was then centrifuged, and the pellet was then subjected to Ficoll-Paque gradient to isolate mononuclear cells. These cells were plated in MEM alpha complete medium containing 10 ng/ml of recombinant murine M-CSF and cultured overnight to separate macrophages (which will attach to the surface of the plate), and the non-adherent cells were harvested to be used as osteoclastic precursors for all the experiments (Fig.2.2).

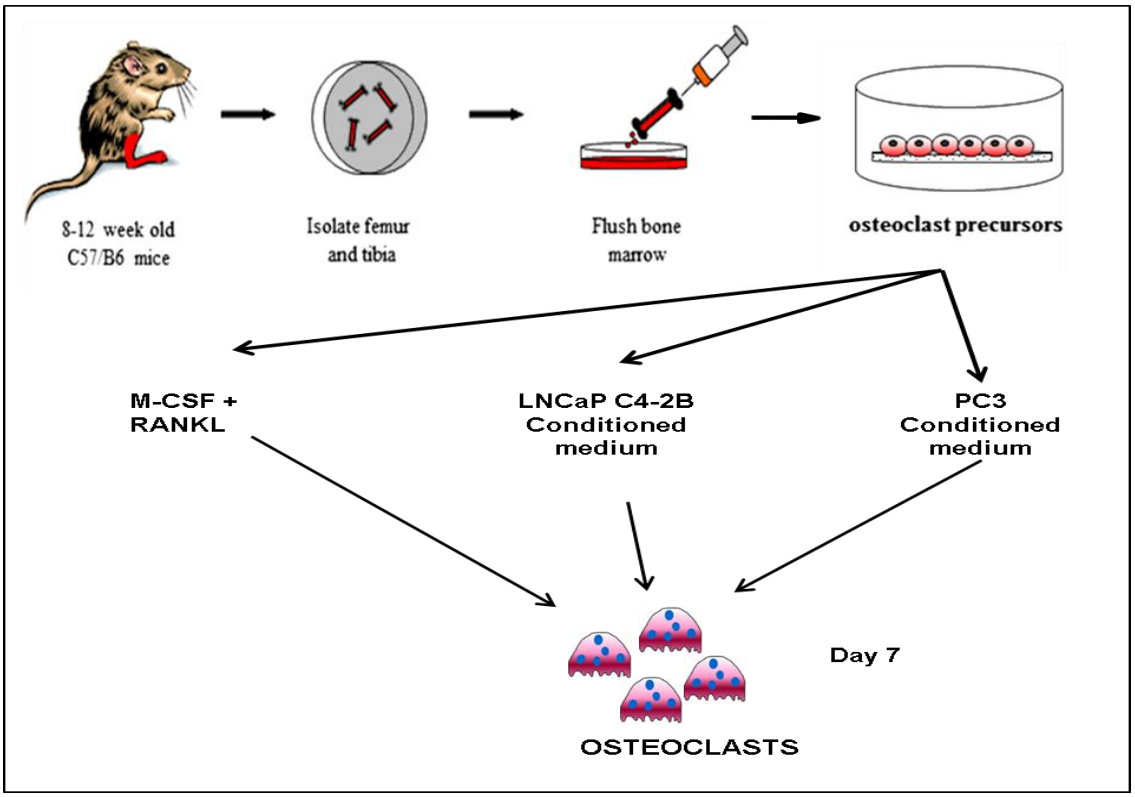


Figure **2.2:** Procedure for isolation of osteoclast precursors and their differentiation under different conditions**.**

For our research study, three different culturing conditions were applied to mimic the osteoclasts in physiological and pathological bone metastasis. To understand the differentiation and function of osteoclasts in healthy bone, the osteoclastic precursors were differentiated into osteoclasts by addition of 20 ng/ml of recombinant M-CSF and 100 ng/ml of recombinant RANK Ligand for seven days with a boost of growth factors every alternate day from the start of the experiment. In the case of bone metastasis, conditioned media collected from metastatic prostate cancer cell lines, PC3 and LNCaP C4-2B (20% v/v) were mixed with MEM alpha complete medium separately. (Various reports have been published on the use of conditioned media in osteoclast differentiation, but none of these reports presented an exact method of conditioned media isolation and use. Hence, standardization was conducted to check their effect on osteoclast differentiation with different concentrations of conditioned media. The osteoclastic precursors are plated in these media separately to differentiate into osteoclasts. Every consecutive day, a boost either with RANKL and M-CSF or conditioned media was added. On day 7, the mature osteoclasts from all the three conditions were then used for isolation of protein or RNA based on the experiment conducted (Fig.2.2).

Depletion of NRP2 using RNA interference and knockout model

After isolation of osteoclastic precursors from C57BL/6 mice, these cells were depleted of NRP2 by using Lonza nucleofector kit (VPA-1007) and nucleofector

2b device. The procedure followed for nucleofection was as per the manufacturer's protocol. 25 nM of NRP2 and scrambled siRNA were used. After nucleofection, the osteoclast precursors were maintained in nucleofector solution containing medium for 8 hrs after which MEM alpha complete medium containing either M-CSF and RANK Ligand or conditioned medium from prostate cancer cell lines was added to each plate and allowed to differentiate for seven days.

Osteoclastic precursors isolated from transgenic mice, CSF1R-Cre; $NRP2^{flox/flox}$ were treated with 0.3 μM/ml of (Z)-4-Hydroxytamoxifen to deplete NRP2 from osteoclasts specifically. A parallel set of osteoclastic precursors where no (Z)-4-Hydroxytamoxifen was added as control for each experiment. The control and knockout osteoclastic precursors were then differentiated into mature osteoclasts (7 days) either with M-CSF and RANK Ligand or conditioned medium from prostate cancer cell lines.

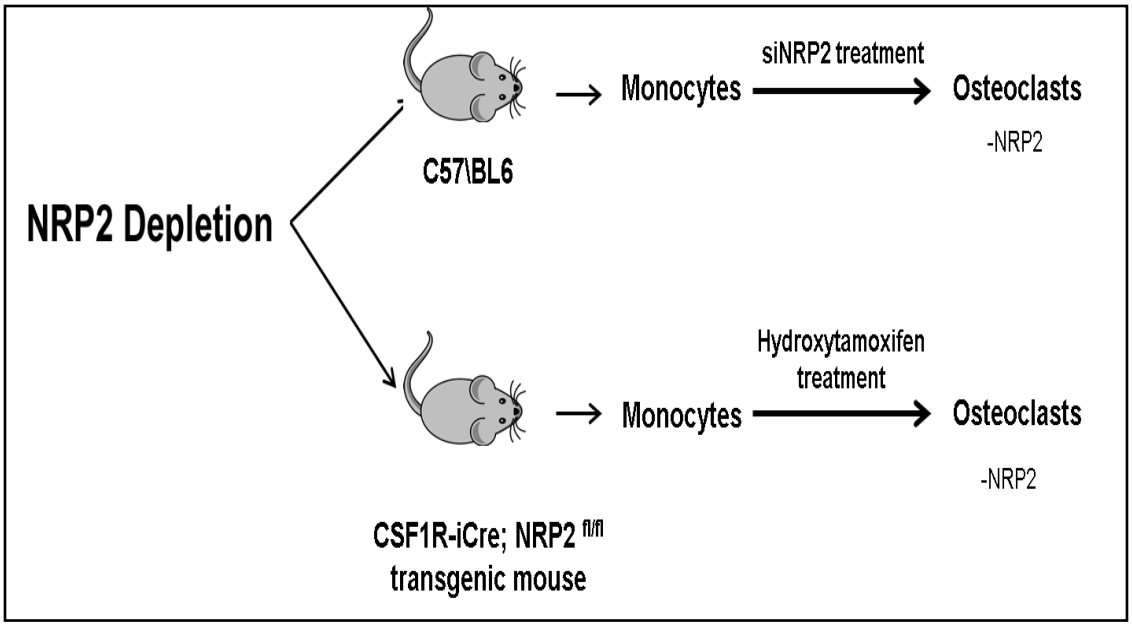


Figure **2.3:** Depletion of NRP2 using siRNA and addition of hydroxytamoxifen

Tartarate-resistant acid phosphatase staining

To confirm the presence of osteoclasts, TRAP staining was conducted. For better understanding of the differentiation pattern, osteoclasts were stained from day 4 to day 7 of differentiation. TRAP staining kit was purchased from CosmoBio (Cat.no. - PMC-AK04F-COS) and stored at 4°C. Briefly, monocytes were differentiated using standard as well as conditioned media treatment in 24- well plate. On day 4, cells were washed with PBS twice to remove any traces of media and unattached cells. The attached osteoclasts were fixed with 10% Formalin at room temperature for 10 mins. The osteoclasts were then washed three times with excess deionized water. The kit contains lyophilized vials of chromogenic substrate and Tartrate-containing buffer. For each vial of substrate, 5ml of Tartrate-containing buffer was added and mixed thoroughly. To each well of 24- well plate, 150 μl of the chromogenic substrate was added and incubated at 37°C for 1-2 Hrs. based on the time taken by the osteoclasts to stain for TRAP. After completion of 1-2 Hrs., the cells were washed with deionized water to remove the substrate and stop the reaction. 2 or more nucleated TRAP-positive cells were counted and compared between control and NRP2-depleted osteoclasts under all three conditions and a mean and standard error of mean was calculated and represented graphically. This procedure was repeated until day 7 of osteoclast differentiation. All experiments were conducted at least thrice for statistical significance.

Pit formation assay

Pit resorption is a function of activated osteoclasts. Osteoassay plates (24 – well) were bought from Corning Inc. (cat.no. - 3987). These plates are coated with in-organic bone biomimetic synthetic surface that allows the assessment of the at-tachment, differentiation, and function of osteoclasts. Monocytes derived from bone marrow of mice were plated in each well of the osteoassay plate under dif-ferent conditions to analyze the efficacy of the conditions inactivation of osteo-clasts. On day 4 along with TRAP staining, the pit formation was also assessed. To visualize the pits, 10% bleach diluted in deionized water was added for 10 mins at room temperature to remove cells from the plate. Each well was washed with deionized water and dried for 3 to 5 hrs at room temperature. Pit clusters were observed and captured using phase contrast microscope. Images obtained were analyzed for the measurement of coated surface resorbed using photoshop (magic wand tool). The percent surface area resorbed was calculated and plotted with standard deviation under different treatment conditions. Pit assays were done thrice for statistical significance.

Immunobloting

For protein analysis, osteoclasts were washed with PBS to remove any traces of media. The cells were lysed with ice cold lysis buffer containing CHAPS buffer pH 7.4 (40 mM HEPES, 0.3% CHAPS, 10 Mm - Glycerophosphate, 10 mM so-dium pyrophosphate, 2 mM EDTA) and combination of protease inhibitors, 20

µg/mL Leupeptin, 10 µg/mL Aprotinin, 1mM PMSF and Halt protease. The cells were scraped using cell scraper and ultrasonicated to lyse the cells.

For separation of nuclear and post-nuclear fraction in osteoclasts, 250 µL of buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 10 µg/mL Leupeptin, 10µg/mL Aprotinin, 3 mM DTT, 1mM PMSF and Halt protease) was added to each sample and incubated on ice for 17 mins. 20 µL of 10% NP- 40 detergent was added to each sample containing buffer A and vortexed for two mins and centrifuged at 14,000 RPM for five mins. The supernatant containing the post-nuclear proteins was separated from the pellet and labeled for protein analysis. The pellet was resuspended in 50 µL of buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 10 µg/mL Leupeptin, 10µg/mL Aprotinin, 3 mM DTT, 1mM PMSF and Halt protease) and vortexed and placed on a rotating rack for 1 hour to dissolve the pellet containing the nuclear proteins. The dissolved pellet was then centrifuged at 14000 RPM for 10 mins. The supernatant containing the nuclear proteins was separated from the pellet which contains DNA.

Total protein was estimated using Bradford reagent and calculated for the required concentration of protein for analysis by western blot. The samples were prepared by the addition of SDS sample buffer containing -mercaptoethanol and boiled at 95 ºC for five mins to denature the protein complexes. The prepared samples were then loaded on a precast 4–20% Mini-PROTEAN® TGX™ Gel (BioRad) and run alongside a protein loading marker ladder as reference. After the completion of the run, the proteins on the gel are transferred on to a PVDF

membrane (Life Technologies) and stained with Ponceau dye for confirmation of proper transfer. The membrane was then blocked in 5% non-fat dry milk in 1X TBST (1X Tris Buffered Saline, 0.1% Tween-20) for at least 30 mins following which primary antibody diluted in 1X PBS was added and incubated overnight at 4ºC with continuous shaking at low speed. On the next day, membrane was washed with 1X TBST for four times for 5min and incubated in appropriate dilution of secondary antibody conjugated with HRP for 1hr in 1X TBST with continuous shaking at low speed at room temperature. The membranes were washed in 1X TBST every 5min for at least 5-7 times, and the protein bands were detected using a combination dilution of SuperSignal™ West Femto Maximum Sensitivity Substrate and SuperSignal™ Pico Maximum Sensitivity Substrate which was captured on an X-ray film. The X-ray film was developed using a Kodak film developer, and the protein bands were identified and compared and presented as an image using ImageJ software.

RNA isolation

On day 7, osteoclasts were washed twice with PBS. Total RNA was isolated by adding 1mL of TRIzol Reagent (ThermoFisher Scientific, CA) per 1 million cells as per manufacturer's protocol and gently scraped using cell scraper and collected in 1.5 mL microcentrifuge tubes and allowed to stand for 5mins at room temperature. Each tube was then added with 300 l of chloroform and mixed well by inverting the tubes for 20 seconds and allowed to stand for 5mins at room temperature following which they were centrifuged at 12000 rpm for 15mins at 4ºC. The clear layer was extracted without disturbing the cellular layer and collected

into fresh tubes. To this, 500 µL isopropanol and 1 µL of Glycogen (RNA graded from Invitrogen) were added and mixed. The samples were allowed to stand for 5mins at room temperature. The samples were then centrifuged at 12000rpm for 15min at 4ºC. The supernatant was discarded without disturbing the pellet and 1mL of 75% ethanol was added to wash the pellet and centrifuged at 7500rpm for 5mins at 4ºC. The wash with 75% ethanol was repeated twice to ensure removal of salts and impurities. The supernatant was decanted, and the pellet was air-dried briefly to remove traces of alcohol. The pellet was resuspended in required amount of RNAase-free DNAase-free distilled water. The concentration and quality of the RNA were analyzed using Nanodrop Spectrophotometer. The RNA was either stored at -80ºC until use or processed immediately for cDNA synthesis.

cDNA was synthesized with Transcriptor First strand cDNA synthesis kit (Roche Diagnostics Corporation) as per the instructions provided by the manufacturers. 1 g RNA was used to generate cDNA.

Real-time quantitative PCR

For real-time PCR, cDNA (50ng) was used, and each reaction was performed in duplicates in 25 µl volume in a 96-well PCR plates using SYBR green detection system (Applied Biosystems Group) in an ABI 7500 Fast and Real-Time PCR (2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 94°C and 1 min at 60°C) with 200-300 nM concentration of primers. The list of the primers used in this study is listed in Table 1. The expression was calculated relative to that of control

cells and normalized with 36B4 measured under the same conditions (Applied Biosystems/Roche, Branchburg, NJ), using the 2–ΔΔCT method.

Gene	Primer sequence
36B4	F: 5’ ATGCAGCAGATCCGCATGT 3’
	R: 5’ TCATGGTGTTCTTGCCCATCA 3’
NRP2	F: 5’ GTGAAGAGTGAAGAGACTACCA 3’
	R: 5’ GCTGAAGTTTTCCCCACACT 3’
DC-STAMP	F: 5’ GTATCGGCTCATCTCCTCCA 3’
	R: 5’ ACTCCTTGGGTTCCTTGCTT 3’
Car II	F: 5’ ATTGGACCTGCCTCACAAGG 3’
	R: 5’ CCACATGAGACACCTGGGTC 3’
MMP9	F: 5’ CGTCTTCCCCTTCGTCTTCC 3’
	R: 5’ TGTCTGCCGGACTCAAAGAC 3’
ATP6V0D2	F: 5’ AGAGGGGTTGCGGTTGTTAG 3’
	R: 5’ GCCAGTGAGCAGGAAGTCAT 3’
RANK	F: 5’ CAAACCTTGGACCAACTGCAC 3’
	R: 5’ TGGTCTCCTCAGTGTCATGGAAG 3’
TRAP	F: 5’ TACTTCACTGGAGTGCACGAT 3’
	R: 5’ GAAGTTCCAGCGCTTGGAGA 3’
Cathepsin K	F: 5’ GAGGGCCAACTCAAGAAGAA 3’
	R: 5’ GCCGTGGCGTTATACATACA 3’

T**able 1:** *List of primers used in real-time PCR.*

Enzyme-linked immunosorbent assay (ELISA)

Human M-CSF and GM-CSF Quantikine ELISA kits were procured from R&D systems (cat.no. - DMC00B, DGM00 respectively) and Human RANKL from Abcam (cat.no. - ab213841) to measure the amount of these cytokines in the conditioned media obtained from PC3 and LNCaP C4-2B cell lines. The assays were conducted according to the standard protocol provided by the manufacturer. Briefly, microplates coated with monoclonal antibody specific for human M-CSF and GM-CSF were supplied with the kits. Standards and samples (Conditioned media) were added into the wells to allow the binding of the growth factors present in the conditioned media. Unbound cytokines were washed, and the antibody specific for these growth factors were added. Following this, the antibody was removed followed by washing, and substrate solution was added to the wells and incubated until color developed. The color development was stopped, and the intensity of the color was measured at 450nm. For wavelength correction, readings were obtained at 540 nm or 570 nm, and these reading were subtracted from readings at 450 to correct optical imperfections of the plate.

Cytokine profiling assay

To detect the expression levels of cytokines and chemokines in the conditioned media, proteome profiler array (human cytokine array panel A, cat.no. ARY005) from Abcam was used. According to the array procedure, the nitrocellulose

membranes containing 36 different capture antibodies was blocked with blocking buffer. In separate sets, 1mL of each conditioned medium was diluted with array buffer and antibody cocktail was added to the solution and incubated for one hr. The blocking buffer was removed from the membranes and the solution containing the conditioned media and the antibody cocktail was added and incubated on a rocking platform shaker overnight at 4°C. On the following day, the membranes were washed with washed buffer thrice and 2 mL of the solution containing streptavidin-HRP was added to the membranes and incubated at room temperature for 30 mins on a rocking platform shaker. The membranes were washed three times with wash buffer and chemiluminescent detection reagent mix were added sequentially. The light produced at each spot in the membrane is proportion to the amount of cytokine bound which was captured on an x-ray film and developed using an x-ray film developer. The positive signals obtained were identified by placing the transparency overlay template on the array image and aligning it with the pairs of reference spots on the array membranes. The identifies cytokines and chemokines were measured for their pixel density to compare the difference in the expression pattern in the conditioned media using imageJ software and represented as a graph with statistical significance.

Immunofluorescence

Osteoclasts grown in the three different conditions were stained for the detection of translocation of NF-B and NFATc1 from cytoplasm to the nucleus. The cells were grown in glass chamber slides for better attachment and convenience of

staining. Based on the requirement of the study, osteoclasts from day 2 or day 3 of differentiation were used for the immunostaining. To prepare the cells for immunofluorescence, osteoclasts were washed with 1X PBS three times and fixed with 4% buffered formaldehyde for 15 mins. The cells were washed thoroughly thrice with 1X PBS. The blocking buffer containing 0.3% TritonX-100 and 5% goat serum in 1X PBS was added for one hour to block nonspecific binding of primary antibody. The primary antibody (1:500 dilution) prepared in the blocking buffer was added to the cells and incubated overnight at 4°C. On the next day, the cells washed three times with 1X PBS containing 0.3% TritonX-100 and incubated in the secondary antibody (Alexa-546 for NF-B and Alexa-660 for NFATc1) diluted 1:1000 in 0.3% TritonX-100 and 5% goat serum in 1X PBS for one hour at 4°C in the dark. After completion of secondary antibody incubation, the cells were washed with 1X PBS containing 0.3% TritonX-100 at least four times to decrease non-specific background. The cells were finally mounted with mounting solution containing DAPI and covered with a glass coverslip and sealed. The cells were stored in 4°C in the dark and were observed using Zeiss LSM 800 with Airyscan microscope located in the UNMC confocal core facility, and data were analyzed and processed with the Zeiss Zen 2010 software. All confocal data were analyzed using Adobe Photoshop and quantified using ImageJ software and graphical illustrations made using GraphPad Prism software.

Statistical significance

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the standard two-tailed Student's t-test using PRISM-6 software (GraphPad Inc.). Statistical comparisons of more than two groups were performed using unpaired student t-test. In all cases, a $P < 0.05$ was considered as highly significant.

CHAPTER 3

NRP2 regulates osteoclastic differentiation and activation in promoting prostate cancer bone metastasis

Introduction

Prostate cancer (PCa) is one of the most frequently diagnosed cancers and is the second leading cause of cancer-related deaths in men in the United States. The incidence of PCa will likely continue to increase with the aging population. Early detection of prostate cancer in patients can lead to successful medical intervention by use of treatment strategies such as radiotherapy and prostatectomy leading to 100% survival rates[11]. However, in patients with the locally advanced stage of PCa, androgen deprivation therapy is the standard treatment strategy[12]. Although the hormonal treatment is effective in inhibiting the progression of PCa, the disease attains resistance and becomes castration-resistant PCa (CRPC) within 18 to 24 months of the therapy[7]. Patients with CRPC have a poor prognosis and a predicted survival rate of fewer than two years from the initial time of progression, comprising a significant portion of the prostate cancer-related deaths per year. Currently, CRPC is an incurable disease and represents a significant clinical hurdle.

Bone is the most common site for the CRPC to metastasize with 70-80% of the advanced stage PCa patients developing skeletal metastases. Patients with bone metastasis suffer from severe bone pain, pathologic fractures, spinal cord and nerve compression syndromes, hypercalcemia, and increased mortality. Radiographic studies of PCa characterized bone metastases promoted by PCa as osteoblastic lesions as opposed to osteolytic lesions with decreased bone mineral density[69]. However, it is lucid from the histological evidence that PCa bone metastases show a complex blend of osteoblastic and osteolytic functions with the

balance shifted to favor pronounced osteoblastic metastasis[13,68,76]. PCa bone metastases are resistant to most of the commonly available therapies. Current treatment strategy for PCa patients with metastatic bone disease includes taxane-based chemotherapy which can effectively limit the progression of the disease for a short-term but eventually relapses within the first year of treatment[68,73,80]. Till date, bone metastases remain to be a frequent and incurable complication in PCa patients and its management is clinically challenging and requires the identification of the new molecular target(s) that can be therapeutically exploited to improve patient outcome.

The occurrence of metastatic bone disease is a result of interaction between cancer cells and the bone cells namely osteoblasts, osteoclasts, and osteocytes which contribute to a vicious cycle that provides fertile soil for the metastatic growth of cancer cells in the bone. Metastatic prostate cancer cells secrete growth factors and cytokines to stimulate bone cells. Osteoblasts are activated due to the release of these growth factors and cytokines. Activated osteoblasts deposit new woven bone which results in the release of osteoblast- or bone-derived factors that stimulate cancer cells to grow and further activate osteoblasts. Therefore, bone metastasis of prostate cancer is predominantly osteoblastic, although activation of osteoclast has been detected in prostate cancer and is important for its metastasis[70,71]. Activated osteoblast in tumor microenvironment can induce osteoclast by releasing RANKL which is a crucial osteoclastic differentiation factor. Osteoclast can be directly activated as well by tumor-derived growth factors. As a result, osteoclastic precursors are recruited to the

resorption site where they differentiate and become active osteoclasts. These active osteoclasts resorb the bone thereby releasing growth factors that facilitate the growth of prostate cancer cells in the bone microenvironment. Osteoclastic markers such as TRAP, CA II, and Cathepsin K are found to be elevated in the serum of bone metastatic prostate cancer patients suggesting an overall increase in the bone remodeling process in bone metastasis induced by prostate cancer cells[69]. Further, a clinical study on bone metastasis in prostate cancer patients reported that nearly 84% of the patients with prostate cancer bone metastasis are associated with lysis and sclerosis suggesting that along with osteoblasts, osteoclasts are also involved in the process of dysregulated bone resorption and formation[68,73,81]. All these studies therefore indicate that a multifaceted interplay between prostate cancer cells and the osteoclasts is instrumental in the initial stages of development of the bone metastasis. Hence, targeting osteoclasts for therapy can contribute to the curbing of prostate cancer cells growth in the bone. However, the knowledge of the exact mechanisms through which the cancer cells affect this interplay is still to be elucidated and can be crucial for either improving the existing therapies or for the development of new effective treatment strategy.

The NRPs are type 1 transmembrane glycoproteins. It belongs to a family of non-tyrosine kinase, cell surface receptors widely functioning in multiple cellular signaling in normal physiology as well as pathological conditions. They are central in VEGF family-dependent angiogenesis and lymphangiogenesis as well as semaphorin-dependent neuronal guidance. They are known for the regulation of

VEGF- and semaphorin-mediated signaling cascade and the cross-talk between these two signaling to enable normal functioning of the fundamental physiological processes such as cardiovascular, neuronal and immune systems[84]. As described in detail in the chapter 3, there exists two members in NRP family, NRP1, and NRP2 which share a conserved domain structure with nearly 40% similarity at amino acid level. They are over-expressed in various neoplasms and correlates with stress-induced cancer survival, progression, metastasis and poor prognosis. NRP1 is well studied in bone physiology and is reported to be osteoprotective via Sema 3A in healthy bone[83].
On the contrary, NRP2 is reported to be expressed by bone cells, but the exact function and the mechanisms are unknown at large. A study by Lieve Verlinder *et al.* showed that NRP2 is expressed by bone cells and a total knockout of NRP2 in mice resulted in decreased osteoblasts and increased osteoclast number[85]. However, this study did not address whether the increase in osteoclast number is a consequence of increased differentiation caused by the deletion of NRP2 and what are the molecular signaling pathways that are regulated by the NRP2 on osteoclasts. In a pathological condition where bone homeostasis is dysfunctional such as prostate cancer-induced bone metastasis, the function of osteoclasts is vital for the metastatic propensity of prostate cancer cells in the bone. Interestingly, our previous studies on NRP2 in metastatic prostate cancer cells suggested that NRP2 promotes survival of cancers against chemotherapies[89]. Although this information is beneficial in targeting the cancer cells, the role of NRP2 in the cancer-induced bone microenvironment especially osteoclasts needs to be ad-

dressed. Having a comprehensive knowledge of the function of NRP2 in osteoclasts in prostate cancer bone metastasis can prove to be beneficial in the development of an effective treatment strategy.

In this section, we focused on understanding how the expression of NRP2 on osteoclasts affect their differentiation and activation from monocytes (precursors of osteoclasts) under physiological as well as pathological conditions, i.e., bone metastatic prostate cancer. With the use of bone-marrow-derived osteoclasts from transgenic mice developed in our laboratory where NRP2 can be depleted explicitly in the myeloid population including monocytes and osteoclasts, we found that NRP2 downregulates osteoclastogenesis and function in physiological as well as by prostate cancer cells, which promotes mixed or osteoblastic lesions. However, osteolytic prostate cancer evades the regulation of NRP2 on osteoclasts. Together, our study suggests that NRP2 negatively regulates the differentiation and functions of osteoclasts, which are bypassed by metastatic prostate cancer cells that promotes osteolysis.

Results

NRP2 is expressed by osteoclasts induced by metastatic prostate cancer.

The expression of NRP2 was investigated during the *in vitro* osteoclast differentiation, and activation under the influence of either RANKL and M-CSF (physiological condition) or treatment with conditioned medium (CM) collected from metastatic prostate cancer cell lines, LNCaP C4-2B and PC3. Being a derivative of LNCaP with its ability to metastasize to bone, LNCaP C4-2B is a prostate cancer cell line that can induce mixed bone lesions with high osteoblastic activity while maintaining a detectable osteolytic function. Therefore, LNCaP C4-2B closely enacts the physiology of metastatic prostate cancer. The other cell line, PC3 is a bone metastatic prostate cancer cell line which promotes high osteolytic lesions with less or no osteoblastic function. Standardizations were conducted to determine the effect of conditioned media from PC3 and LNCaP C4-2B on osteoclast differentiation and activation (data not shown). Under the conditions of physiological (RANKL and M-CSF) and pathological influence (conditioned medium from PC3 and LNCaP C4-2B simultaneously), we tested whether NRP2 is expressed in osteoclasts differentiated from its precursors obtained from the bone marrow of C57BL6 mice's forelimbs and hindlimbs. We found that osteoclasts expressed NRP2 in all the three conditions. This observation became apparent when we performed a time course study (0-6 days) in the differentiating osteoclasts in all the three conditions and found that NRP2 expression increases with time both at transcriptional and protein levels (Fig.3.1, 3.2). In fact, the expression of NRP2 in RANKL and M-CSF differentiated osteoclasts is higher than its expression in the

osteoclasts induced by the prostate cancer CM (Fig.3.3). Furthermore, the mouse osteoclastic precursors did not express NRP2 at protein and mRNA levels.

To confirm the presence and function of multinucleated osteoclasts, TRAP staining and activity assay were conducted. We observed that with time an increase in TRAP activity along with the formation of giant multinucleated TRAP-positive osteoclasts upon treatment with RANKL and M-CSF became evident (Fig.3.4 A). In comparison to the physiological condition, we found small multinucleated (3 or 4 nuclei) osteoclasts in PC3 which is more osteolytic in nature (Fig.3.6 A). In the presence of LNCaP C4-2B CM which shows low osteolysis function, differentiation of osteoclasts was limited to 1 or 2 nucleated cells with rare 3-nucleated osteoclasts in the later days of differentiation (Fig.3.5 A). The TRAP activity of osteoclasts in the CM of PC3 and LNCaP C4-2B was comparable but higher than the untreated osteoclast precursors (Fig.3.7 B).

The central role of osteoclasts is to degrade bone and release the growth factors that are essential for the differentiation of osteoblasts in healthy bone as well aid cancer cells in their survival and progression in the bone. The activation of osteoclasts is confirmed by their ability to resorb bone or bone mimetic coated on the surface of tissue culture plate and form pits. The resorptive function of osteoclasts increases with time as they fuse and increase their diameter thereby covering more bone surface area resulting in bigger pits. To evaluate the activation of osteoclasts, we performed pit resorption assay by plating osteoclastic precursors on the osteoassay plate which has bone mimetic layered on its surface. On

this surface, the precursors attach and differentiate into osteoclasts and actively resorb the bone mimetic forming pits under physiological as well as pathological conditions. In an *in vitro* setup, the activation of osteoclasts starts mostly at day 4 of osteoclastogenesis leading to pit resorption. We found that at day 4 of differentiation, osteoclasts activated by the function of RANKL and M-CSF formed moderate pits, but the size of the pits increases gradually with time. By day 6 of differentiation where the osteoclasts are highly active, nearly 30% of the surface area of the plate was resorbed (Fig.3.4 B). In the case of osteoclast differentiation induced by PC3 CM, we observed small pits scattered all over the surface of the osteoassay plate (Fig.3.6 B). This observation suggests that osteolytic PC3 differentiates osteoclasts but allow a moderate level of osteoclast fusion which is sufficient to make the bone porous rather than resorb bone. On the contrary to the PC3-induced osteolysis, conditioned media from LNCaP C4-2B induced differentiation of osteoclasts but the osteoclasts formed are either not active or have a very low level of resorption capability (Fig.3.5 B). Comparing the function of osteoclasts differentiated by physiological with the prostate cancer-induced condition, the resorbed area is higher in physiological condition followed by moderate resorption in the PC3 and no or less resorption in osteoclasts activated by LNCaP C4-2B CM (Fig.3.5 B, Fig.3.6 B).

Osteoclast differentiation results in the expression of enzymes and proteins that aid in their resorptive function. To understand whether the osteoclastic markers are expressed by the osteoclasts, we evaluated the osteoclastic markers such as RANK, TRAP and Cathepsin K at mRNA level in all the three conditions. Our re-

al-time PCR data suggests that in comparison to the untreated osteoclastic precursors, all the osteoclastic markers were upregulated in the conditions of RANKL and M-CSF (Fig.3.7 A). We observed a 20-fold increase in RANK, 900-fold in TRAP and 6000-fold in Cathepsin K mRNA expression in the osteoclasts treated with RANKL and M-CSF (Fig.3.7 A). With a high expression of these markers, osteoclasts in RANKL and M-CSF condition can actively resorb bone. In the PC3 CM-treated osteoclasts, the expression of some of these markers increased. Specifically, the expression of TRAP and Cathepsin K were increased by 150-fold and 180-fold respectively. However, the expression of RANK increased only by 3-fold in the osteoclasts induced by PC3 CM. Interestingly, we observed a two-fold, 40-fold and 90-fold increase in RANK, TRAP and Cathepsin K respectively in LNCaP C4-2B CM-induced osteoclasts (Fig.3.7 A). Together, our data suggest that NRP2 is expressed by osteoclasts induced either by RANKL and M-CSF or CM from PC3 and LNCaP C4-2B.

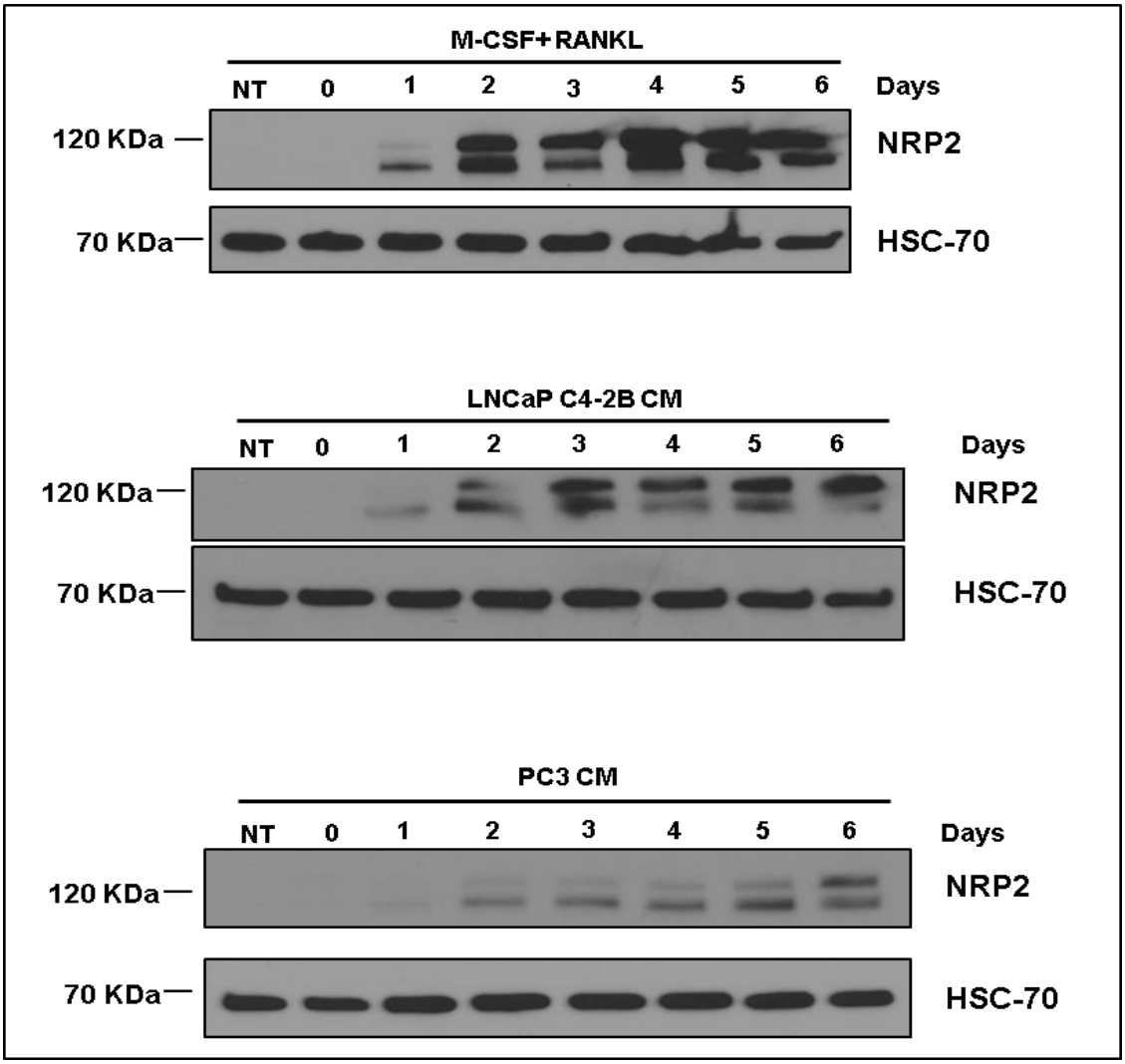


Figure **3.1**: Time course of NRP2 expression at protein level in osteoclasts induced by RANKL and M-CSF, LNCaP C4-2B CM and PC3 CM

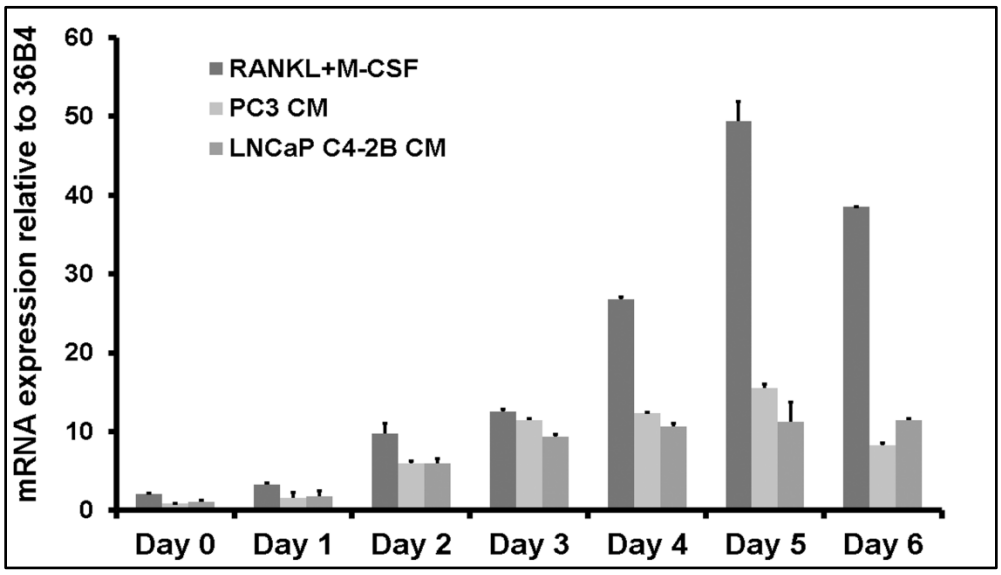


Figure **3.2**: Time course of NRP2 expression at mRNA level in osteoclasts induced by RANKL and M-CSF, LNCaP C4-2B CM and PC3 CM

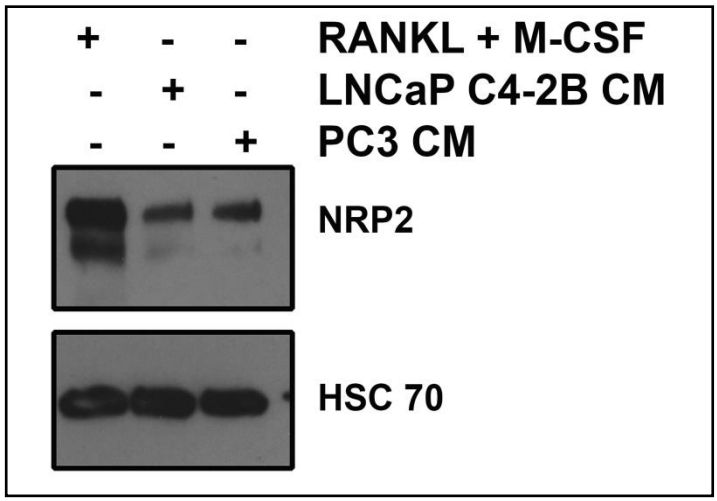


Figure **3.3**: Comparison of NRP2 expression in osteoclasts induced by RANKL and M-CSF, LNCaP C4-2B CM and PC3 CM at day 3 of osteoclast differentiation.

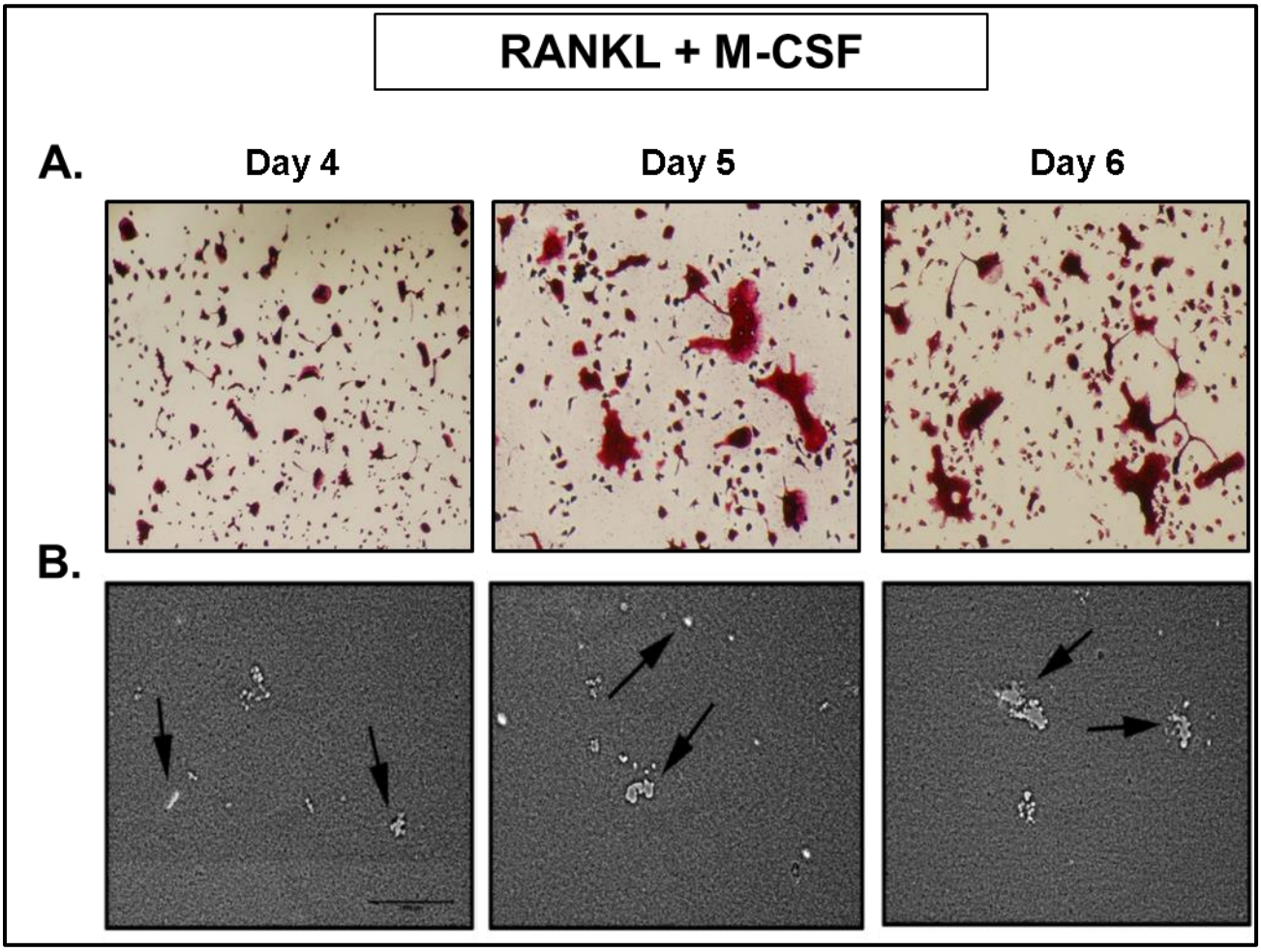


Figure **3.4**: Osteoclast differentiation and function in RANKL+M-CSF at different days **A.** TRAP staining **B.** Pit resorption.

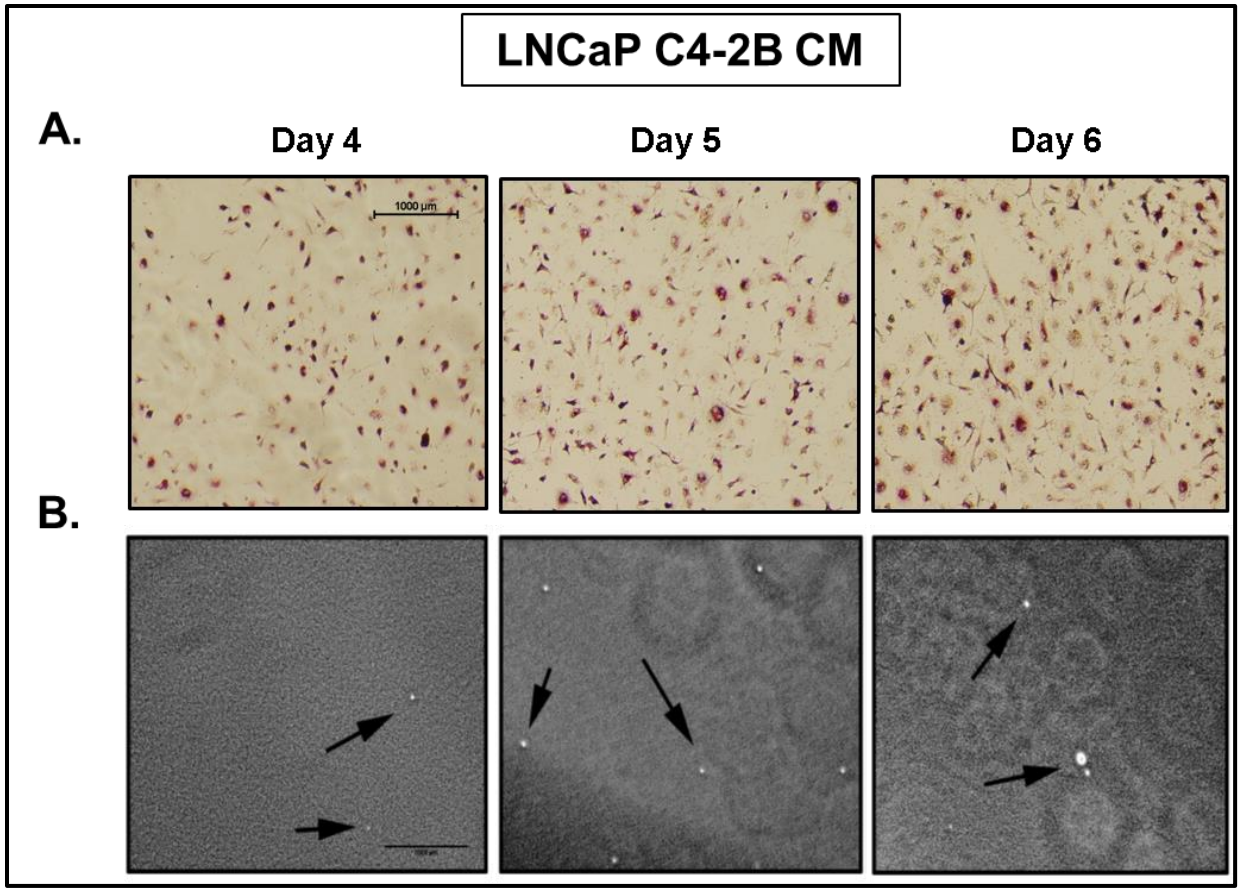


Figure **3.5**: Osteoclast differentiation and function in LNCaP C4-2B CM at different days **A.** TRAP staining **B.** Pit resorption

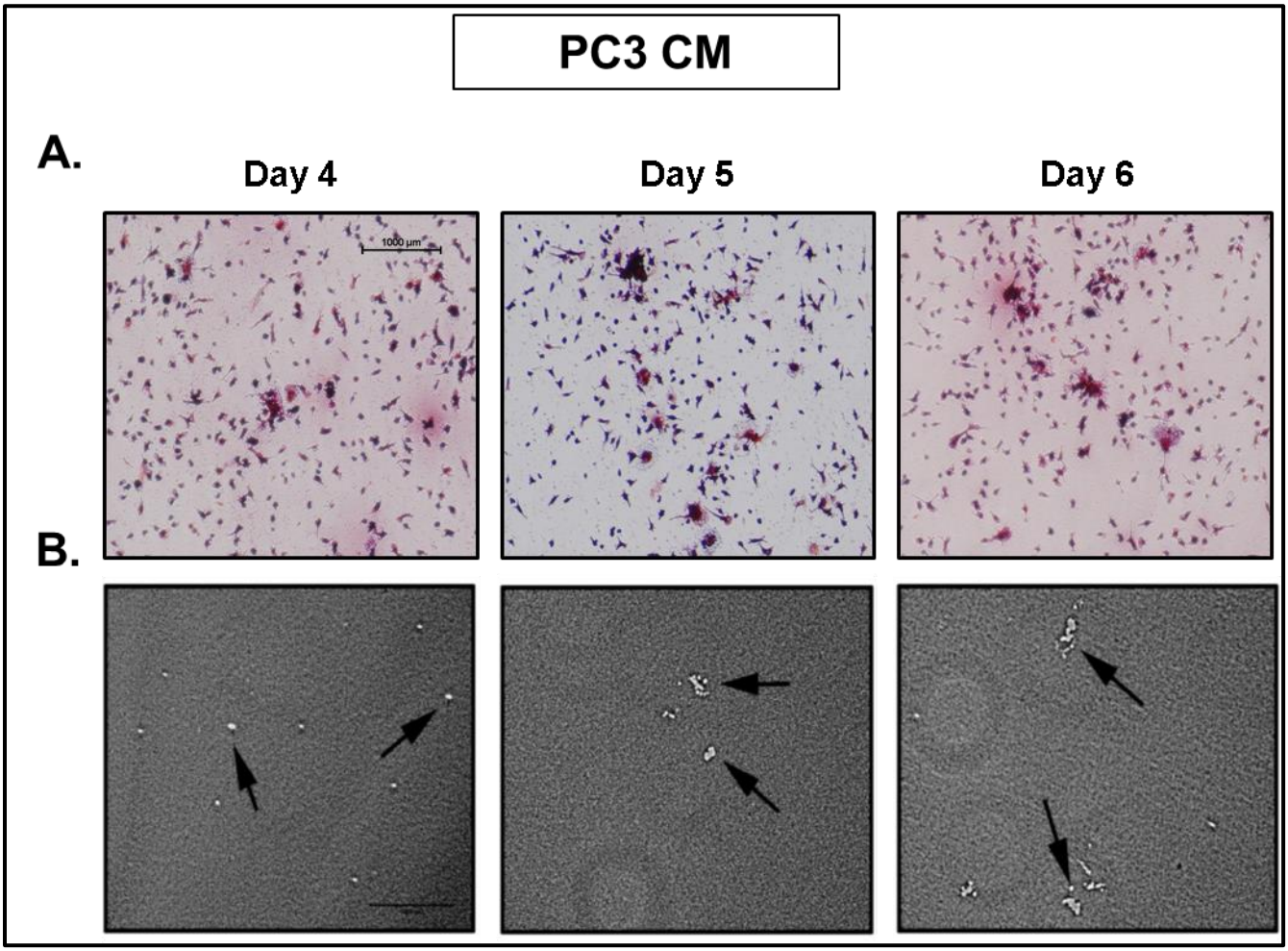


Figure **3.6**: Osteoclast differentiation and function in PC3 CM at different days **A.** TRAP staining **B.** Pit resorption

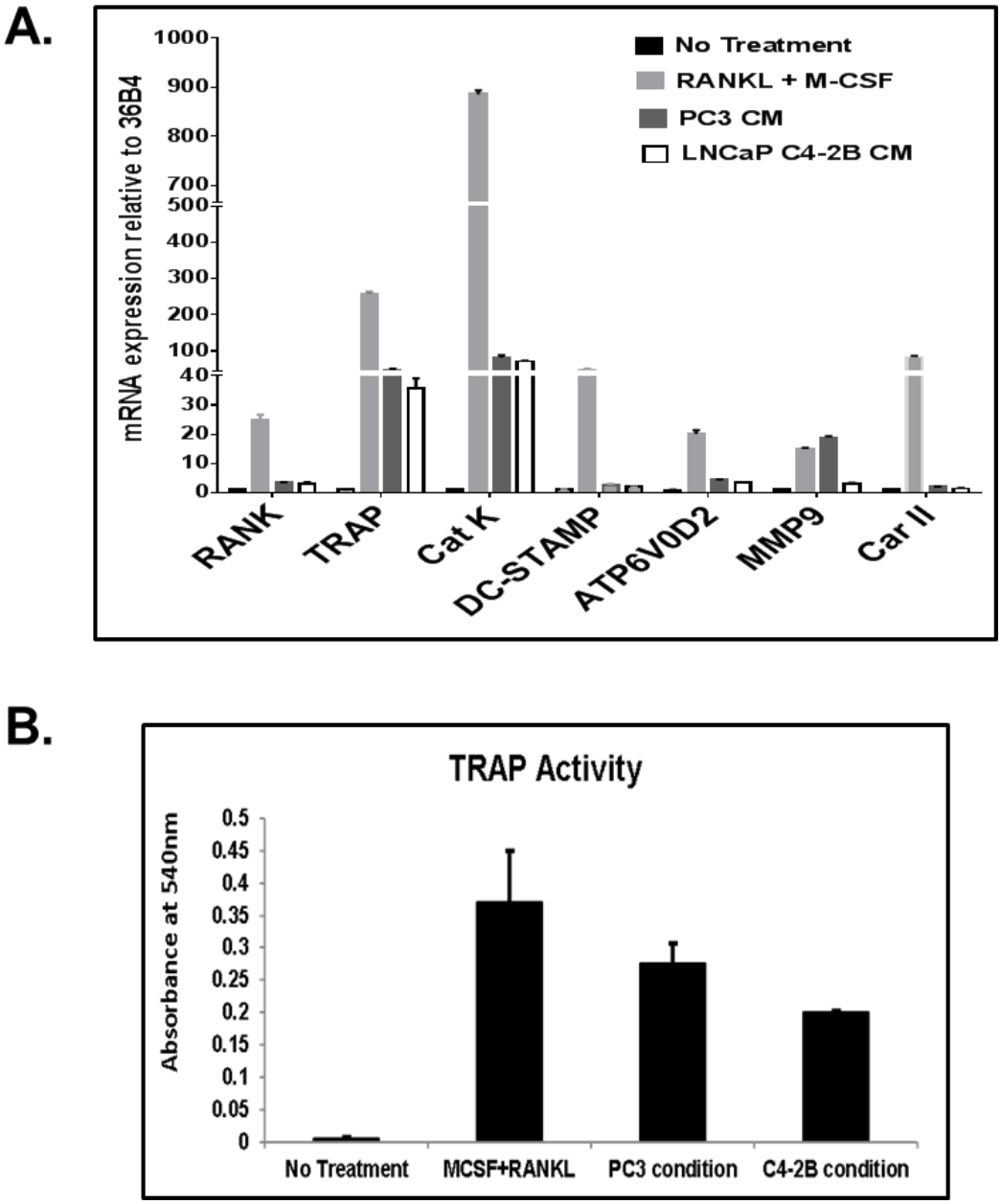


Figure **3.7**: Osteoclast differentiation and function in RANLK+M-CSF, PC3 CM and LNCaP C4-2B CM **A.** mRNA expression of osteoclastic genes. RANK, TRAP & Cathepsin K **B.** TRAP enzymatic activity measured at 540 nm.

Depletion of NRP2 in osteoclast precursor cells upregulates osteoclast differentiation and function.

To understand the significance of NRP2 in osteoclasts, we depleted NRP2 in osteoclast precursors and induced these cells to differentiate into osteoclasts in physiological and metastatic prostate cancer conditions. Depletion of NRP2 in osteoclast precursors was conducted either by transfection with siRNA against NRP2 or knockout NRP2 from osteoclast precursors isolated from a transgenic mouse model generated in our lab, CSF1R-cre; NRP2 Flox/Flox where NRP2 is depleted explicitly in osteoclast precursors by the addition of 4-hydroxytamoxifen. Using these methods, ~80% and 90% depletion of NRP2 was achieved in siRNA and hydroxytamoxifen addition respectively (Fig. 3.8 B, Fig 3.10 B). Depletion of NRP2 and treatment with RANKL and M-CSF in the osteoclastic precursors resulted in a drastic increase in osteoclast differentiation and activation (Fig. 3.8 A, Fig 3.10 A). We found extremely large multinucleated structures in NRP2-depleted osteoclasts in the TRAP staining and increased TRAP activity (Fig. 3.8 C, Fig 3.10 C). The number of osteoclasts per well of a 24-well plate increased by 2-fold in NRP-2 depleted condition than in control (Fig. 3.11). On the day 4 of differentiation, osteoclasts in NRP2-reduced state started to fuse with each other and form a polykaryon. By day 5, these multinucleated osteoclasts started resorbing the surface of the osteoassay plate actively. We observed a significant increase in the area of the resorbed pit in NRP2- depleted osteoclasts in comparison to the control in the pit assay (Fig. 3.13). By day 6, we found that nearly 75% of the surface of the osteoassay plate was resorbed due to deletion of NRP2. As

a result of hyperactivation of osteoclasts in NRP2 nullified condition, the pits formed are more prominent in size when compared to the control osteoclasts treated with RANKL and M-CSF (Fig. 3.13). A striking difference was also observed in the expression of osteoclastic markers. In comparison to the normal osteoclasts, the NRP2 depleted osteoclasts showed a significant increase in the expression of osteoclast markers. RANK expression increased by 10 fold in NRP2 knocked out osteoclasts compared to the NRP2 expressing cells. The expression of TRAP increased 5 fold while Cathepsin K showed a 2.5-fold increase in expression due to NRP2 depletion. Other osteoclast markers such as DC-STAMP, ATP6V0D2, carbonic anhydrase II (Car II), matrix, ATP6i and metalloprotease 9 (MMP9) also showed elevated expression profiles in the absence of NRP2 in osteoclasts. (Fig. 3.9, Fig. 3.12)

Similar to RANKL and MCSF treatment, the depletion of NRP2 in osteoclasts induced by LNCaP C4-2B CM resulted in an escalation of osteoclast differentiation and function. TRAP staining showed an increase in the fusion of osteoclasts even by day 4 leading to the formation of giant, multinucleated cells (Fig. 3.14 A, Fig. 3.16 A). Also, the number of osteoclasts that are attached to the plate is higher in NRP2 devoid osteoclasts (Fig. 3.17). However, when we compared the NRP2 depleted osteoclasts induced by RANKL and M-CSF and LNCaP C4-2B CM, the rate of osteoclast differentiation is much higher in RANKL and M-CSF followed by LNCaP C4-2B CM. The increase in the osteoclastogenesis as a result of NRP2 depletion caused an intensification of the osteoclastic resorption. In contrast to the NRP2 expressing osteoclasts induced by LNCaP C4-2B CM, the

NRP2 deleted osteoclasts showed increased resorptive function. Nearly 45% increase in pit resorption was observed as a consequence of NRP2 ablation by day 6 (Fig. 3.19). Further, mRNA expression analysis of NRP2 expressing as well as depleted osteoclasts in the LNCaP C4-2B CM showed an exponential increase in the expression of osteoclast-associated genes in NRP2 deprived condition compared to the control. Increase in TRAP (25-fold), Cathepsin K (80-fold), DC-STAMP (1000-fold), ATP6V0D2 (21-fold), Car II (33-fold), ATP6i (2-fold) and MMP9 (11-fold) were observed as an effect of depletion of NRP2 in osteoclasts differentiated by LNCaP C4-2B CM (Fig. 3.15, Fig. 3.18). An interesting observation was made when comparing TRAP staining of NRP2 depleted osteoclasts in LNCaP C4-2B CM in siRNA and addition of Hydroxytamoxifen. Although we observed an increase in the number of osteoclasts attached to the plate in both treatments compared to their respective controls, we observed less fusion in transient knockdown of NRP2 in comparison to prominent fusion in NRP2 knockout in LNCaP C4-2B CM (Fig. 3.14, Fig. 3.16).

Interestingly, in PC3 CM-induced osteoclasts, depletion of NRP2 did not show any changes in the osteolytic differentiation and activation. No apparent difference was observed in the TRAP staining after NRP2 reduction compared to the control osteoclasts induced by PC3 CM (Fig. 3.20, Fig. 3.22). There seems to be a slight increase in the number of osteoclasts attached to the plate after NRP2 deletion in PC3 CM (Fig. 3.23). However, the resorption function of the NRP2 downregulated osteoclasts in PC3 CM did not differ from their control counterparts (Fig. 3.25). In case of the osteoclastic markers, deletion of NRP2 caused

either no change or decrease in the expression of osteoclastic genes. We found no difference in the expression of RANK, TRAP, Cathepsin K and Car II while nearly 50% reduced expression was observed in MMP9, DC-STAMP, ATP6V0D2, and ATP6i in NRP2 depleted osteoclasts in PC3 CM (Fig. 3.21, Fig. 3.24). Together, the data suggest that NRP2 removal in osteoclasts induced by osteolytic PC3 CM did not show any evidence of increased or reduced osteoclastogenesis.

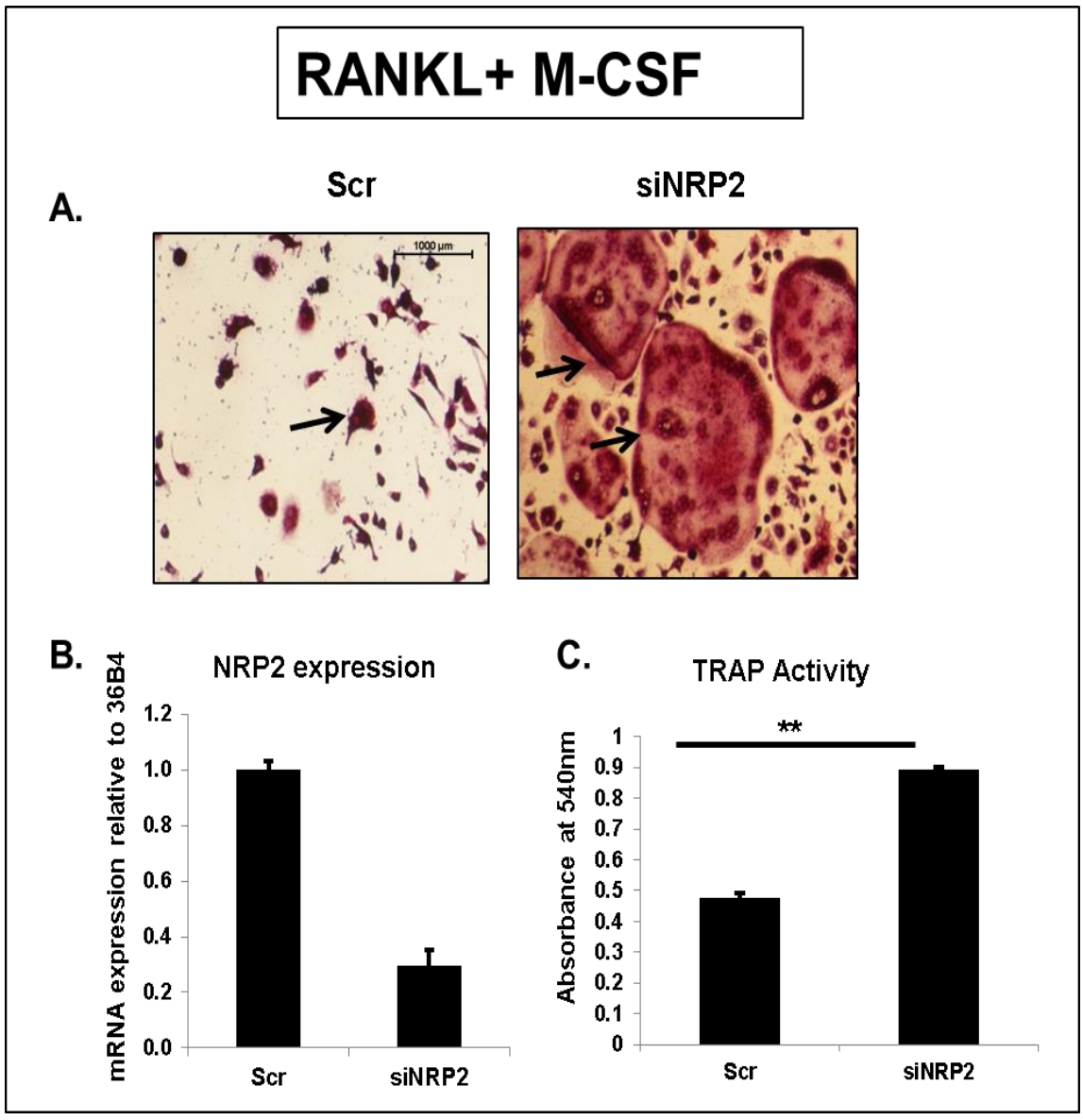


Figure **3.8**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by RANKL and M-CSF. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

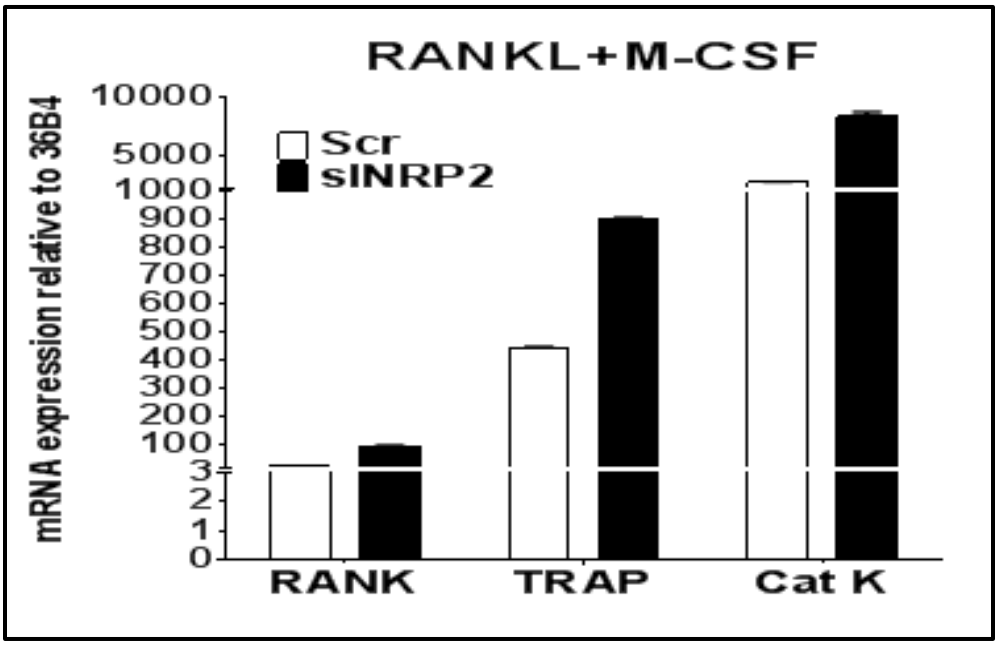


Figure **3.9**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by RANKL and M-CSF. Comparison of osteoclastic gene expression in scrambled and NRP2 knockdown osteoclasts

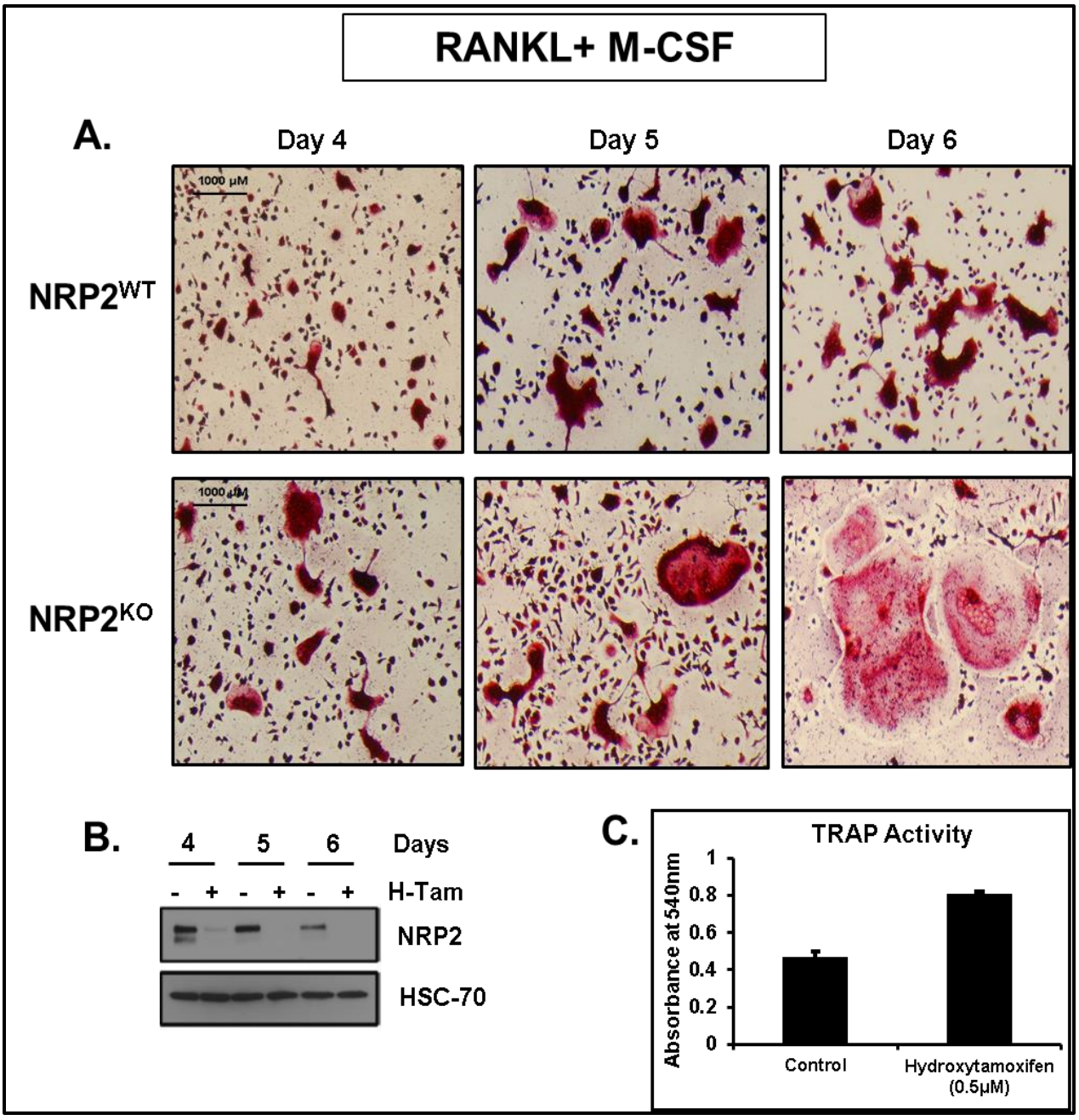


Figure **3.10**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by RANKL and M-CSF. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

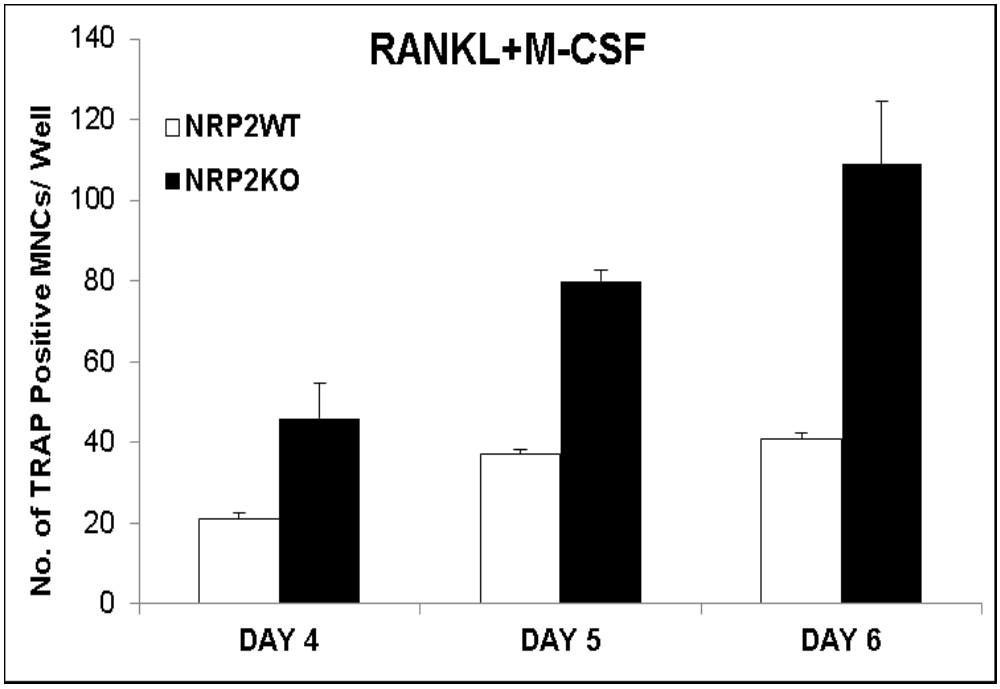


Figure **3.11**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by RANKL and M-CSF. Graphical representation of number of multinucleated osteoclasts per well of a 24-well plate.

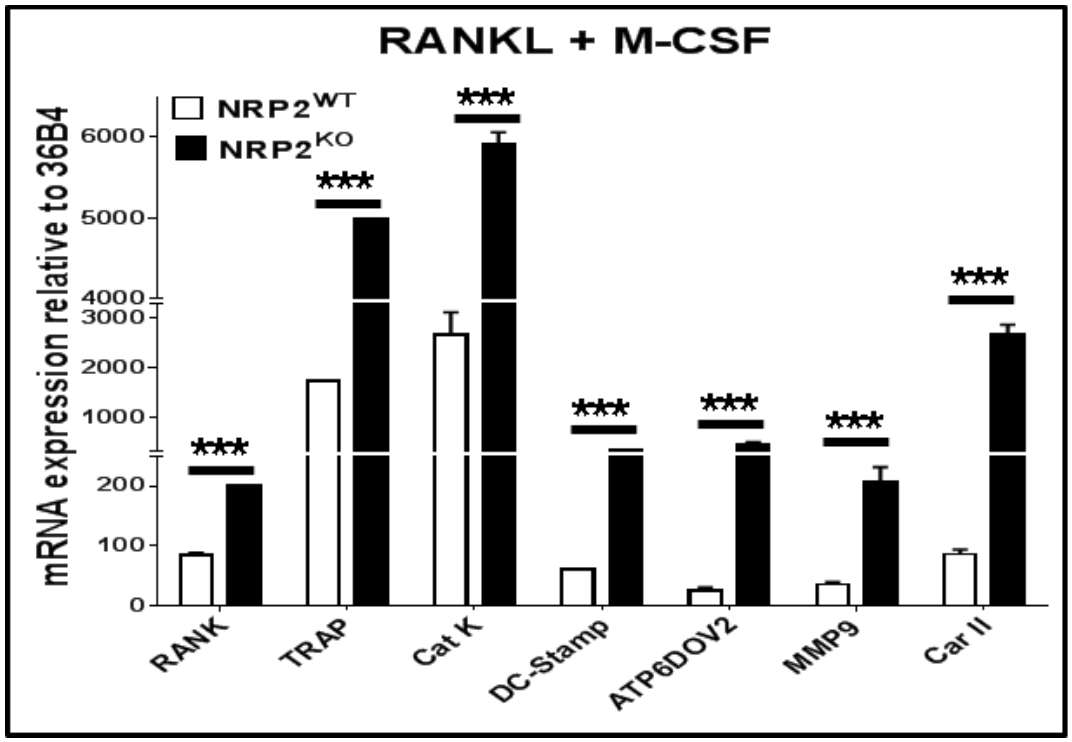


Figure **3.12**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by RANKL and M-CSF. Comparison of osteoclastic gene expression in control and NRP2 knockout osteoclasts.

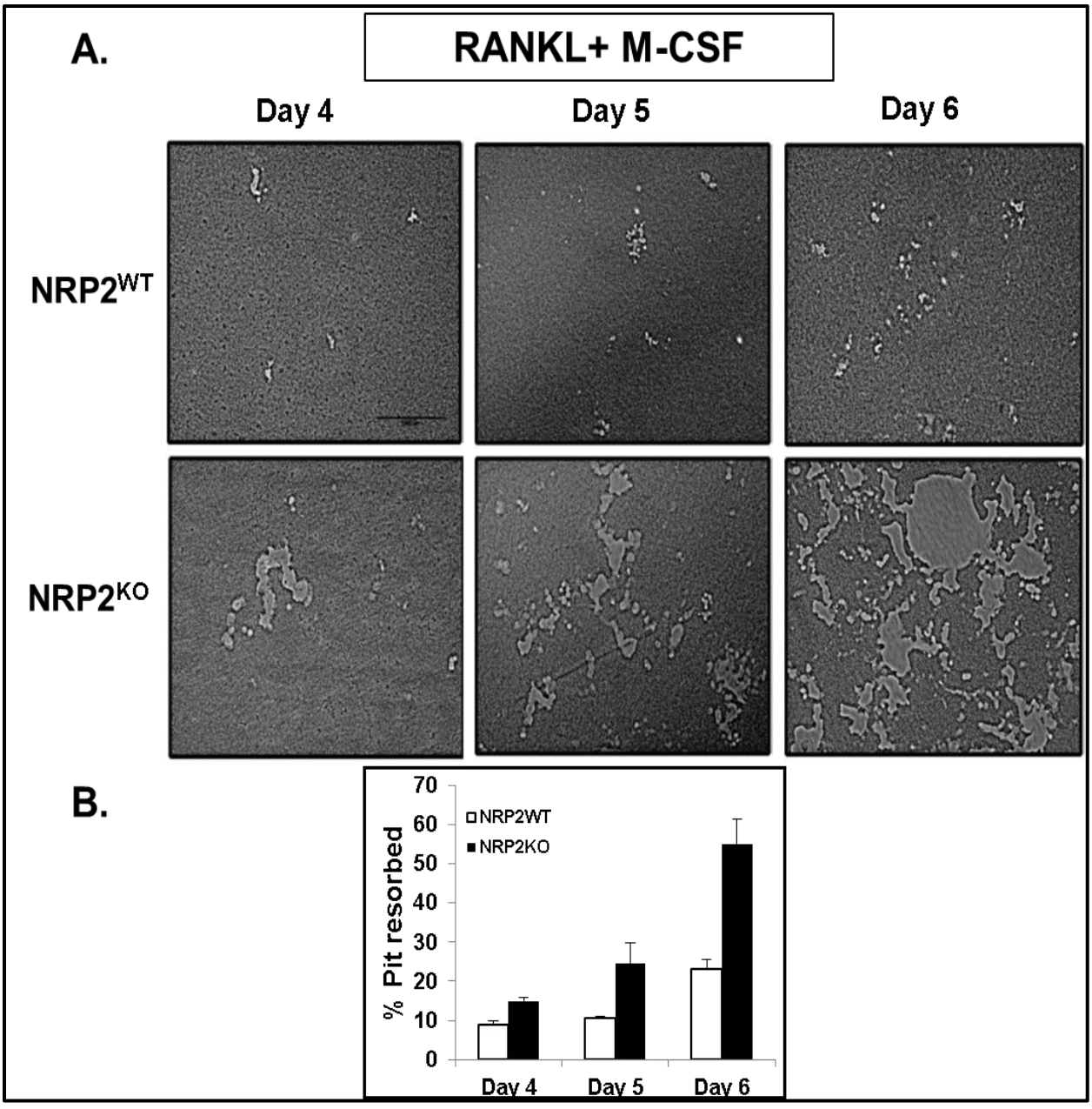


Figure **3.13**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by RANKL and M-CSF. **A.** Pit resorption on osteoassay plate. **B.** Comparison of Pit resorption function in control and NRP2 knockout osteoclasts.

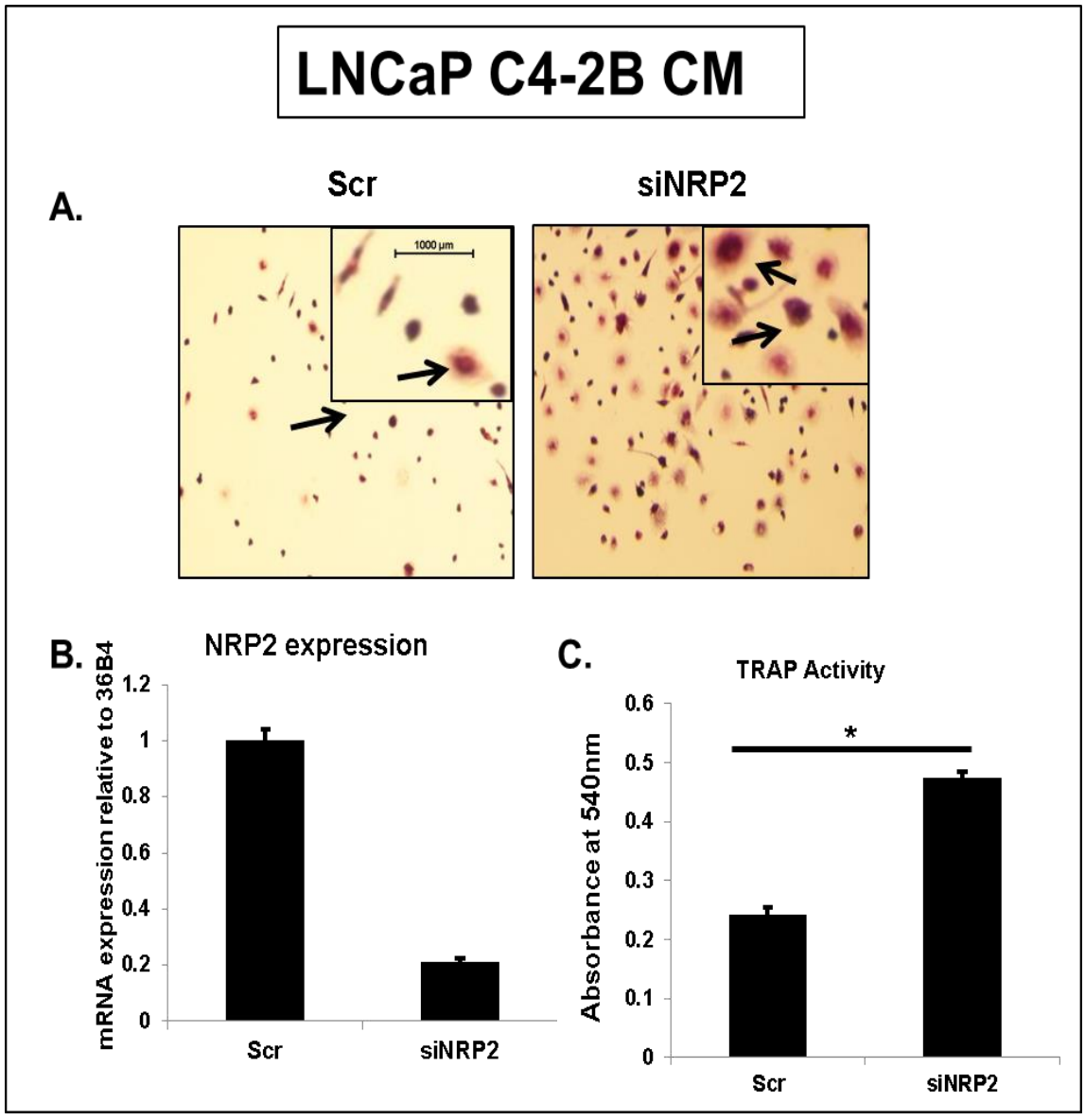


Figure **3.14**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by LNCaP C4-2B CM. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

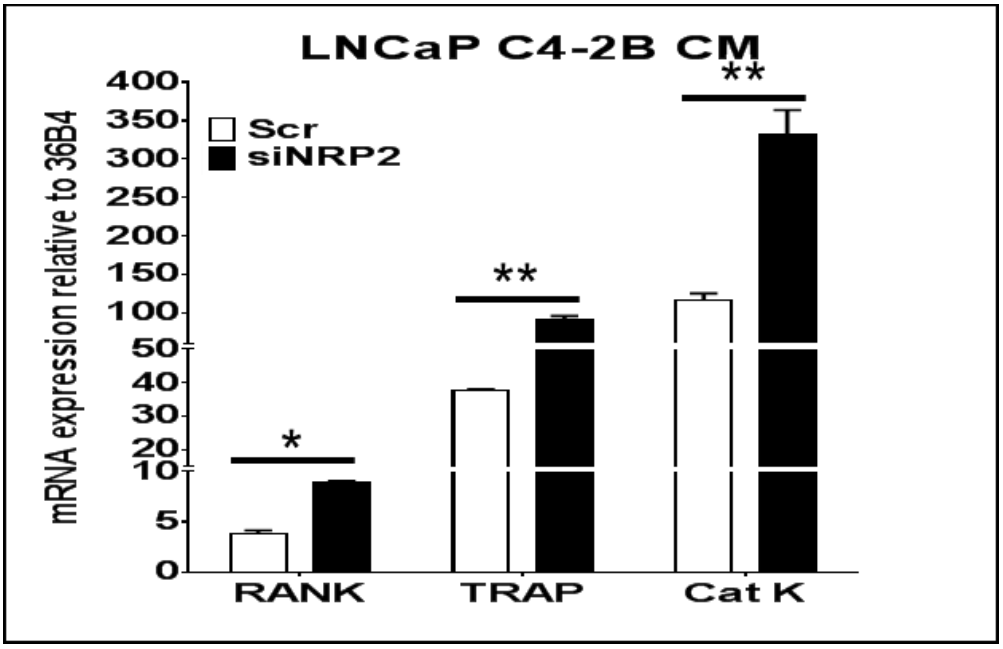


Figure **3.15**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by LNCaP C4-2B CM. Comparison of osteoclastic gene expression in scrambled and NRP2 knockdown osteoclasts

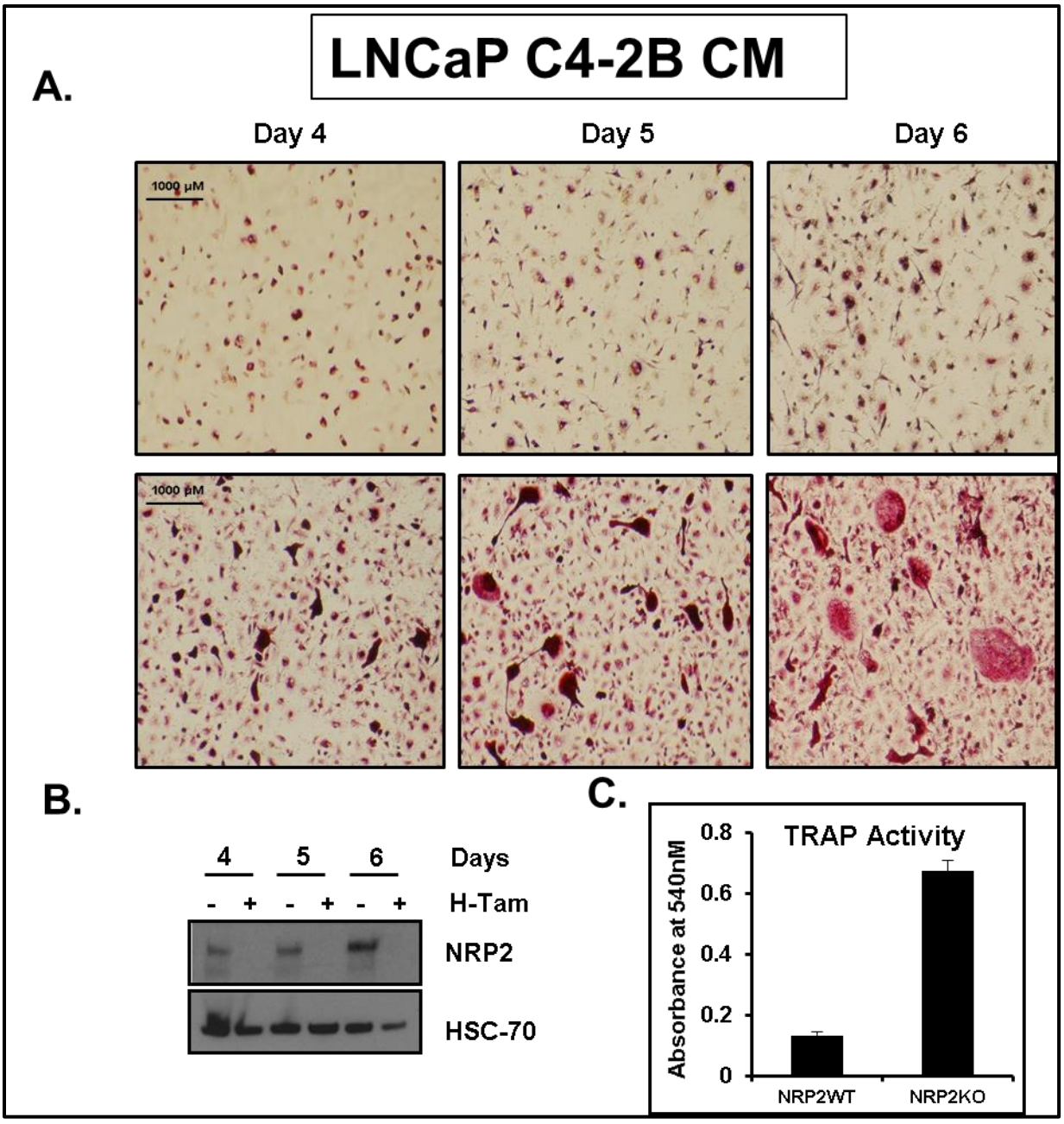


Figure **3.16**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by LNCaP C4-2B CM. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

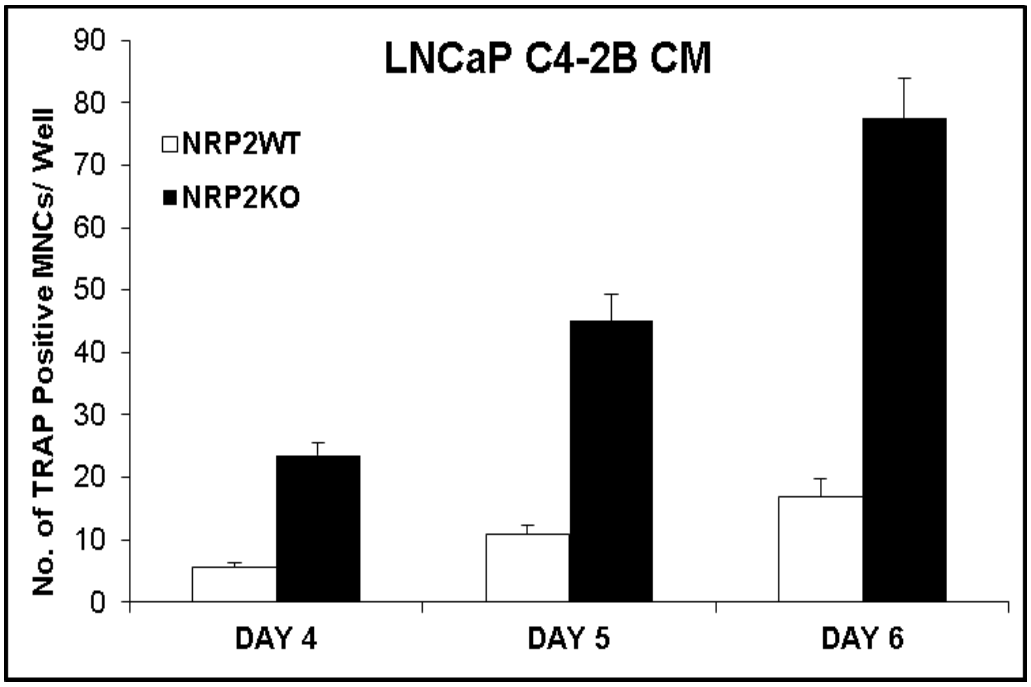


Figure **3.17**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by LNCaP C4-2B CM. Graphical representation of number of multinucleated osteoclasts per well of a 24-well plate.

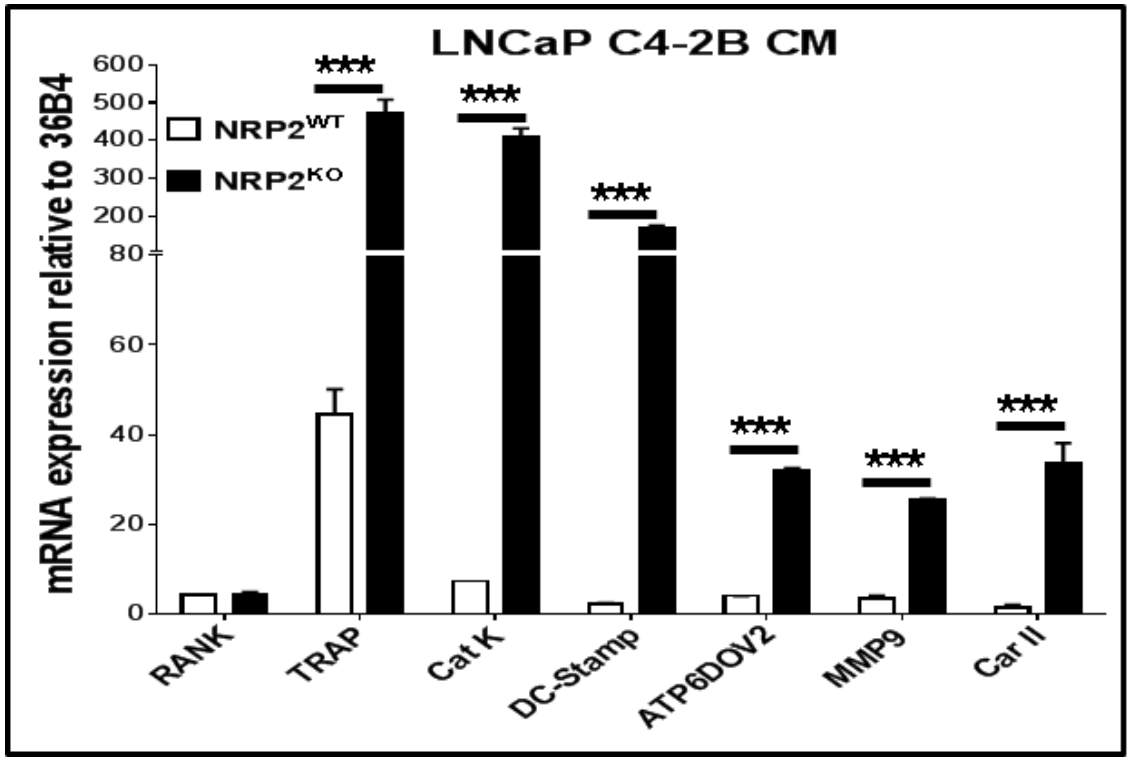


Figure **3.18**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by LNCaP C4-2B CM. Comparison of osteoclastic gene expression in control and NRP2 knockout osteoclasts

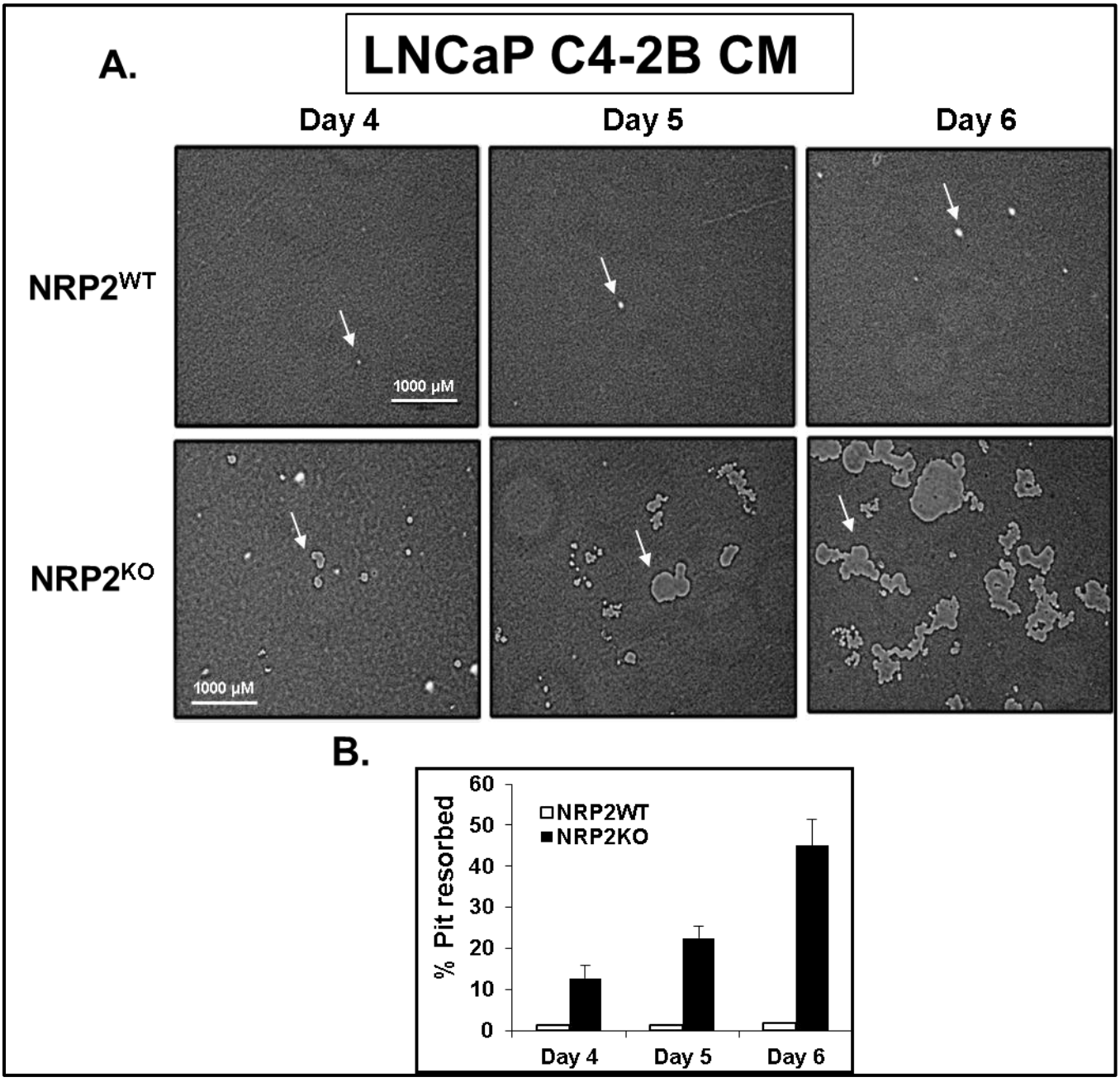


Figure **3.19**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by LNCaP C4-2B CM. **A.** Pit resorption on osteoassay plate **B.** Comparison of Pit resorption function in control and NRP2 knockout osteoclasts.

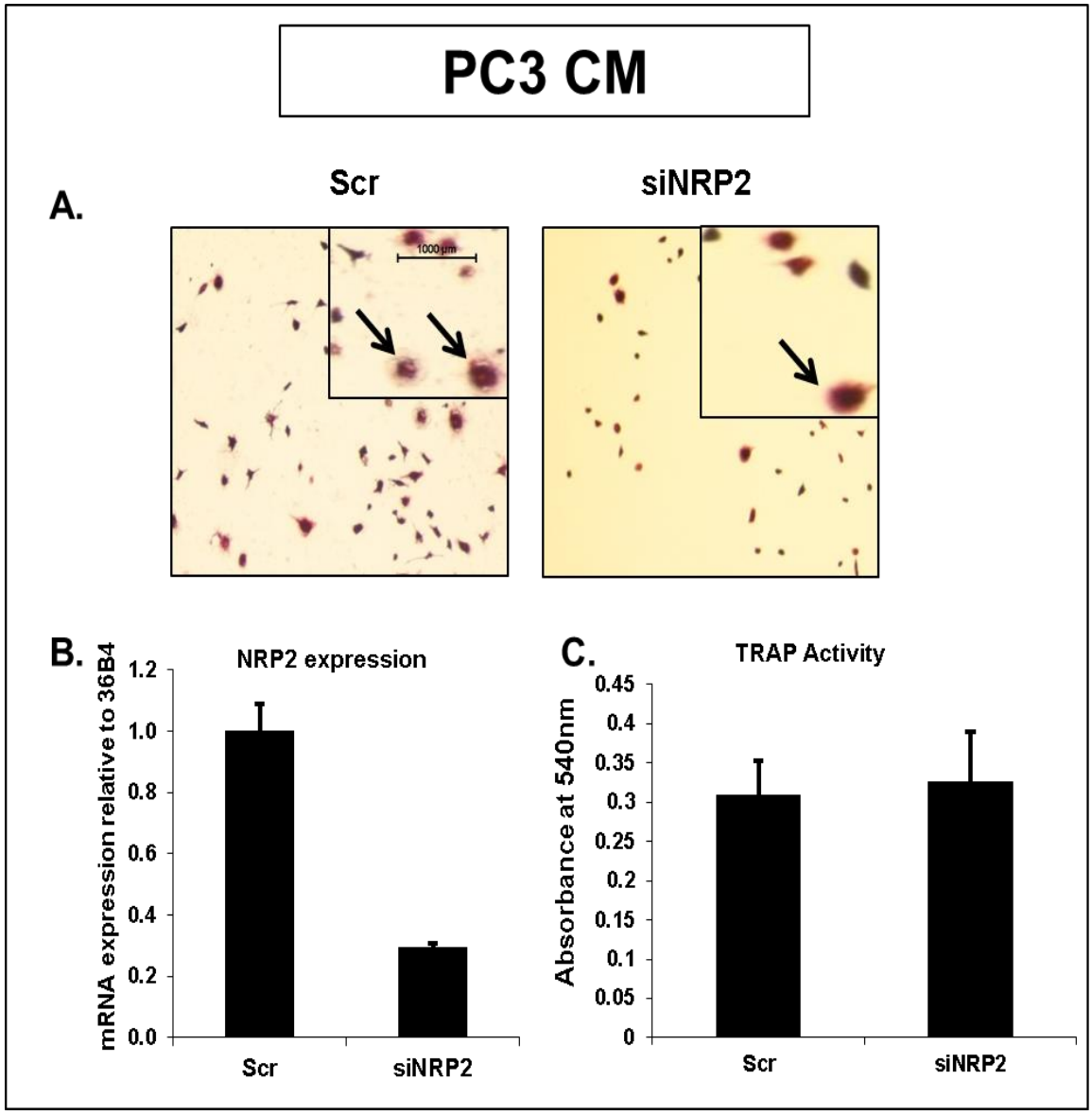


Figure **3.20**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by PC3 CM. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

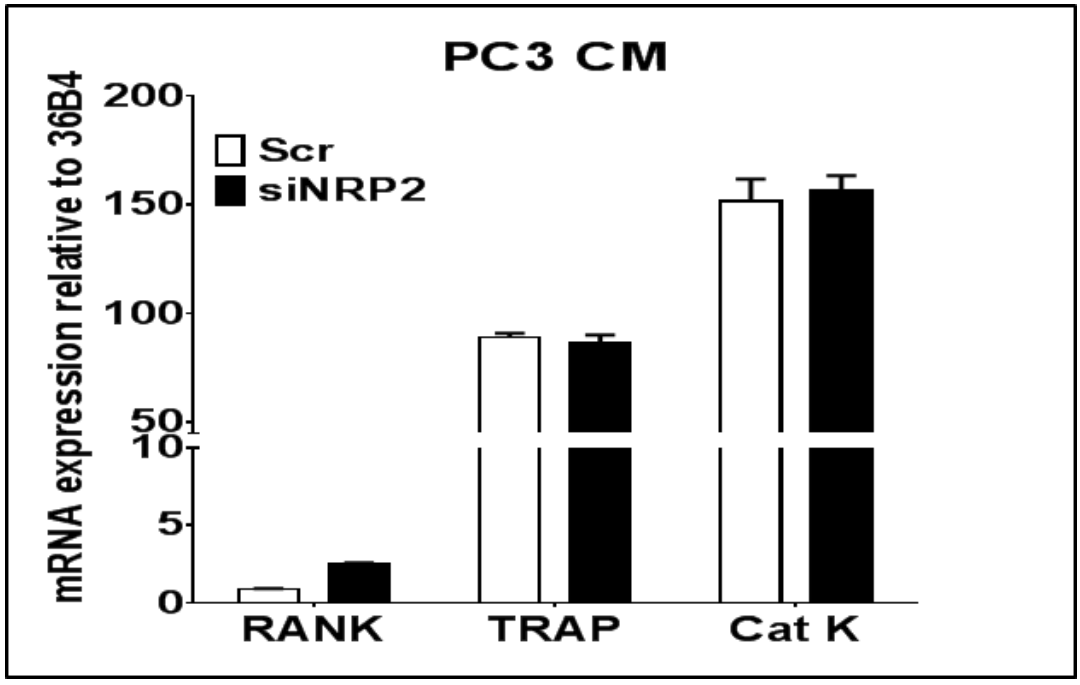


Figure **3.21**: Effect of depletion of NRP2 in osteoclast precursors by siRNA against NRP2 and induced by PC3 CM. Comparison of osteoclastic gene expression in scrambled and NRP2 knockdown osteoclasts.

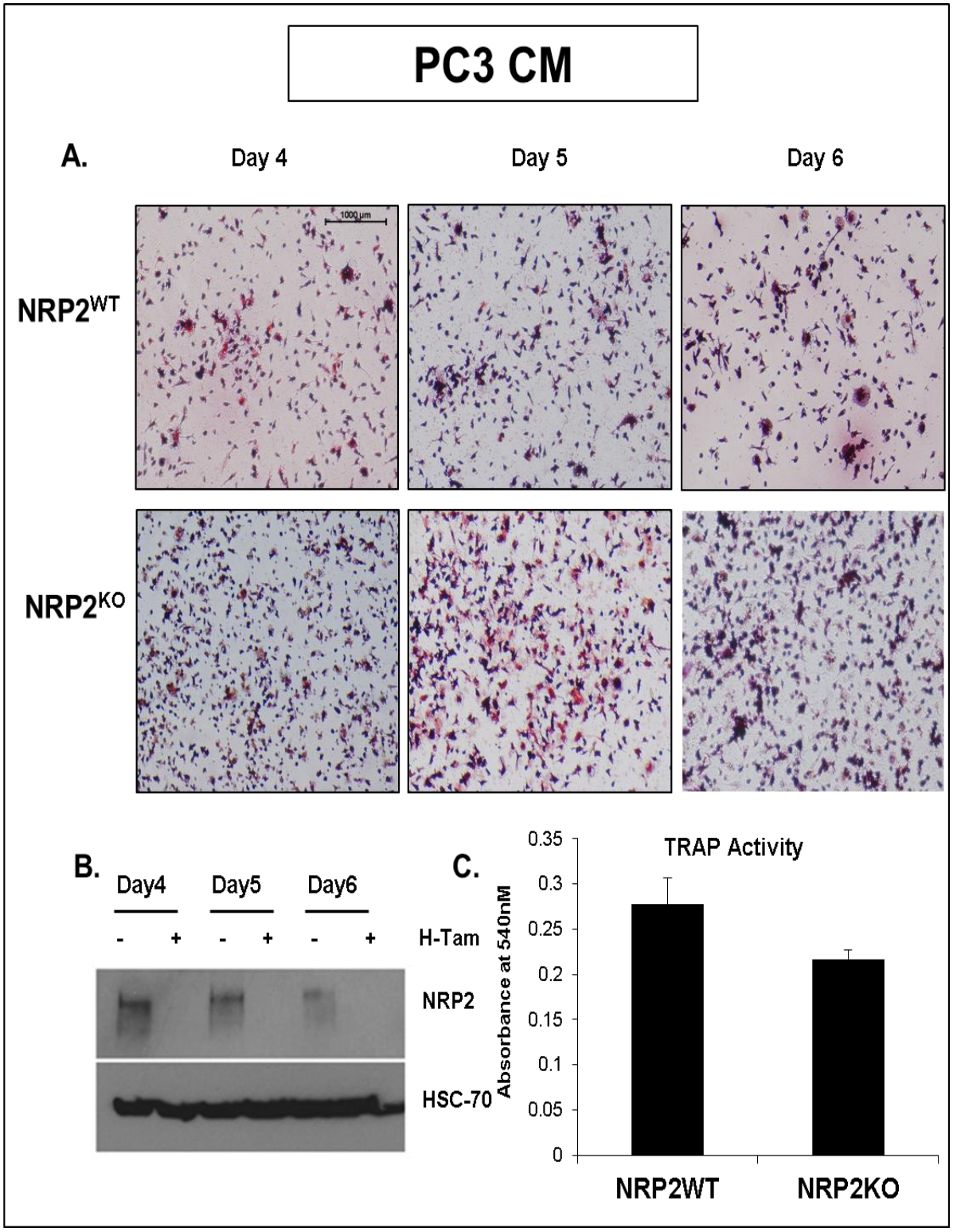


Figure **3.22**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, $NRP2^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by PC3 CM. **A.** TRAP staining. **B.** Efficiency of NRP2 depletion in osteoclastic precursors **C.** TRAP activity.

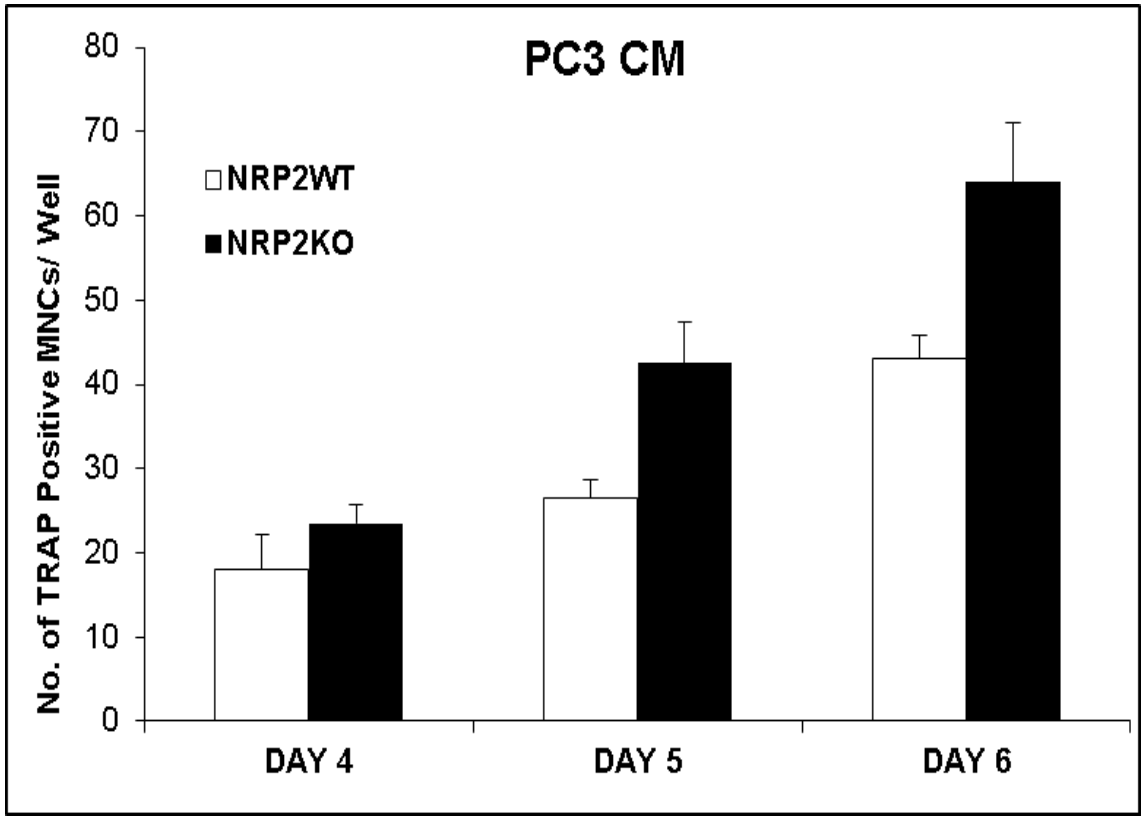


Figure **3.23**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by PC3 CM. Graphical representation of number of multinucleated osteoclasts per well of a 24-well plate.

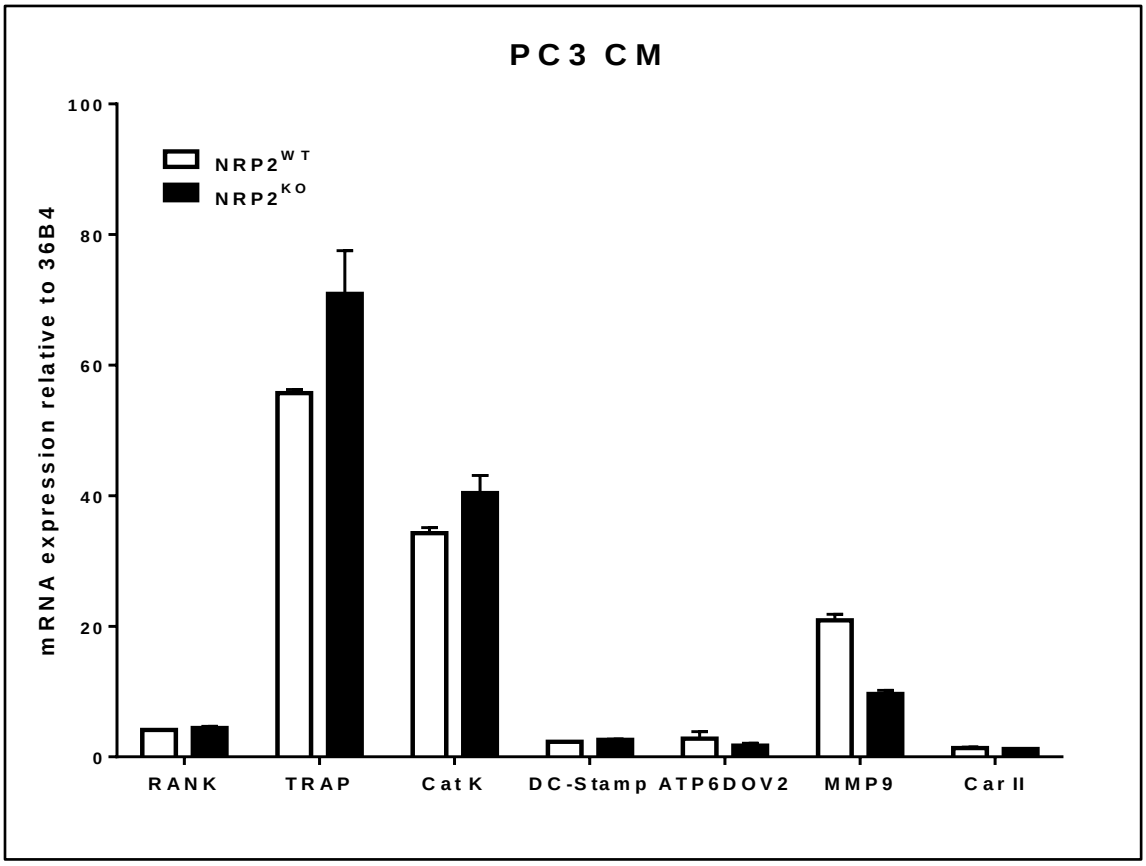


Figure **3.24**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by PC3 CM. Comparison of osteoclastic gene expression in control and NRP2 knockout osteoclasts

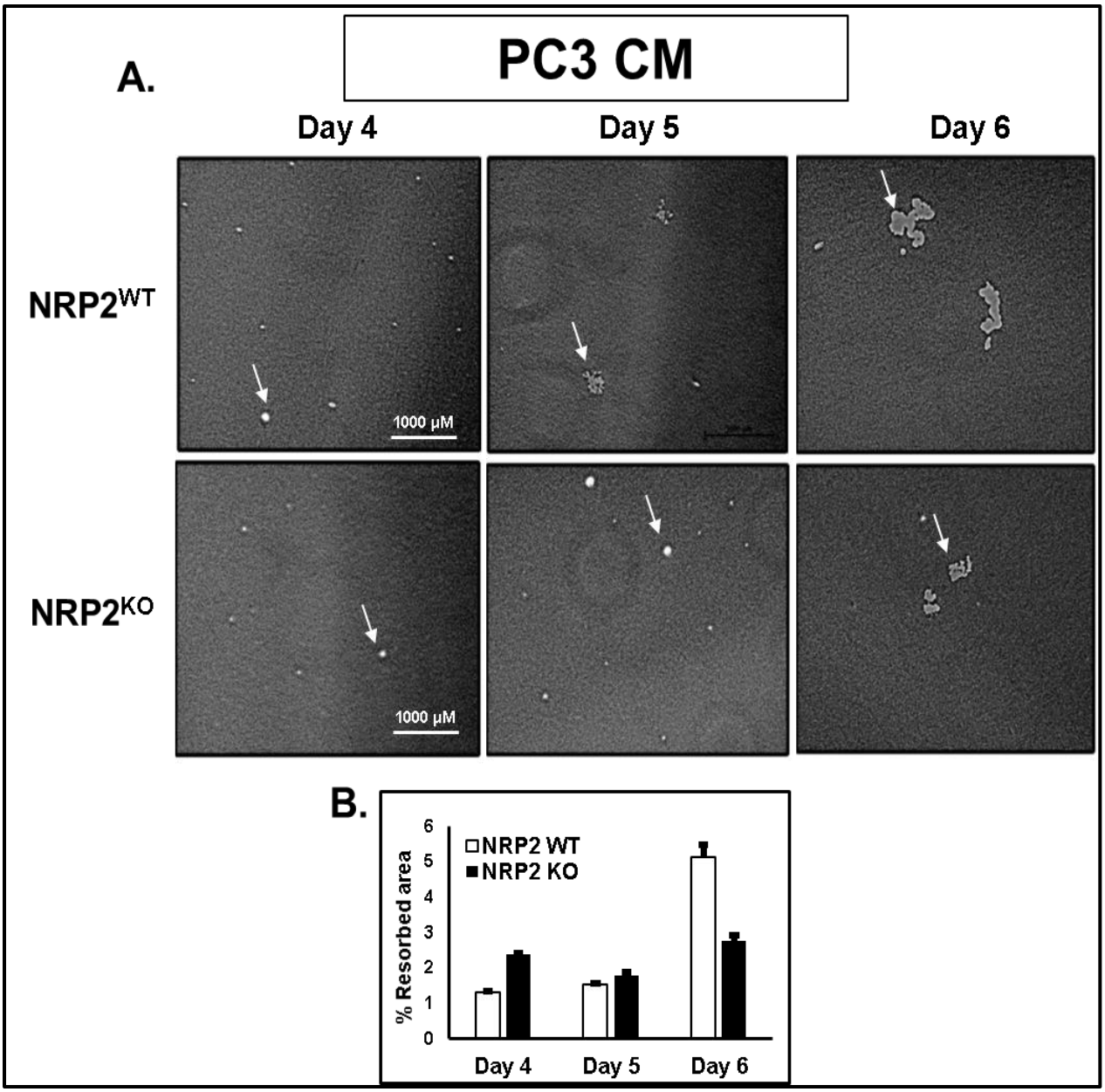


Figure **3.25**: Effect of depletion of NRP2 in osteoclast precursors isolated from transgenic mice, NRP2$^{Flox/Flox}$; CSF1R-Cre by addition of 4-hydroxytamoxifen and induced by PC3 CM. **A.** Pit resorption on osteoassay plate **B.** Comparison of Pit resorption function in control and NRP2 knockout osteoclasts.

Discussion

Understanding the osteoclasts physiology in prostate cancer is imperative for the development of a novel therapy. Current information on NRP2 in osteoclasts is limited and no study till date has addressed the role of NRP2 in osteoclasts involved in the development of bone metastasis in prostate cancer patients. In the present section of the study, we have reported the expression of NRP2 in physiological and pathological condition, prostate cancer bone metastasis. Our results showed the expression of NRP2 in the osteoclast precursors from mice does not express NRP2. However, the levels of NRP2 increase during the differentiation of precursors into osteoclasts. The expression of NRP2 is highly induced by the treatment of positive inducers such as RANKL and M-CSF which supports the earlier report by Leive Verlinden *et. al*.[85] However, no reports have been published till date on NRP2 in a pathological condition such as prostate cancer bone metastasis. Our study is the first to report the expression of NRP2 in osteoclasts promoted by bone metastatic prostate cancer. Our results showed a robust increase in the expression of NRP2 in osteoclasts differentiated by PC3 CM and LNCaP C4-2B CM. In fact, the NRP2 expression was quite evident in the time course analysis of differentiating osteoclasts under the influence of CM from prostate cancer cell lines. However, comparison of the expression of NRP2 during the osteoclast differentiation in normal and prostate cancer bone metastasis showed that the NRP2 expression was elevated in osteoclasts influenced by RANKL and M-CSF than in the CM from the prostate cancer cell line. The ex-

pression of NRP2 in osteoclasts promoted by the PC CM and LNCaP C4-2B CM were comparable.

TRAP staining of osteoclasts in healthy and pathological bone conditions *in vitro* showed that RANKL and M-CSF induced osteoclastic differentiation with high efficacy followed by moderate differentiation in PC3 CM and decreased differentiation in LNCaP C4-2B CM. This data again support the earlier reports on CM of the PC3 being purely osteolytic while LNCaP C4-2B showed predominant osteogenic and limited osteolytic function. Resorption of the bone is the central function of osteoclasts. Our pit assays suggest that the formation of pits as a result of bone mimetic resorption were higher in osteoclasts activated by RANKL and M-CSF. The pits formed by PC3 CM are smaller but increased in number which again supports our TRAP staining of osteoclasts in PC3 CM. Being osteolytic, PC3 may promote the formation of 3-4 nucleated osteoclasts to actively degrade bone by forming small pits and thereby cover more surface area than expending energy reserves to create large multinucleated osteoclasts. This event can make the bone porous and weak leading to fractures in human prostate cancer patients. In contrast to PC3 CM, LNCaP C4-2B CM-induced the formation of 2-3 or single-nucleated osteoclasts observed by TRAP staining and the pit formation by these osteoclasts are minimal in size and less in number suggesting that resorptive function of LNCaP C4-2B CM promoted osteoclasts is low. The role of LNCaP C4-2B in bone cells is to induce predominantly osteogenic differentiation with low osteolytic function, and our data also confirms this fact. Also, the mRNA analysis of the expression of osteoclastic genes indicated that osteoclasts in-

duced by RANKL and M-CSF expressed elevated levels of these genes followed by increased expression in PC3 CM (but not to the levels as in healthy osteoclasts) and moderate levels in LNCaP C4-2B CM.

With the information that NRP2 is expressed in the physiological as well as in prostate cancer-induced osteoclastogenesis, a question was asked. What is the function of NRP2 in osteoclasts in physiological and prostate cancer bone metastasis? To address this, we depleted NRP2 in the osteoclast precursors and induced their differentiation in all the three conditions. Intriguingly, NRP2 depletion in RANKL and M-CSF influenced osteoclasts showed a drastic increase in osteoclast differentiation and activation. The NRP2 –depleted osteoclasts showed increased expression of osteoclastic genes as well as increased resorptive capability. Together, our results in RANKL and M-CSF condition indicated that NRP2 downregulates the differentiation and function of osteoclasts in healthy bone.

Another interesting observation came to our focus upon depletion of NRP2 in osteoclastic precursors followed by differentiation with LNCaP C4-2B CM. With high osteogenic differentiation with nominal osteolysis, the functions of LNCaP C4-2B closely depict prostate cancer cells in the bone microenvironment. In the absence of NRP2, osteoclasts induced by LNCaP C4-2B CM showed a substantial increase in the differentiation and function of osteoclasts. Large, multinucleated osteoclasts in comparison to the control were observed in NRP2 depleted osteoclasts upon addition of LNCaP C4-2B CM. Further, the resorbing capacity of these cells increased exponentially from their control counterparts. We also

found an increase in the expression of osteoclastic genes as a result of deletion of NRP2. This information further reinforces our findings in RANKL and M-CSF conditions. Cumulatively, NRP2 functions as a negative regulator of osteoclasts induced by the RANKL and M-CSF and in prostate cancer bone metastasis.

Contradicting our findings in RANKL and M-CSF and LNCaP C4-2B CM, PC3 which is purely osteolytic, did not display any change in the osteoclast differentiation and activation upon removal of NRP2. The NRP2-ablated osteoclasts showed no noticeable difference in the TRAP staining when compared to the control osteoclasts stimulated by PC3 CM. Also, the resorption function of osteoclasts did not differ due to NRP2 deletion and no change in the expression of osteoclast genes was observed. These data imply that PC3 CM-induced osteoclasts evade the regulation of NRP2.

In summary, NRP2 functions as negative regulator of osteoclasts in healthy as well as osteoclasts induced by prostate cancer cells that promote predominantly osteoblastic metastasis. We have identified a novel inhibitory role of NRP2 in physiology and pathology of osteoclasts. However, osteolytic PC3 bypass the NRP2 regulation. With the function of NRP2 established, we will address the exact molecular pathway that NRP2 functions through to regulate osteoclastogenesis which will be the focus of the next chapter.

CHAPTER 4

NRP2 regulates the functions of NFATc1 and NFκB in osteoclasts

Introduction

Osteoclasts are the major players in the skeletal development in an embryo as well as homeostasis in an adult. They are central to the fundamental processes of bone, i.e., endochondral ossification during early development, bone fabrication during the growth phase and bone remodeling in adulthood. The coordinated functioning of osteoclasts and osteoblasts imparts rigidity and strength. Osteoclasts are explicitly found on the bone matrix to resorb the bone surface. Osteoclasts are formed by the differentiation of myeloid cells of monocyte/macrophage lineage derived from the hematopoietic stem cells along with cues from the surrounding bone microenvironment[24,25,29,34]. Osteoclasts undergo different stages such as early commitment of hematopoietic stem cells to monocytes, differentiation, fusion, and activation depending upon the requirements of the bone microenvironment. The formation and survival of the osteoclasts and their unique ability to resorb bone are controlled by complex signaling pathways leading to the activation of various transcription factors. During the process of differentiation of hematopoietic stem cells to bone resorbing osteoclasts, transcription factors such as PU.1 and MITF play crucial roles. In response to M-CSF, PU.1 signals the early determination of bone marrow progenitors to myeloid cells by stimulating the expression of CSF1R, the receptor of M-CSF[19,26,33,34,39]. PU.1 also regulates the proliferation of monocytes to induce osteoclastic differentiation. Activation of MITF by M-CSF is vital for the induction of Bcl-2 to promote survival of the osteoclastic precursors before their migration and attachment to the target bone resorption site.

During the initiation stage of differentiation, the RANKL/RANK signaling induces a complex signaling cascade resulting in the expression of osteoclastic genes essential for their resorptive function. The osteoclastic genes' expression is cumulatively coordinated by the action of transcription factors stimulated by RANKL/RANK signaling. Binding of RANKL to RANK receptor promotes the activation of NF-κB which translocates to the nucleus to promote transcription of osteoclastic genes. NF-κB is the first transcription factor activated during differentiation of osteoclasts via the RANKL/RANK and M-CSF/c-Fms signaling. In differentiating osteoclasts, both the canonical as well as non-canonical signal activation of NF-κB is reported to co-occur. RANKL/RANK binding stimulates the recruitment of TRAF6 and p62 leading to the complex formation and their subsequent activation. The TRAF6-p62 complex further induces the activation of PKC that then phosphorylates IKK. The active IKK releases NF-κB from the complex of IκB by signaling the ubiquitination of IκB. The free NF-κB p65 and p50 components translocate to the nucleus from cytoplasm for gene expression. Parallel to this, NF-κB is activated by the function of NIK to induce IKKα to process p100 to p52 via the proteasomal function. The RelB- p52 complex translocates to the nucleus for gene transcription. The non-canonical NF-κB signaling is prolonged and sustained than the canonical pathway due to the slow translocation of the RelB-p52 complex into the nucleus. The differentiating osteoclasts favor which NF-κB pathway is based on the requirement and stage of differentiation of the cells. Once in the nucleus, the functional NF-κB promotes the transcription of a key transcription factor, NFATc1[34,38,46].

NFATc1 is central to the differentiation and function of osteoclasts. Its expression is induced by the recruitment and binding of NF-κB and NFATc2 whose expression and activation occurs in monocytic precursors. The induction of NFATc1 expression is also promoted by the association of DAP12/ ITAM co-receptor with RANK to costimulate it. After activation, the DAP12/ ITAM/ RANK complex synergistically recruits Syk to induce BTK, Tec, and PI3K which sequentially prompts PLCγ to drive Ca^{2+} oscillations. Ca^{2+} stimulates calcineurin to dephosphorylate NFATC1 to enable its translocation into the nucleus for transcription of osteoclast genes[37,46]. Ca^{2+}-mediated activation of NFATc1 also triggers the autoamplification loop of NFATc1 and ensures a sustained NFATc1-dependent transcriptional function. c-Fos is also recruited to the promoter site of NFATc1 and is reported to be indispensable for the early induction of NFATc1. However, the exact mechanism of action of c-Fos on NFATc1 is still to be elucidated. NFATc1 is known to be master transcription factor which in cooperation with transcriptional factors such as AP-1, PU.1, MITF, NF-κB and others to transcribe osteoclastic genes. Nearly 70% of the osteoclast genes that are functionally important are expressed through the function of NFATc1 but with different transcription binding partners[31]. Further, the cooperation of NFATc1 with varying transcription factors leads to expression of different osteoclastic genes and also the expression of genes may vary with different binding partners under physiological and pathological conditions. It is still a question unanswered as to what transcriptional factors bind along with NFATc1 to what gene promoter site to promote transcription under a specific condition or disease.

The expression of NFATc1-regulated osteoclastic genes that are necessary for the resorptive function are TRAP, Cathepsin K, CA II, CIC7, LTBP3, ATP6i (H^+ ATPase proton pump), serpin peptidase inhibitor, clade D, member 1 (Serpind1) and MMP9. It also directly activates the genes associated with fusion of osteoclasts such as ATP6V0D2, OC-STAMP, and DC-STAMP. Proteins involved in cytoskeleton rearrangement and exocytosis such as Rho C, Rab38, Na+/H^+ exchanger-like domain-containing protein 2 (Nhedc2*)* and adenylate cyclase 3 (Adcy3) are NFATc1-dependent. Further, NFATc1 is a crucial regulator of osteoclast migration and adhesion as it induces the expression of αvβ3 integrin and c-Src necessary for the formation of ruffled borders and F-actin ring arrangement[37]. Together, NFATc1 is important for the formation of mature osteoclasts through direct regulation of various genes involved in osteoclast differentiation and activation. Interestingly, a recent study showed that osteoclastogenesis induced by metastatic prostate cancer-derived factors increases the basal levels of Ca^{2+} causing Ca^{2+} fluctuations which subsequently promote the translocation of NFATc1 into the nucleus. Inhibition of NFATc1 signaling decreases the ability of bone metastatic prostate cancer cells to promote osteoclast differentiation and activation[27].

In the previous chapter, we showed the expression of NRP2 in healthy as well as prostate cancer-induced osteoclasts and that the depletion of NRP2 caused a dysregulation leading to hyperactivation of osteoclasts in RANKL and M-CSF and LNCaP C4-2B CM. The hyperactivated osteoclasts in NRP2 devoid state evaluated for the expression of osteoclastic genes showed elevated mRNA levels of

TRAP, cathepsin K, DC-STAMP, ATP6V0D2, CA II, MMP9 and ATP6i in both the conditions. In this chapter, we studied how NRP2 governs the osteoclastic gene expression to inhibit osteoclast differentiation and activation. Here, we focused on identifying which key transcription factors are involved in the regulation of osteoclasts by NRP2. Our results implicate that NRP2 critically inhibits NFATc1 and NF-κB in osteoclasts differentiated by RANKL and M-CSF. In LNCaP C4-2B CM, NRP2 depletion although caused an upregulation of NFATC1 expression and its activity, no increase in NF-κB function was observed. Our results therefore suggested activation of transcription factors in osteoclasts is condition-dependent and further pointed out the molecular mechanisms by which NRP2 negatively regulates the osteoclastic activity in each condition.

Results

NRP2 regulates the expression and function of NFATC1 and NF-κB in RANKL/MCSF treated osteoclasts.

Our previous results discussed in chapter 3 implicated that the depletion of NRP2 in RANKL/MCSF-treated osteoclasts leads to their hyperactivation. The function of osteoclasts is mainly dependent on the expression and activation of the transcription factor, NFATc1. Hence, we evaluated whether NRP2 has any effect on NFATc1 expression and activation during osteoclast differentiation. In RANKL and M-CSF treatment, protein analysis at day 2 and day 3 of osteoclastic differentiation showed that the total protein of NFATc1 increases in NRP2-depleted cells compared to the controls (Fig 4.1 B, Fig. 4.2 B). We then investigated whether NFATc1 translocates to the nucleus for its activity in the presence or absence of NRP2. Using immunofluorescence microscopy, we evaluated the expression of NFATc1 in the nucleus in differentiating osteoclasts under RANKL and M-CSF at day 2 and day 3. We observed bright cells with high expression of NFATc1 in control as well as NRP2-ablated osteoclastic precursors. Interestingly, we observed in each field very few NFATc1-expressing cells (Fig 4.1 A, Fig. 4.2 A). However, the total expression of NFATc1 in the nucleus calculated as corrected total cell fluorescence (CTCF) increases by 2-fold in the NRP2-depleted osteoclasts than in the NRP2-expressing cells in day 2 and day 3 (Fig 4.1 C, Fig. 4.2 C). Additionally, the total number of NFATc1 expressing cells in the NRP-2 deleted condition increases than in control condition. These data advocates that

presence of NRP2 negatively regulates the osteoclast differentiation and activation by restraining the expression and translocation of NFATc1 in osteoclasts.

Although our data suggest the NRP2 regulation of NFATc1 which is central to osteoclast differentiation and function, NFATc1 cannot work alone in the transcription of osteoclast genes. The coordinated function of NFATc1 with other factors such as NF-κB has been widely studied. NF-κB is the first transcription factor that is activated by the signaling of RANKL/RANK during the early differentiation of osteoclasts. Hence, we investigated whether NRP2 conducts its regulation via NF-κB in osteoclasts. For this, we depleted NRP2 in osteoclastic precursors and analyzed the NF-κB protein localization via fractionation of nuclear and post-nuclear proteins (membrane and cytoplasmic proteins) in RANKL and M-CSF. We detected high expression of NF-κB in the control and NRP2-depleted cells in RANKL and M-CSF condition. In comparison to the control, the nuclear expression of NF-κB was increased in NRP2-depleted osteoclasts (Fig 4.3). These data were confirmed by the observations from immunofluorescence staining of NF-κB. We examined the osteoclasts induced by RANKL and M-CSF for the localization of NF-κB in the nucleus. NRP2 removal in osteoclasts resulted in an increase in the nuclear localization of NF-κB (Fig 4.4). In summary, RANKL/MCSF-treated osteoclasts express NRP2 to control the expression and localization of NFATc1 and NF-κB during osteoclastic gene transcription. Absence of NRP2 in osteoclasts leads to their hyperactivation because of increased activity of NFATc1 and NFκB.

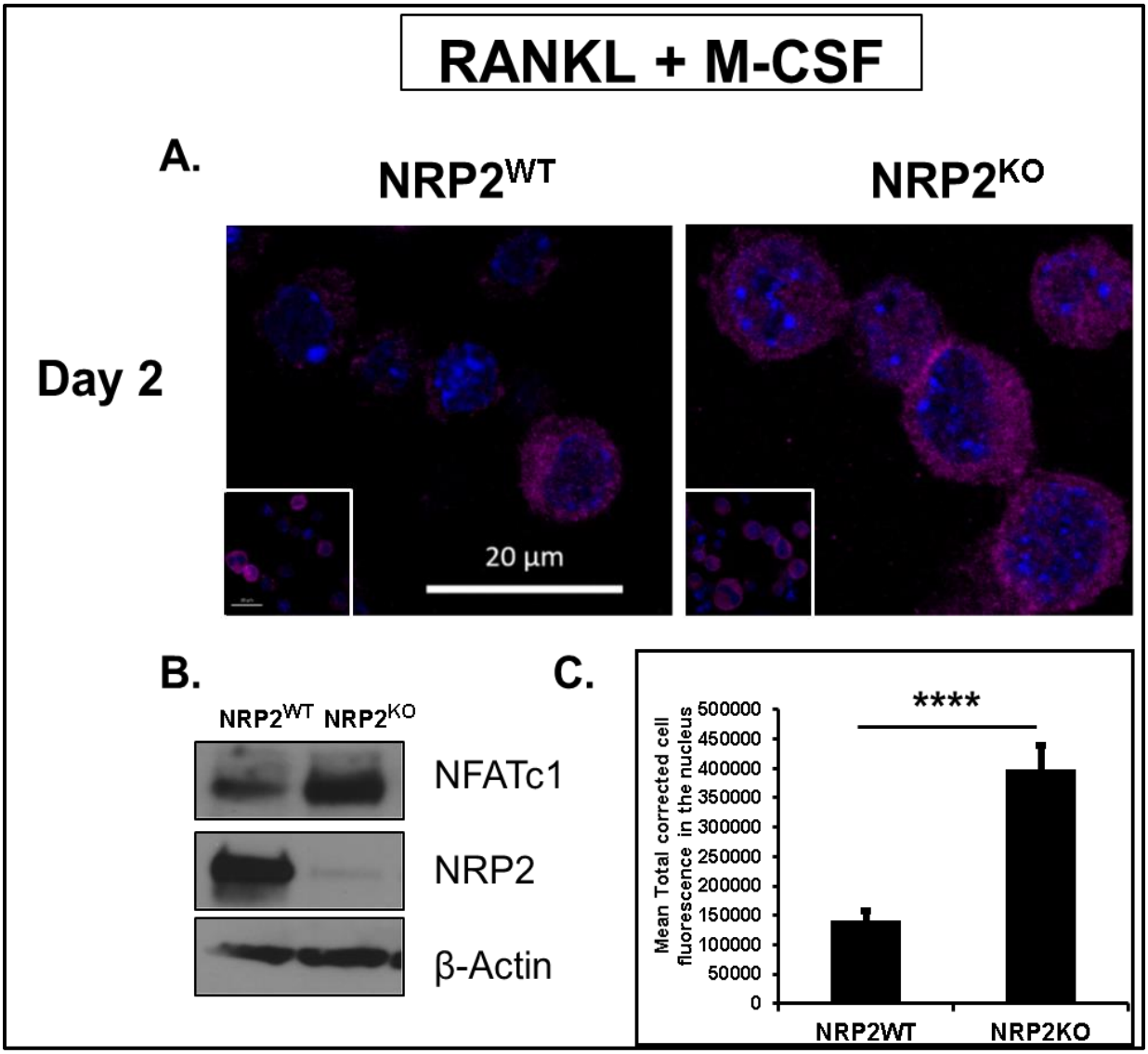


Figure **4.1**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by RANKL and M-CSF at day 2. **A**. Immunofluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue). **B.** Protein analysis of NFATc1 comparing its total expression in control and NRP2 knockout osteoclasts. **C.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knockout osteoclasts.

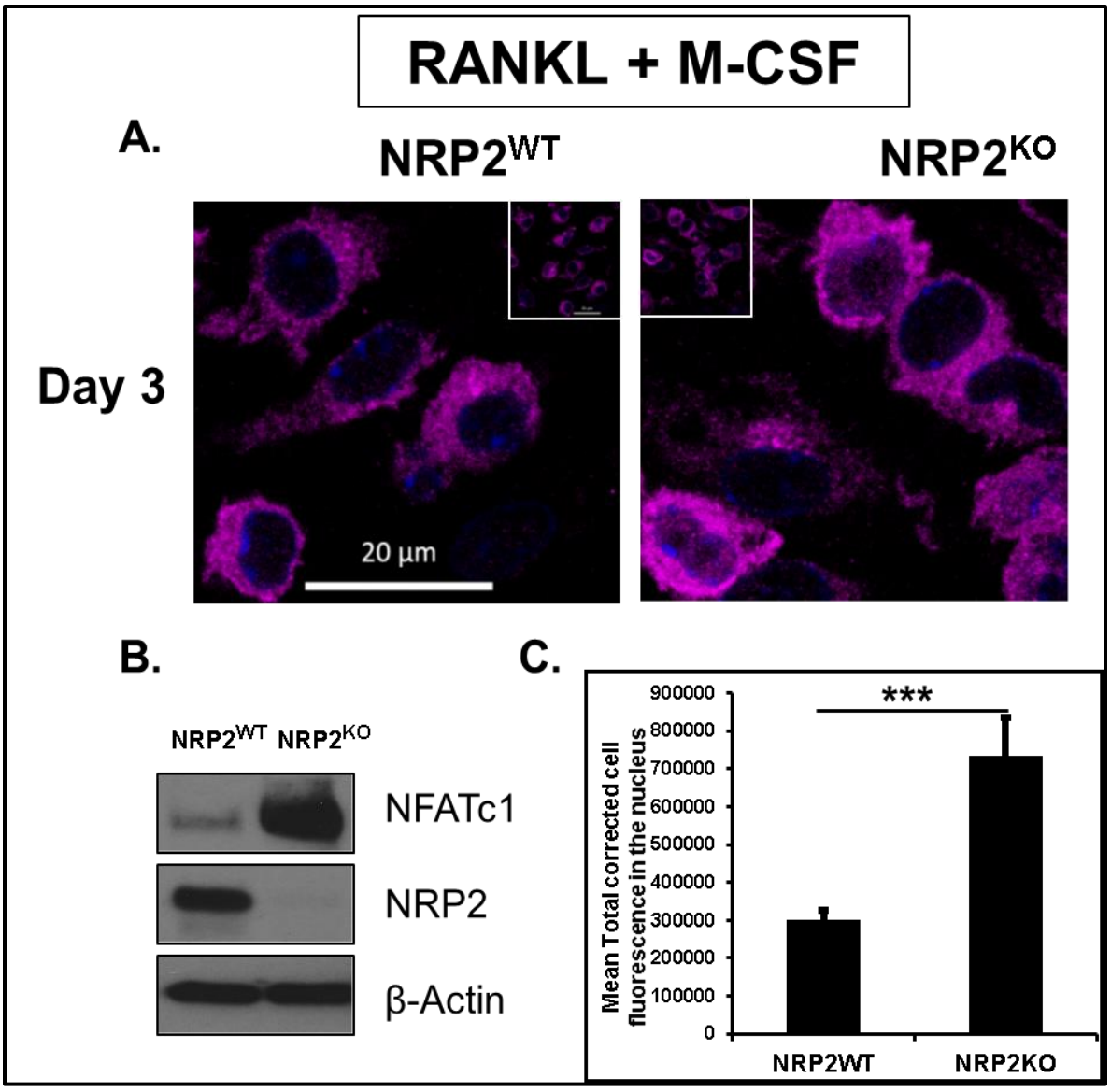


Figure **4.2**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by RANKL and M-CSF at day 3. **A**. Immuno-fluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue). **B.** Protein analysis of NFATc1 comparing its total expression in control and NRP2 knockout osteoclasts. **C.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knockout osteoclasts.

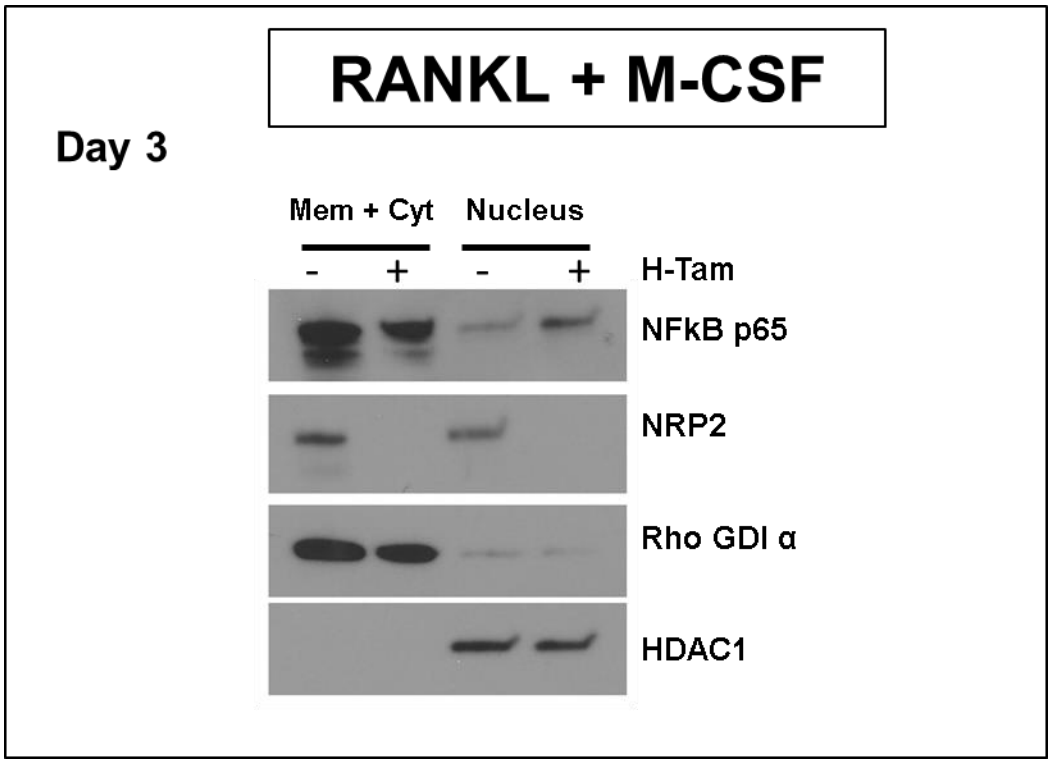


Figure **4.3**: Localization of NFκB in the nucleus following depletion of NRP2 in osteoclast precursors induced by RANKL and M-CSF at day 3. Analysis of NFκB protein in the nuclear and post nuclear fractions in control and NRP2 knockout osteoclasts

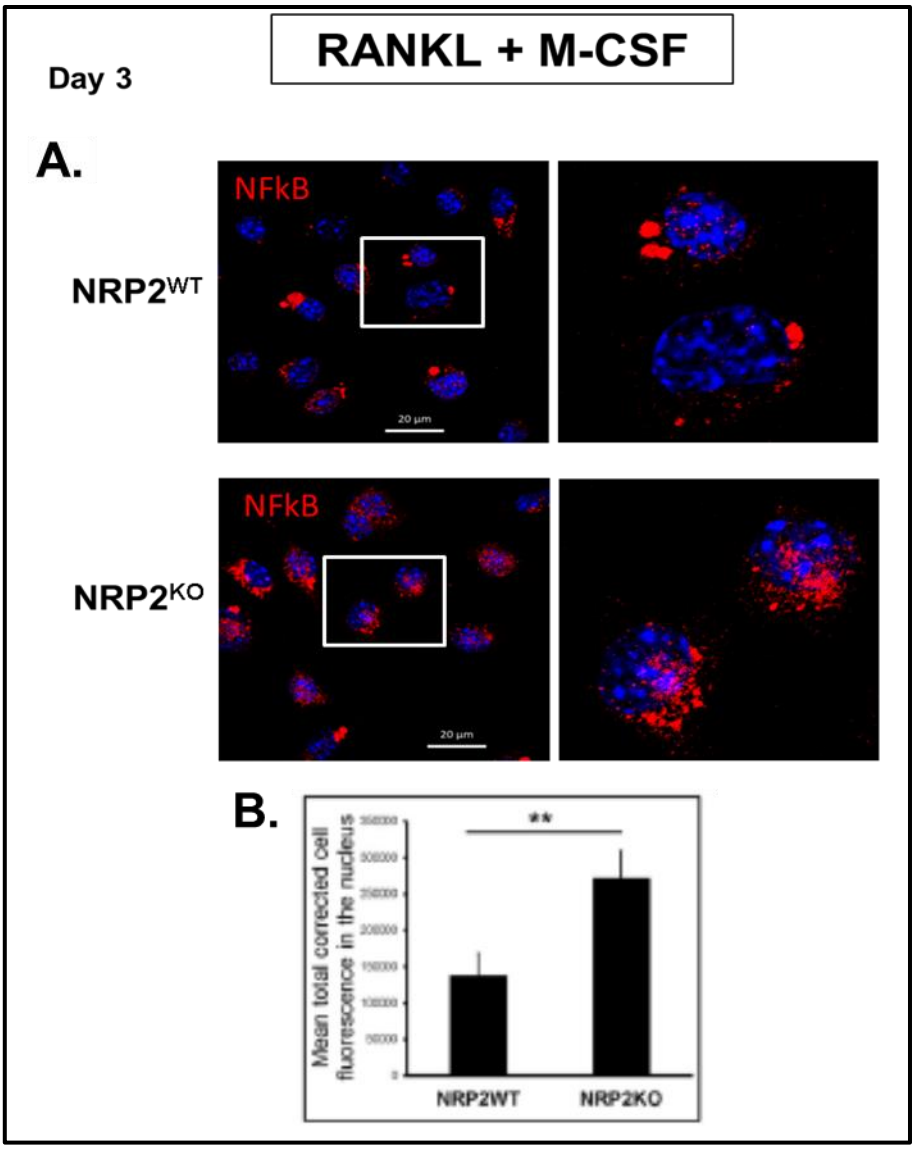


Figure **4.4**: Localization of NFκB in the nucleus following depletion of NRP2 in osteoclast precursors induced by RANKL and M-CSF at day 3. **A**. Immunofluorescence staining of NFκB (Alex 540, red) and nucleus with DAPI (blue). **B.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFκB in the nucleus between control and NRP2 knockout osteoclasts.

NRP2 controls osteoclastogenesis via NFATc1 but not by NFκB in the CM of osteoblastic prostate cancer cells, LNCaP C4-2B. .

NFATc1 has been reported to be crucial for osteoclastogenesis promoted by metastatic prostate cancer. To understand the signaling mechanism through which NRP2 regulates the functions of osteoclasts in prostate cancer bone, we depleted NRP2 in osteoclasts and treated with CM from LNCaP C4-2B to induce osteoclast differentiation. As previously described, LNCaP C4-2B promotes both osteoblastic and osteoclastic differentiation similar to what is observed in prostate cancer patients with bone metastasis. We analyzed the expression and activation of NFATc1 at day 2 and day 3 of osteoclastogenesis. We did not observe NFATc1 at protein level in day 2 in both the control and NRP2 depleted osteoclasts. In day 3 at the protein level, we found that LNCaP C4-2B-derived factors increase the total expression of NFATc1 in NRP2-depleted osteoclasts in comparison to the NRP2 expressing osteoclasts (Fig. 4.6 B). Further confirmation came from the immunofluorescence studies where we stained osteoclasts with NFATc1 antibody to evaluate its expression and translocation into the nucleus. Surprisingly, a contrasting difference was observed in the expression and localization of NFATc1 in NRP2-depleted osteoclasts in LNCaP C4-2B CM. Similar to our protein analysis; we found expression of NFATc1 is more in NRP2-depleted osteoclasts. Interestingly, the control cells show no or less localization of NFATc1 in the nucleus suggesting NFATc1 is mainly in the cytoplasm in osteoclasts when treated with CM of LNCaP C4-2B cells. However, when we depleted NRP2 in these osteoclasts in LNCaP C4-2B CM, we observed osteoclasts that are highly

expressing NFATc1 (Fig. 4.5 A, Fig. 4.6 A). More importantly, the localization of NFATc1 in the nucleus increased by 6- and 14-fold at day 2 and day 3 respectively due to the decrease in NRP2 (Fig. 4.5 B, Fig. 4.6 C). These data cumulatively prove that NRP2 negatively regulates NFATc1 in osteoclasts during their differentiation by LNCaP C4-2B CM.

We further evaluated the status of NF-κB after depletion of NRP2 in osteoclasts following its treatment with LNCaP C4-2B CM. The NRP2-expressing osteoclasts differentiated by LNCaP C4-2B -derived factors showed the presence of NF-κB in the nucleus. However, removal of NRP2 caused a drastic decrease in the nuclear localization of NF-κB (Fig. 4.7). This data contradicts our findings in RANKL and M-CSF induced osteoclasts suggesting that the increase in osteoclastic activity following NRP2 depletion in osteoclasts differentiated by LNCaP C4-2B CM is not dependent on NF-κB, rather work centrally through NFATc1.

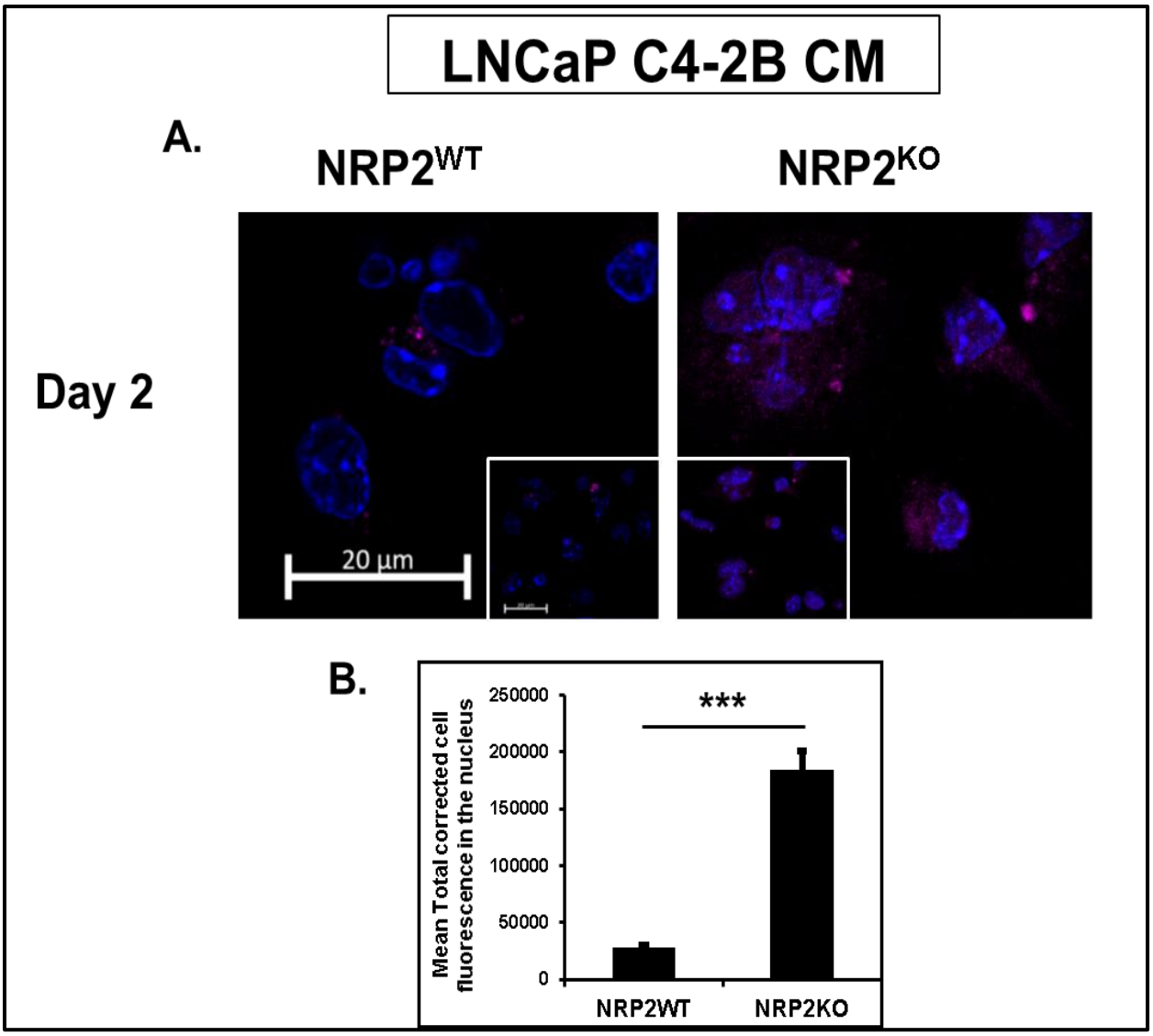


Figure **4.5**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by LNCaP C4-2B CM at day 2. **A**. Immunofluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue) **B.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knock-out osteoclasts.

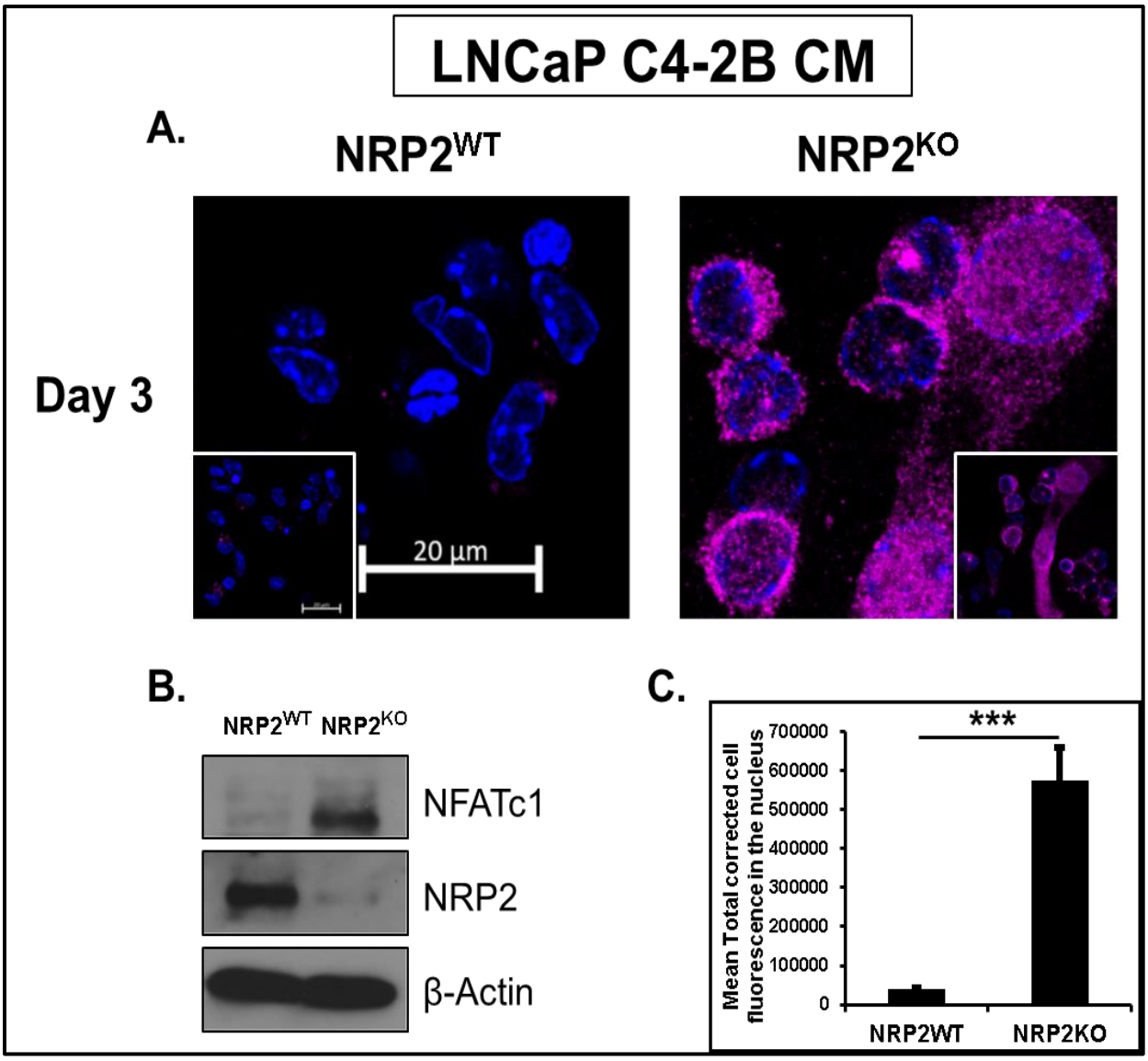


Figure **4.6**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by LNCaP C4-2B CM at day 3. **A**. Immunofluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue). **B.** Protein analysis of NFATc1 comparing its total expression in control and NRP2 knockout osteoclasts. **C.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knockout osteoclasts.

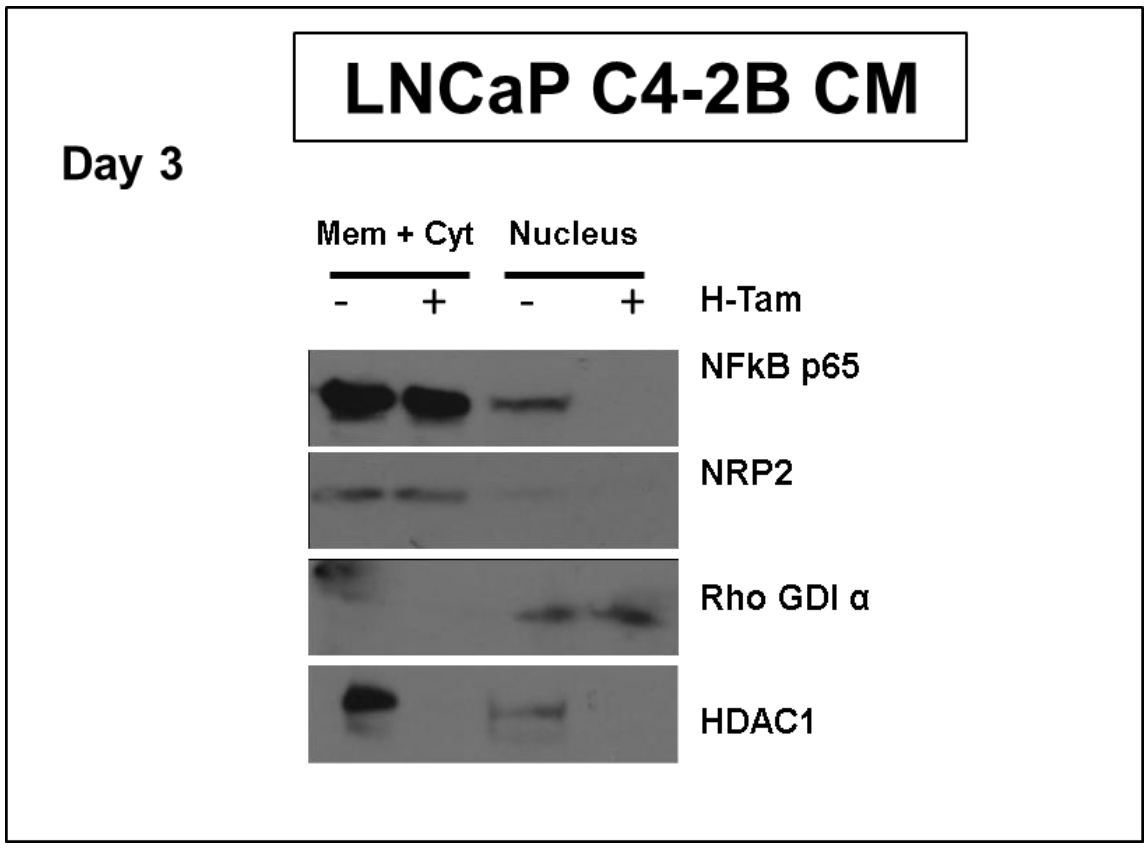


Figure **4.7**: Localization of NFκB in the nucleus following depletion of NRP2 in osteoclast precursors induced by LNCaP C4-2B CM at day 3. Analysis of NFκB protein in the nuclear and post nuclear fractions in control and NRP2 knockout osteoclasts

Discussion

Resorption by osteoclasts is central to the functionality of the bone. The shape, size, and strength of the bone are modulated by osteoclasts in coordination with osteoblasts. The differentiation and activation of osteoclasts require different genes at different stages working in a specific framework to enable the functioning of osteoclasts at a determined space and location. Any change in the expression of these genes can lead to a pathological conditions observed frequently during prostate cancer bone metastasis. The expression of these genes is controlled by the function of transcription factors which function in a sequential manner to manage the requirement of the osteoclast based on its differentiation and activation status. Many transcription factors have been widely studied in osteoclasts in context to bone remodeling under physiological condition. Major transcription factors that are reported to function in the release of osteoclastic genes are NFATc1, NF-κB, PU.1, AP1, and MITF. Any change or loss of function in these transcription factors is detrimental for the differentiation and activation of osteoclasts. Previous reports on signaling in osteoclasts have implicated the requirement of RANKL/RANK, M-CSF/ c-Fms as well as DAP12/ FcR and interleukins such as IL-1, IL-6, and IL-8 in association with unknown signaling partners to activate these transcription factors.

Despite extensive research on the regulation of transcription factors in osteoclasts, not many regulators has been reported to date that can control the function of osteoclast-specific transcription factors. In this study, we report for the first time that NRP2 regulates the activation of key transcription factors, NFATC1 and

NF-κB in osteoclasts. Our data indicate that NRP2 depletion increases the translocation of important transcription factor, NFATc1 into the nucleus to transcribe a range of osteoclastic genes. Moreover, protein expression of NFATc1 is also increased upon NRP2 depletion. Interesting facts were releveled by the immunofluorescence staining of NFATc1 in osteoclasts induced by RANKL and M-CSF. We observed few osteoclasts in a given area exhibit high expression of NFATc1. During osteoclast fusion, it was reported that some of the osteoclasts act as mother cell and form fusion bridges to recruits nearby mononuclear osteoclasts for fusion. To form the fusion bridge, the mother cell increases its fusion - associated genes which are in turn transcribed by NFATc1[40,44]. Hence to compensate for the increase in demand for fusion genes, we believe the mother cell increases the expression of NFATc1 and thereby activates it to localize in the nucleus for gene transcription. Interestingly, depletion of NRP2 further enhanced the number of osteoclasts that are high in NFATc1 expression in RANKL and M-CSF condition. Therefore, we deduce from our findings that NRP2 by controlling the expression and localization of NFATc1 in the RANKL and M-CSF-induced osteoclasts maintains an optimum level of osteoclast activation required for its physiological function. In the absence of NRP2, osteoclast becomes hyperactivated because of increased localization of NFATc1. We predict that depletion of NRP2 may thus lead to pathological conditions with enhanced osteolytic activity in bone. Our results further indicated that NFκB, which is known to bind cooperatively with NFATc1 for osteoclastic gene activation, was regulated similarly by

NRP2. Together, they can induce robust activation of osteoclast following the depletion of NRP2.

The osteolysis induced by prostate cancer CM has been reported to induce different osteoclastic genes. Although these genes are actively transcribed by the function of various transcription factors, no study has reported the involvement of transcription factors in the promotion of osteoclast differentiation and function in cancerous condition. Furthermore, not many mediators have been disclosed to regulate the activation of osteoclasts. Our study marks the first reporting of a regulator that controls the activation of transcription factors in osteoclast in prostate cancer bone metastasis. Our experimental evidence supports the regulation of NFATc1 by NRP2 in LNCaP C4-2B treated osteoclasts. The total expression of NFATc1, as well as the nuclear localization of NFATc1 increased in the absence of NRP2 in osteoclasts differentiated by LNCaP C4-2B CM suggesting that NRP2 restricts the activation and nuclear localization of NFATc1 in these osteoclasts under this condition. In addition to these findings, we observed high expression of NFATc1 in some of the osteoclasts which may function as mother cell similar to the results reported previously in RANKL and M-CSF treatment. However, to our surprise, NF-κB nuclear localization was inhibited in NRP2 depleted osteoclasts in LNCaP C4-2B CM suggesting a different regulation in osteoclast when induced by prostate cancer cells. We speculate that NFκB has limited influence in promoting osteoclastic activity in the presence of CM of LNCaP C4-2B cells. This is because osteoclastic activity is significantly low in osteoclasts when differentiated by CM of LNCaP C4-2B, despite the presence of detectable level of

NFκB in the nucleus. Moreover, NRP2 depletion although can increase the osteolytic activity in this condition, we detected no NFκB in the nucleus. The question is then what can be the dominant transcription factor/s that drive the osteoclastogenesis when stimulated by LNCAP C4-2B especially when NRP2 was depleted. Our data indicated that NFATc1 can be the factor as it is not present in a significant level in NRP2-expressing osteoclasts when treated with CM of LNCaP C4-2B and thus can explain the low activity of the osteoclasts in this condition. Interestingly, NFATc1 protein level is not only increased in NRP2-depleted osteoclasts but there is almost 3-fold increase in its translocation in the nucleus, which correlates with the increase in osteolytic activity. Our results therefore suggested differential regulation of transcription factors by NRP2 in osteoclasts, which are based upon the way they are differentiated. We believe that both NFATc1 and NFκB are important for inducing osteoclastogenesis in RANKL and MCSF-treated conditions thus explaining the robust osteolytic activity in comparison to when treated with CM of cancer cells. The osteolytic activity has enhanced further upon NRP2 depletion as both the transcription factors can localize more in the nucleus. On the other hand the osteolytic activity is low in osteoclasts when treated with CM of LNCaP C4-2B, because of less NFATc1 in the nucleus even detectable level of NFκB is present. The activity was increased following NRP2 depletion as NFATc1 started entering into the nucleus, although NFκB was reduced during the same condition. Since NFATc1 and NFκB are known to act cooperatively to enhance robust osteolytic activity, the absence of NFκB in the nucleus during LNCaP C4-2B CM treated condition might explain why the in-

creased osteolytic activity was not comparable with the activity that we observed in RANKL/MCSF-treated osteoclasts following the depletion of NRP2.

CHAPTER 5

Elucidation of how PC3 CM evades the regulation of NRP2 regulation in prostate cancer bone metastasis

Introduction

In humans, prostate cancer bone metastasis is identified as mixed lesions with both osteoblastic and osteolytic phenotypes. Clinically, the progression of prostate cancer-induced osteoblastic metastases is slow compared to osteolytic lesions. The occurrence of osteolytic lesions is rare but once established; it progresses at a rapid rate to degrade bone rendering it weak and prone to skeletal complications such as fractures, brittleness, hypercalcemia, and anemia. Till date, the pathophysiology of osteolytic bone metastasis is still not well understood. It is clear from the research conducted in the past three decades that osteoclast activation is often a predominant phenomenon in osteolytic prostate cancer. In our study, we utilized CM collected from PC3 which is a known osteolysis promoting cell line. Many previous reports have confirmed the osteolytic function of PC3 in prostate cancer bone metastasis both in *in vitro* as well as *in vivo* model systems. The secreted factors from PC3 can induce osteoclast differentiation. These osteoclasts are capable of releasing proteins necessary for the resorption of bone. However, reports have suggested the release of MMP9 by PC3 CM-induced osteoclasts and no precise information is available on the other genes involved in osteoclastic differentiation, activation, and function.

We reported earlier the signaling molecule central to this study, NRP2 is expressed in the osteoclasts differentiated by PC3 CM and that removal of NRP2 in osteoclastic precursors showed no evident changes in the osteoclastogenesis induced by PC3 CM. This raises a question as to how do PC3 CM-induced osteoclasts evade the function of NRP2. In this section, we attempted to address this

aspect. Our initial evidence came from the TRAP staining of osteoclasts differentiated by PC3 CM. It suggested that PC3 CM did not promote fusion of osteoclasts irrespective of the presence or absence of NRP2. Even with many inducers, the osteoclasts do not form multinucleated cells. The studies performed in this chapter have addressed those issues by providing the underlying molecular mechanisms that regulate the fusion of osteoclasts in PC3 CM.

Results

Osteoclastic NFATc1 and NF-κB are not affected by PC3 CM

Our previous results discussed in chapter 3 implicated NRP2 is expressed in the osteoclasts in prostate cancer bone metastasis. The deletion of NRP2 affected the differentiation and function of osteoclasts in LNCaP C4-2B CM. However, no difference was observed in the osteoclasts depleted of NRP2 and treated with PC3 CM. Although NRP2 is expressed by PC3 CM-treated osteoclasts, removal of NRP2 did not affect differentiation or function suggesting that PC3 CM promotes signaling that bypasses the control by NRP2 in osteoclasts.

In osteolytic prostate cancer such as PC3, NFATc1 is reported to be expressed and functional in osteoclast differentiation. Hence, we checked the status of NFATc1 in osteoclasts induced by PC3 CM. We found very low or no expression of the total NFATc1 in the PC3 CM treated osteoclasts and no change in the total protein was observed after depletion of NRP2 (data not shown). Furthermore, in immunofluorescence staining, we observed the localization of NFATc1 in the cytoplasm but not in the nucleus in both the NRP-2 expressed as well as depleted osteoclasts in PC3 CM (Fig 5.1, Fig. 5.2). Hence, this suggests that NRP2 does not regulate NFATc1 in PC3 CM-induced osteoclasts.

NF-κB is reported to be involved in the osteoclastic gene regulation. Hence, we analyzed the NF-κB protein localization in PC3 CM. We compared the control and NRP2 knockout osteoclasts to understand whether NF-κB is vital for the

functions of osteoclasts in PC3 CM. We observed NF-κB localization in the cytosolic compartment but completely absent in the nucleus in NRP2-expressing and knockout osteoclasts (Fig. 5.3). This data implicates that PC3 CM induces NF-κB expression in osteoclasts, but it is rendered inactive in both NRP2-expressing or knockout conditions as it is localized in the cytosol of the osteoclasts. Together, NFATc1 and NF-κB do not function in the osteoclastic gene expression in PC3 CM.

With the information that NFATc1 and NF-κB are not the transcription modulators of osteoclastic genes in PC3 CM, we attempted to identify other transcription factors involved in the osteoclastic gene expression induced by PC3 CM. We assessed the expression of transcription factors that have been reported to play a role in osteoclastic gene expression at mRNA level. PC3-derived factors induced the expression of high expression of PU.1, ATF family (ATF1, ATF2, and ATF4), c-Fos, c-Jun, and TFE3. In addition to these factors, TAL1 which is a regulator of DC-STAMP is highly expressed in osteoclasts induced by PC3 CM (data not shown). Although we reported the expression of many transcription factors involved in osteoclastogenesis in prostate cancer, the expression at mRNA level does not necessarily imply their activation in osteoclasts. Hence, further studies are imperative in addressing the activation of these genes in osteolytic prostate cancer.

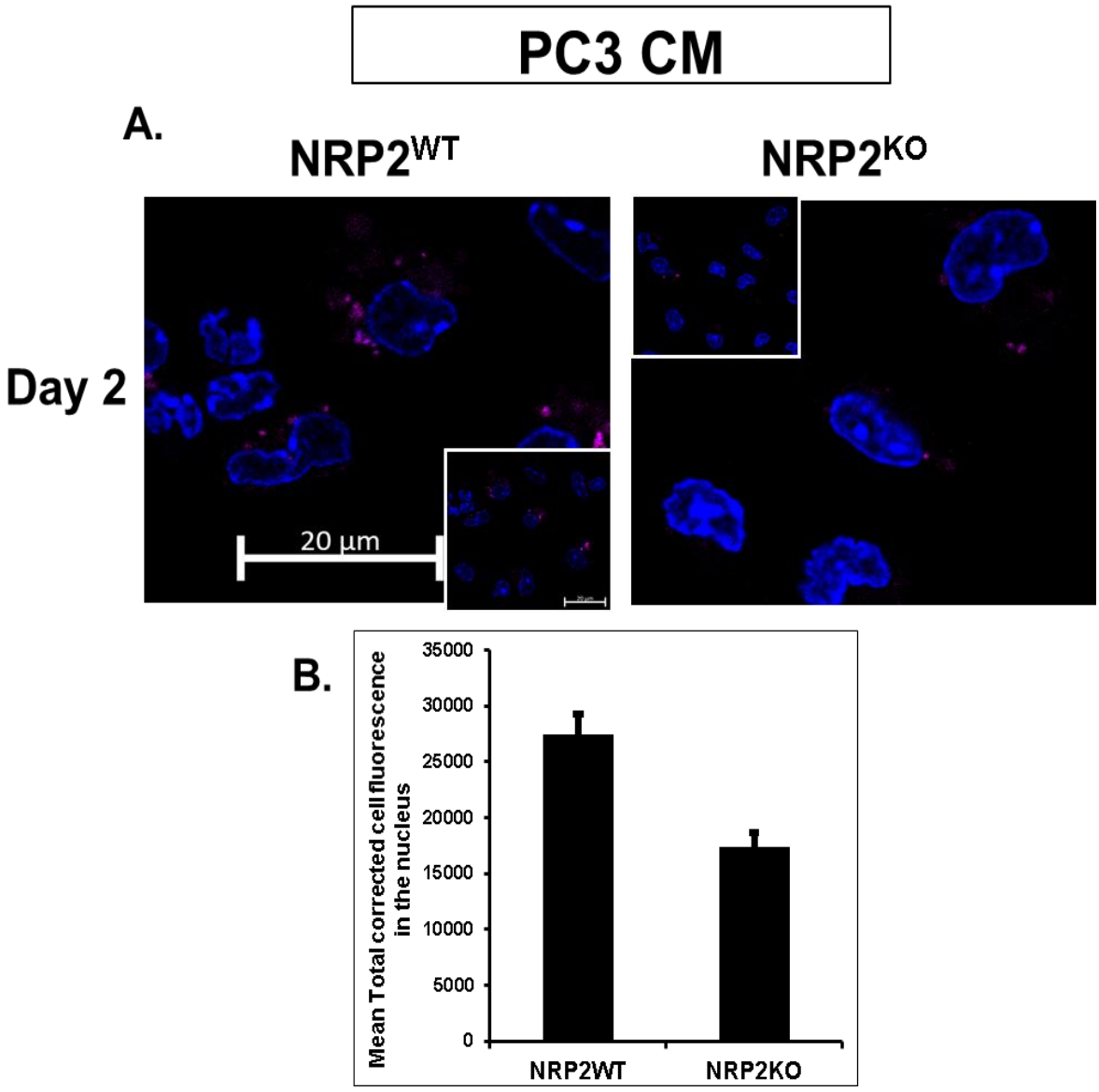


Figure **5.1**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by PC3 CM at day 2. **A**. Immunofluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue). **B.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knockout osteoclasts.

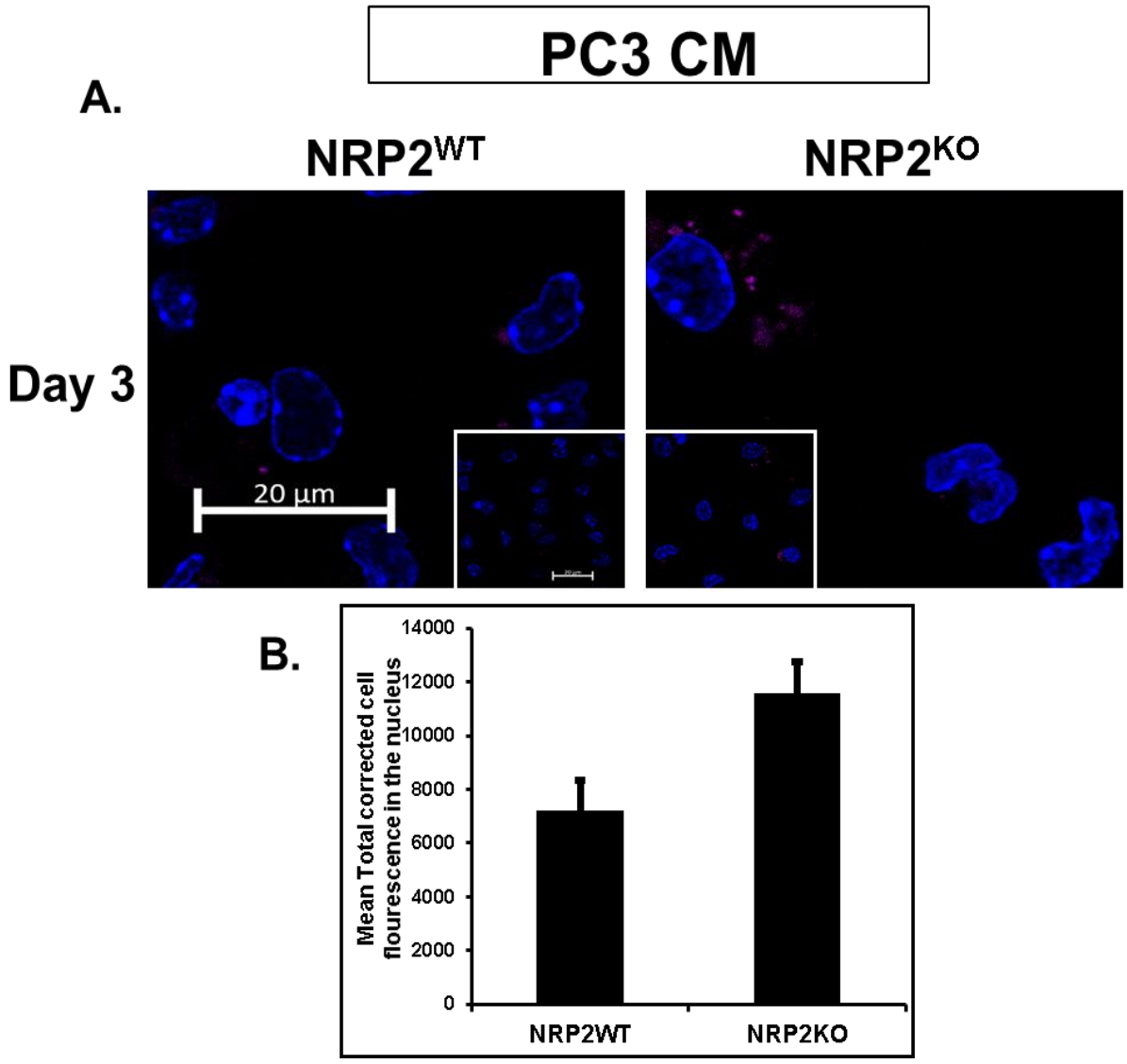


Figure **5.2**: Localization of NFATc1 in the nucleus following depletion of NRP2 in osteoclast precursors induced by PC3 CM at day 3. **A**. Immunofluorescence staining of NFATc1 (Alex 660, pink) and nucleus with DAPI (blue). **B.** Graphical representation of the corrected total cell fluorescence (CTCF) of NFATc1 in the nucleus between control and NRP2 knockout osteoclasts.

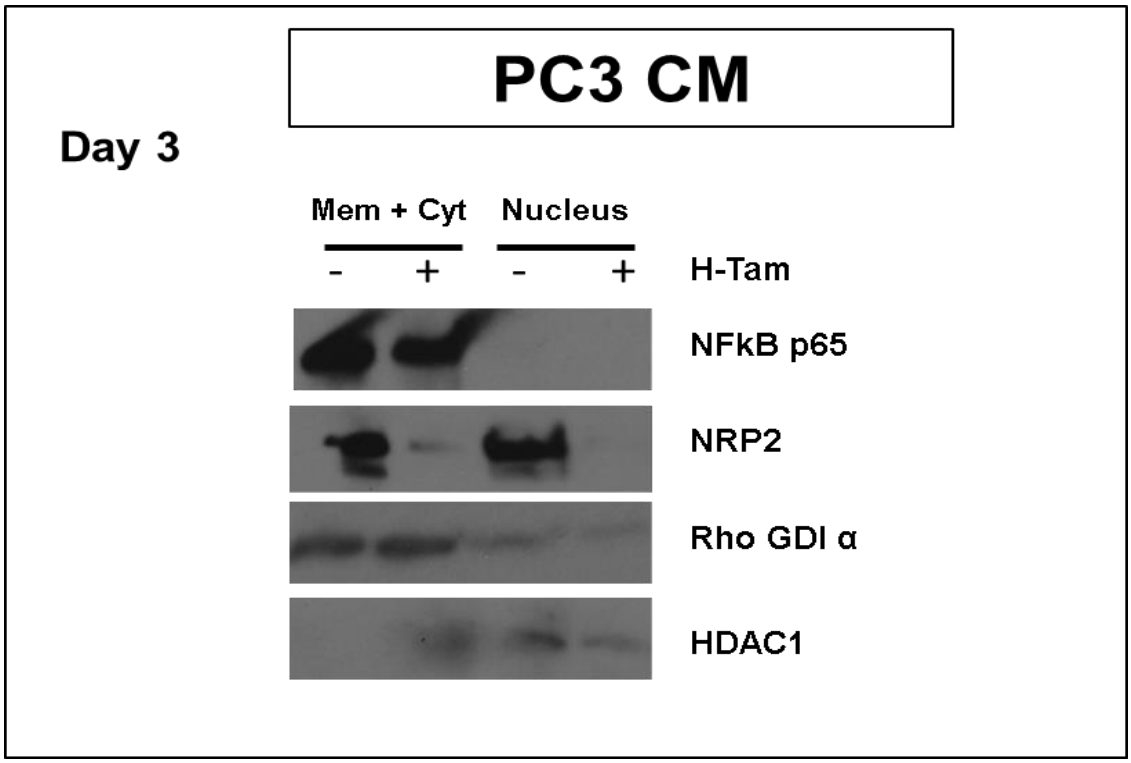


Figure **5.3**: Localization of NFκB in the nucleus following depletion of NRP2 in osteoclast precursors induced by PC3 CM at day 3. Analysis of NFκB protein in the nuclear and post nuclear fractions in control and NRP2 knockout osteoclasts.

GM-CSF is highly detected in CM from PC3.

Our TRAP staining data on PC3 CM-induced osteoclasts showed that these osteoclasts do not differentiate into large multinucleated cells but are restricted to either 2 or 3 nucleated osteoclasts. Also, the pits formed by these osteoclasts are small and high in number. This indicates that PC3 CM induces osteoclast to an extent that they are active even in 2-3 nucleated state with moderate expression of osteoclastic genes sufficient for the resorptive function. It also implicates that the differentiation and function of osteoclasts in PC3 CM differ from those in LNCaP C4-2B CM and that NRP2 does not play any role in these osteoclasts.

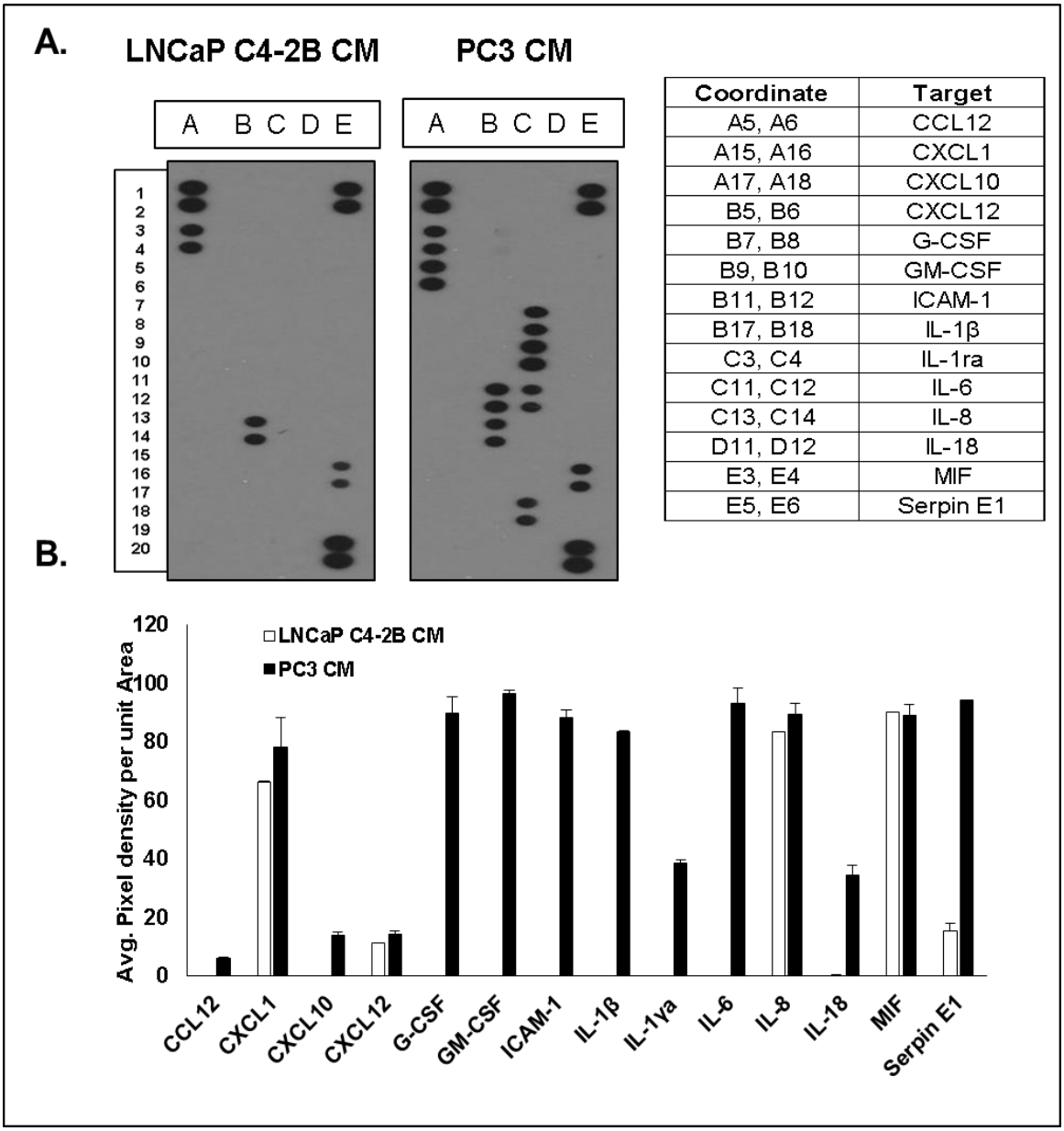
Cytokines released by prostate cancer cells have been reported to play crucial roles in the disease progression and metastasis. Secretion of certain cytokines is cell-specific and is dependent on their requirement in the microenvironment. Prostate cancer cells are known to release various growth factors and signaling molecules to promote their survival and progression in the bone microenvironment. It is understood from the functions of PC3 and LNCaP C4-2B CM that they secrete different factors to regulate the functioning of osteoclasts. However, it is still unknown what secreted factors from these cell lines govern the variance in their functions in osteoclasts. Hence, we evaluated the CM of PC3 and LNCaP C4-2B for the secreted factors. As it is well-known that RANKL signaling is central to osteoclast differentiation, we analyzed the levels of RANKL in the CM of PC3 and LNCaP C4-2B. We detected very low levels of RANKL nearly 1 pg/ml and 2 pg/ml in PC3 and LNCaP C4-2B CM respectively (Fig. 5.5). This suggests that RANKL signaling is not functional in prostate cancer-induced osteoclasts. In

a metastatic bone microenvironment, RANKL is released by other cells such as osteoblasts. Hence, we elevated the differentiation and function of osteoclasts in PC3 CM by supplementing with RANKL at a concentration of 50 ng/mL. We observed that even at day 6 of differentiation, the osteoclasts looked similar in non-treated and RANKL-treated condition in the presence of PC3 CM. Also, no change was observed after depletion of NRP2 in these conditions. This suggests that RANKL/RANK pathway is not central to the osteoclasts differentiated in the presence of PC3 CM (Fig. 5.6).

We next checked the levels of another potent growth factor, M-CSF which is a determinant in the commitment of myeloid progenitors into osteoclast and macrophage precursors. It is also involved in osteoclastic differentiation via the RANKL signaling. ELISA of the CM from PC3 and LNCaP C4-2B was conducted to analyze the concentration of M-CSF. We detected around 500 pg/ml of M-CSF in LNCaP C4-2B CM and 600 pg/ml in PC3 CM (Fig. 5.7). The presence of M-CSF in the CM of prostate cancer cell lines indicate that the early commitment of myeloid progenitors to osteoclastic precursors occurs confirmed by TRAP staining where we observed mononucleated TRAP-positive cells, but the differentiation of osteoclasts is not solely mediated by M-CSF and requires the signaling from other secreted cytokines and growth factors.

To evaluate the presence of osteoclast promoting factors in the CM of prostate cancer cell lines, we conducted a cytokine array where we analyzed the presence of 36 different cytokines in the CM. Interestingly, we found few cytokines in LNCaP C4-2B CM. CXCL1, CXCL12, IL-8, MIF, and Serpin E1 were detected in

LNCaP C4-2B CM. Of these, IL-8 and CXCL1 which are positive inducers of osteoclasts were found to be present in high levels. On the contrary in PC3 CM, we detected several osteoclast inducing factors that were reported in the literature. We observed CCL12, CXCL1, CXCL10, CXCL12, G-CSF, GM-CSF, IL-1β, IL-1F3, IL-6, IL-8, IL-18, MIF, and Serpin E1 in the PC3 CM. Among these secreted factors, CXCL1, G-CSF, GM-CSF, IL-1β, IL-6, and IL-8 which are reported to have functions in osteoclast differentiation were found to be highly present. Interestingly, GM-CSF is a potent inhibitor of early osteoclastic differentiation and fusion and is present at a prominent level in the PC3 CM (Fig. 5.4). An ELISA to analyze the levels of GM-CSF in PC3 and LNCaP C4-2B CM was conducted. We observed very low level of GM-CSF in LNCaP C4-2B CM and very high concertation of 590 pg/ml was detected in PC3 CM (Fig. 5.8). GM-CSF in high concentrations can induce immature dendritic cell commitment which by the function of c-Fos can differentiate into TRAP-positive osteoclasts. There is a possibility that PC3 CM-induced osteoclasts are derived from immature dendritic cells rather their osteoclastic precursors and this switch in precursors of osteoclasts can be related to the evasion of NRP2 regulation in PC3 CM osteoclasts.



Coordinate	Target
A5, A6	CCL12
A15, A16	CXCL1
A17, A18	CXCL10
B5, B6	CXCL12
B7, B8	G-CSF
B9, B10	GM-CSF
B11, B12	ICAM-1
B17, B18	IL-1β
C3, C4	IL-1ra
C11, C12	IL-6
C13, C14	IL-8
D11, D12	IL-18
E3, E4	MIF
E5, E6	Serpin E1

Figure **5.4**: Cytokine analysis of LNCaP C4-2B and PC3 CM. **A.** Immunoblot and table showing cytokine array profile in CM**. B.** Graphical illustration of the comparison of cytokines identified in the CM of LNCaP C4-2B and PC3.

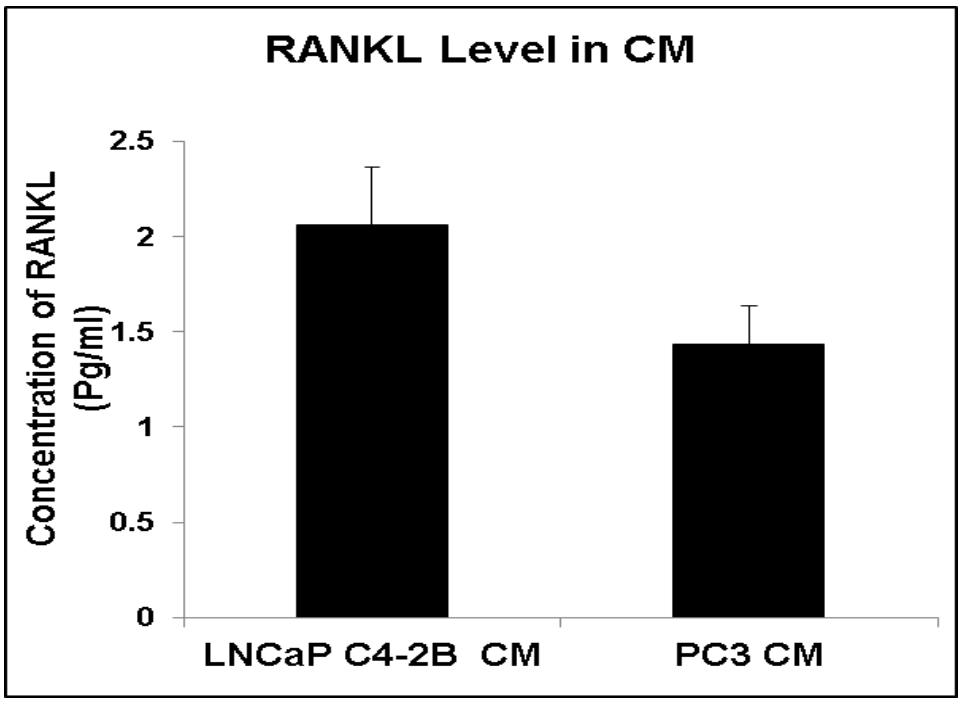


Figure **5.5**: Graphical representation of RANKL concentration (pg/ml) in LNCaP C42B and PC3 CM.

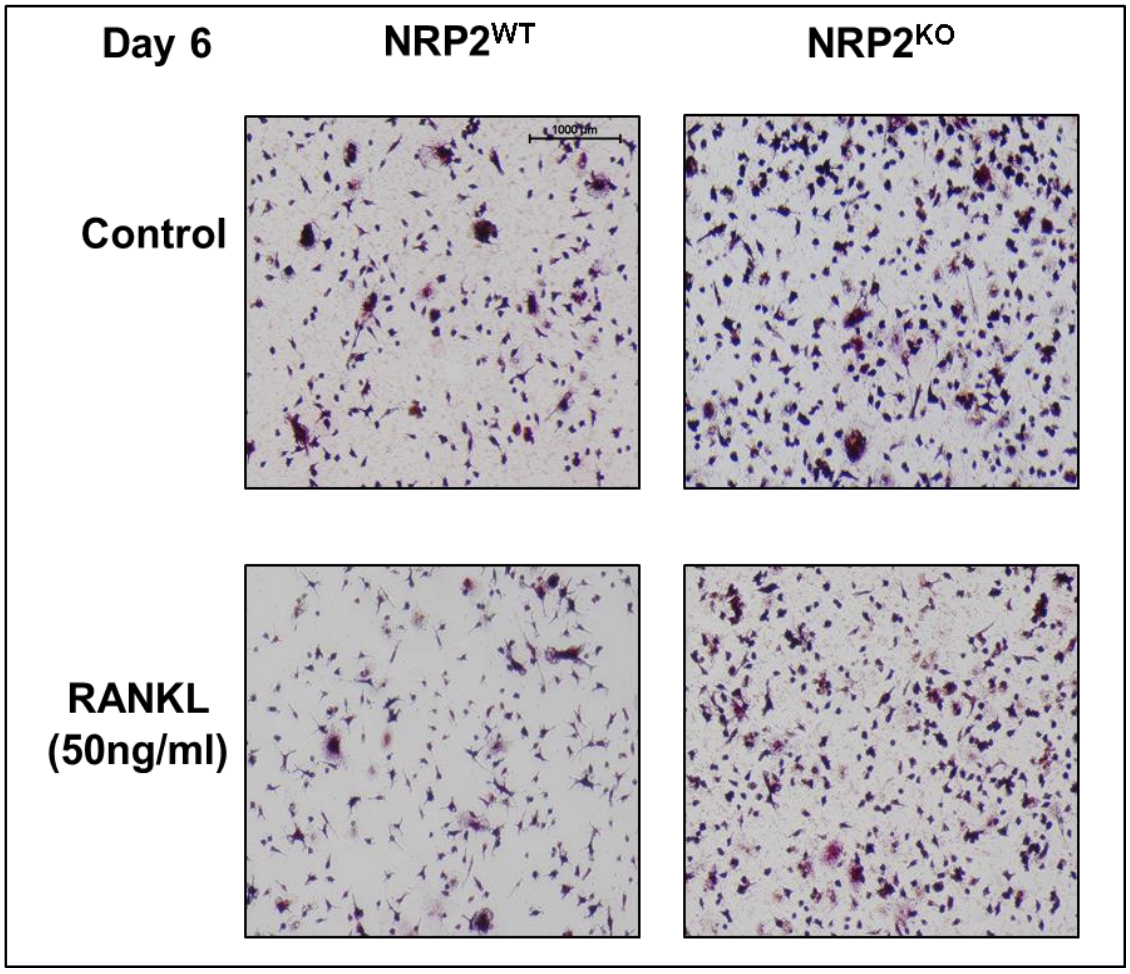


Figure **5.6**: TRAP staining of osteoclasts at day 6 in control and NRP2 depleted state in PC3 CM without and with RANKL (50 ng/mL).

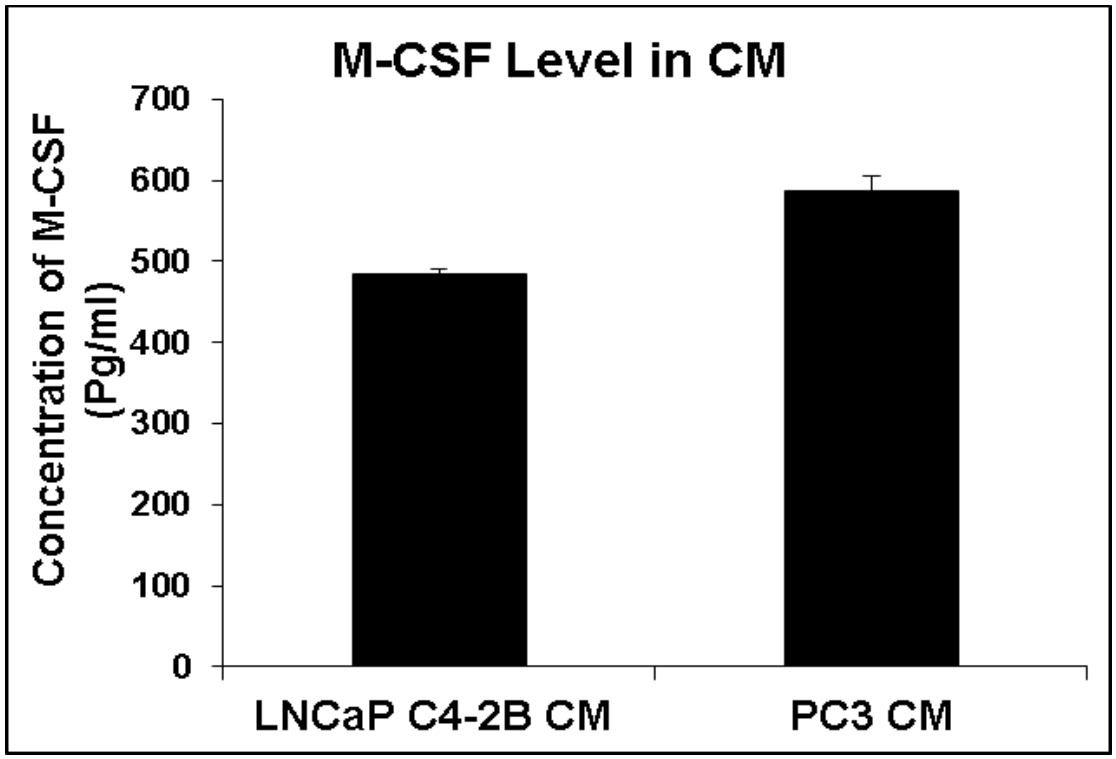


Figure **5.7**: Graphical representation of M-CSF levels in LNCaP C42B and PC3 CM

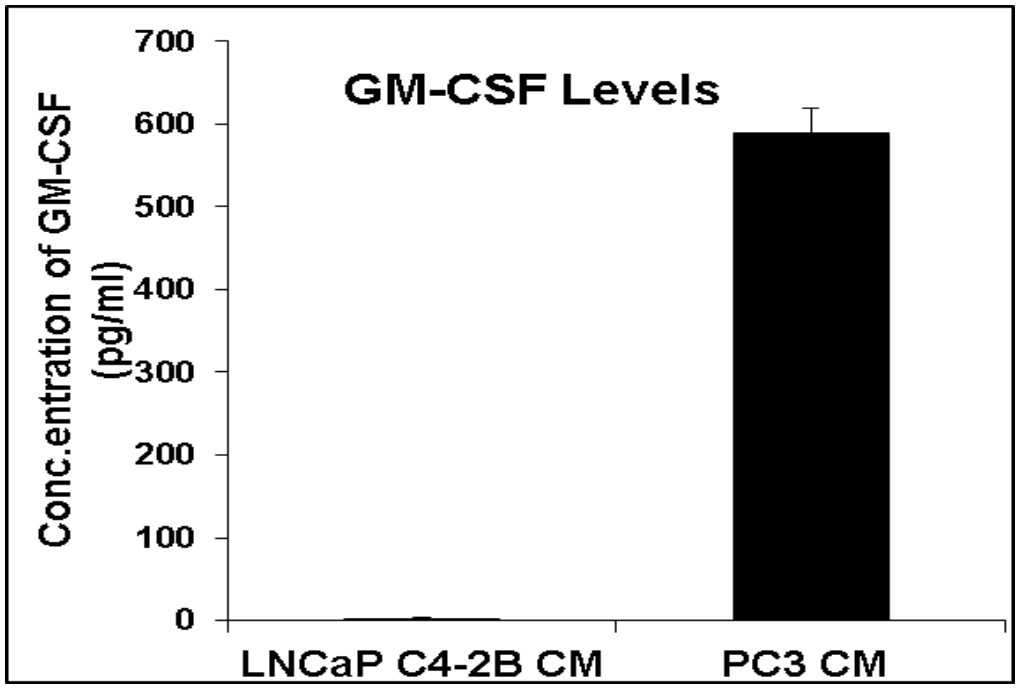


Figure **5.8**: Graphical representation of GM-CSF levels in LNCaP C42B and PC3 CM

GM-CSF inhibits osteoclast differentiation and fusion in healthy osteoclasts and LNCaP C4-2B induced osteoclasts.

To test whether high GM-CSF levels can inhibit osteoclast differentiation and fusion, we treated osteoclasts with varying concentrations of GM-CSF in conditions of RANKL and M-CSF. On day 2 of osteoclastic differentiation, we observed a decrease in the no. of TRAP-positive osteoclasts which can occur due to the commitment of myeloid precursors to immature dendritic cells rather than osteoclastic precursors (Fig. 5.9). Our observations also indicate the presence of mononuclear cells suggesting a delay in the fusion of these osteoclasts in the presence of GM-CSF in comparison to the control. However, at day 3 of differentiation, the number of TRAP-positive osteoclasts in GM-CSF treated condition were comparable to the untreated osteoclasts, but the delay in the fusion as characterized by the mononuclear osteoclasts persisted in the GM-CSF treated state (Fig. 5.10). We observed similar effect in osteoclasts depleted of NRP2 and treated with RANKL and M-CSF (Fig. 5.9, Fig. 5.10). These findings indicate that GM-CSF inhibited the early differentiation and fusion of osteoclasts and removal of NRP2 did not rescue the cells from the inhibition. However, in the later stages of osteoclast differentiation, GM-CSF inhibited the fusion of osteoclasts but did not restrict the osteoclast differentiation.

LNCaP C4-2B has low osteolytic function which is depicted in their differentiation ability of osteoclasts. Depletion of NRP2 increased the osteolytic capability of the osteoclasts in this condition. The osteoclasts induced by LNCaP C4-2B CM are generally mononucleated or rarely binucleated, but NRP2 removal caused the

cells to fuse and become functional multinucleated osteoclasts and further the secreted GM-CSF levels in the CM of LNCaP C4-2B is low. In such a condition, we wanted to test whether the addition of GM-CSF can inhibit the osteoclastic differentiation and function in NRP2 depleted osteoclasts in LNCaP C4-2B CM. We observed significant reduction of osteolytic activity upon GMCSF addition in NRP2-depleted osteoclasts in LNCaP C4-2B CM during early differentiation. At day2, we observed a decrease in the total number of TRAP positive mononuclear osteoclasts in GM-CSF treatment (Fig. 5.11). Also, we witnessed a delay in the fusion of osteoclasts in NRP2-depleted cells indicating that GM-CSF inhibits osteoclast differentiation and fusion in NRP2 depleted osteoclasts during early differentiation. Similar to osteoclasts in RANKL and M-CSF condition, GM-CSF did not inhibit differentiation at day 3 but inhibited osteoclast fusion (Fig. 5.12).

Taking together all these results, we can deduce that GM-CSF is an inhibitor of early osteoclast differentiation and fusion. The secretion of GM-CSF by PC3 may contribute to the maintenance of osteoclasts in mononucleated state and thus help the cells to bypass the regulation of NRP2.

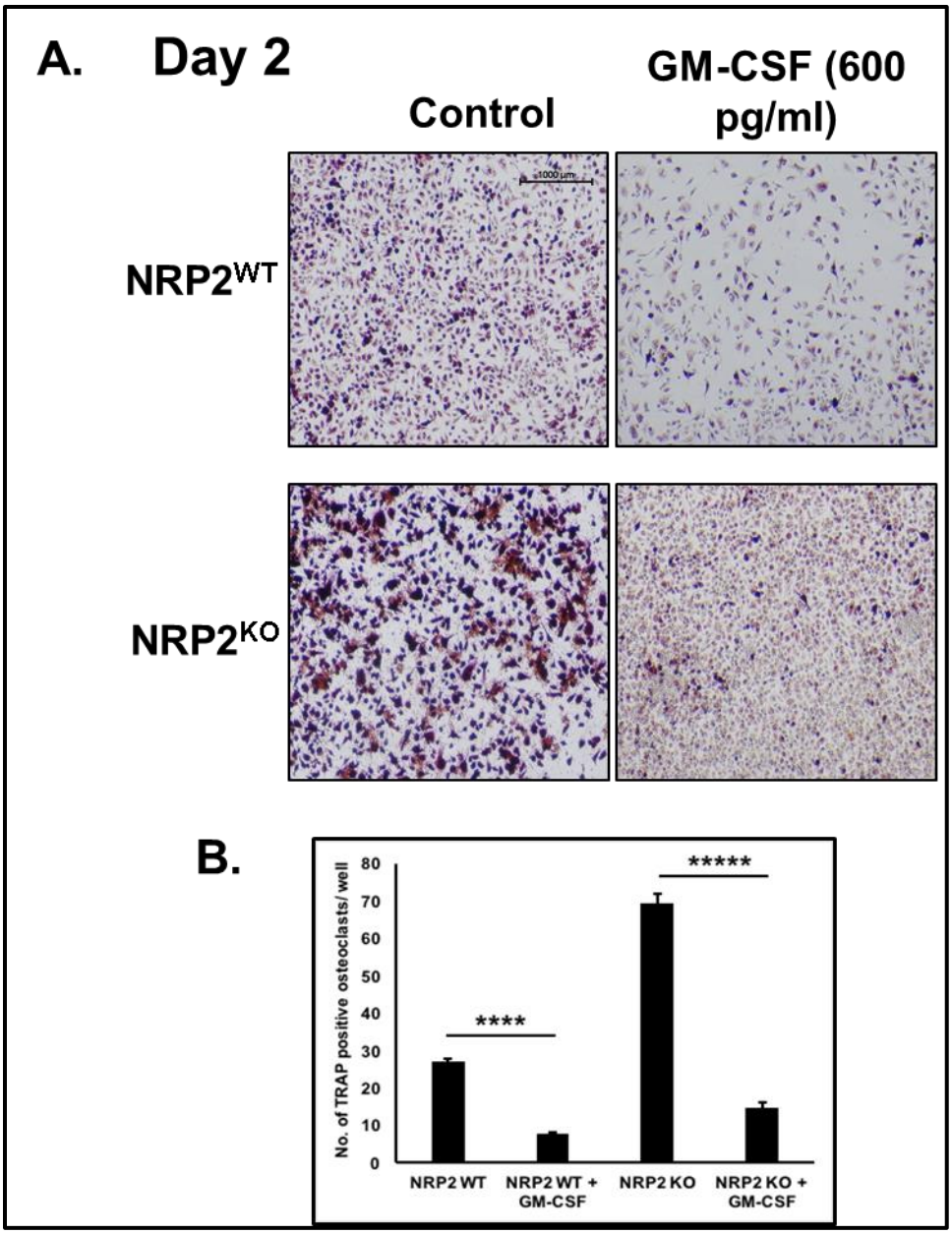


Figure **5.9**: **A.** TRAP staining of osteoclasts at day 2 in control and NRP2 depleted state in RANKL and M-CSF without and with GM-CSF (600 pg/mL). **B.** Graphical representation of the quantification and comparison of TRAP-positive MNCs/well in control and GM-CSF treated osteoclasts in $NRP2^{WT}$ and $NRP2^{KO}$ osteoclasts.

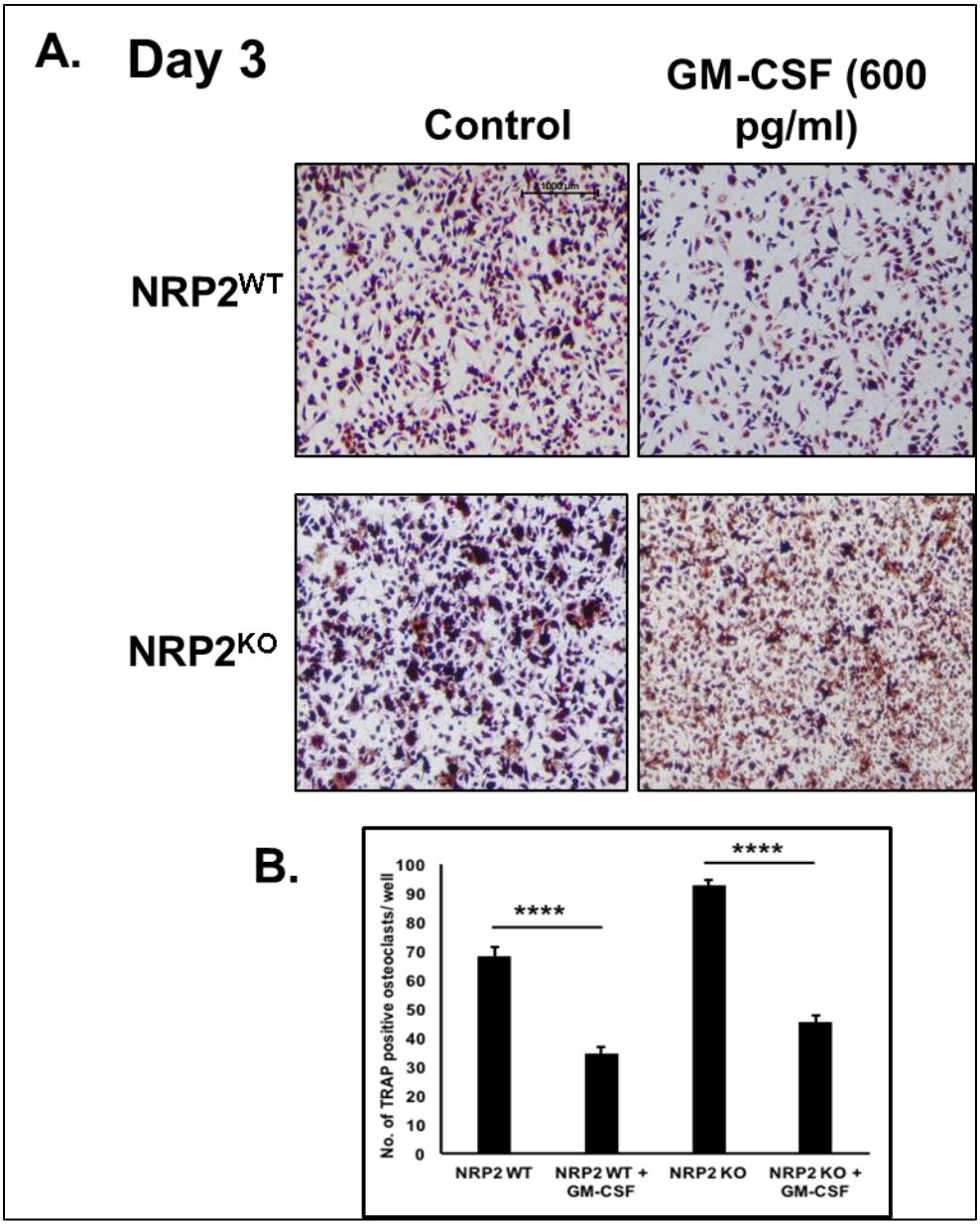


Figure **5.10**: **A.** TRAP staining of osteoclasts at day 3 in control and NRP2 depleted state in RANKL and M-CSF without and with GM-CSF (600 pg/mL). **B.** Graphical representation of the quantification and comparison of TRAP-positive MNCs/well in control and GM-CSF treated osteoclasts in $NRP2^{WT}$ and $NRP2^{KO}$ osteoclasts.

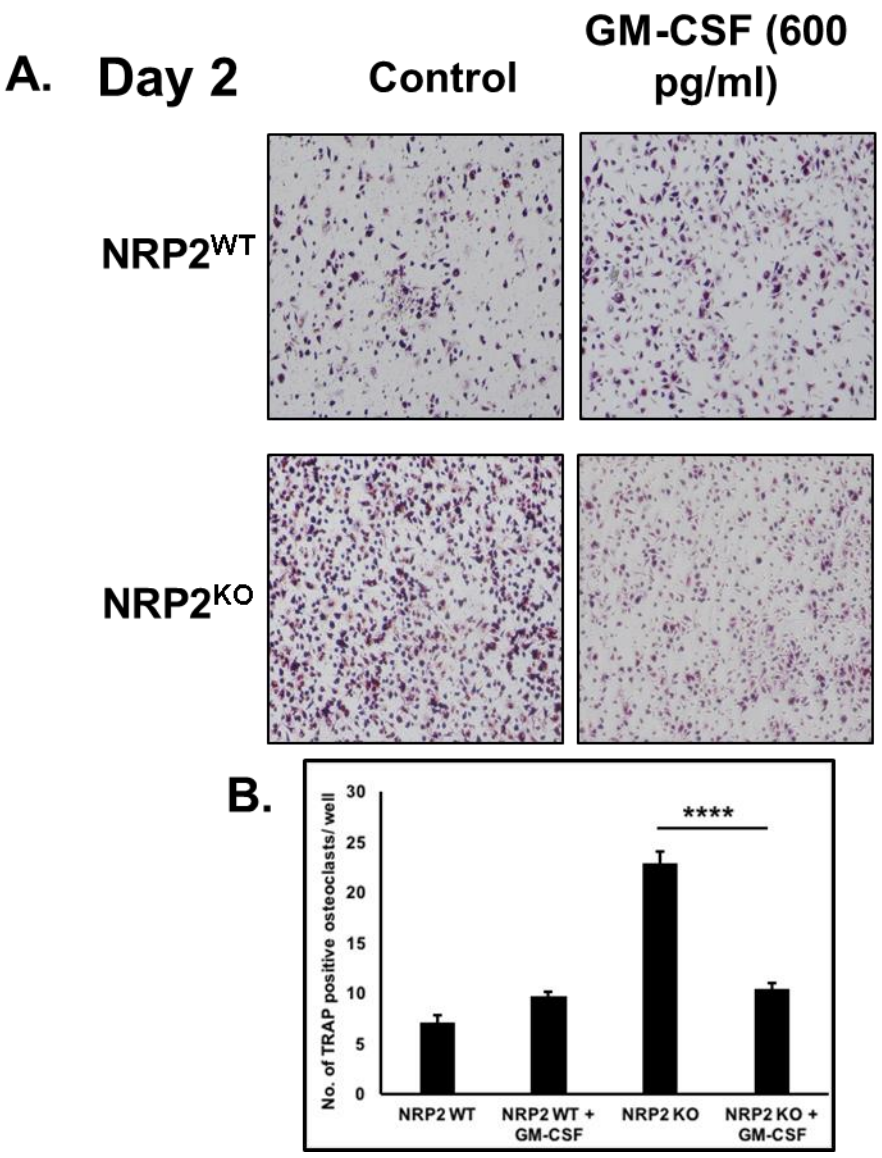


Figure **5.11**: **A.**TRAP staining of osteoclasts at day 2 in control and NRP2 depleted state in LNCaP C4-2B CM without and with GM-CSF (600 pg/mL). **B.** Graphical representation of the quantification and comparison of TRAP-positive MNCs/well in control and GM-CSF treated osteoclasts in $NRP2^{WT}$ and $NRP2^{KO}$ osteoclasts.

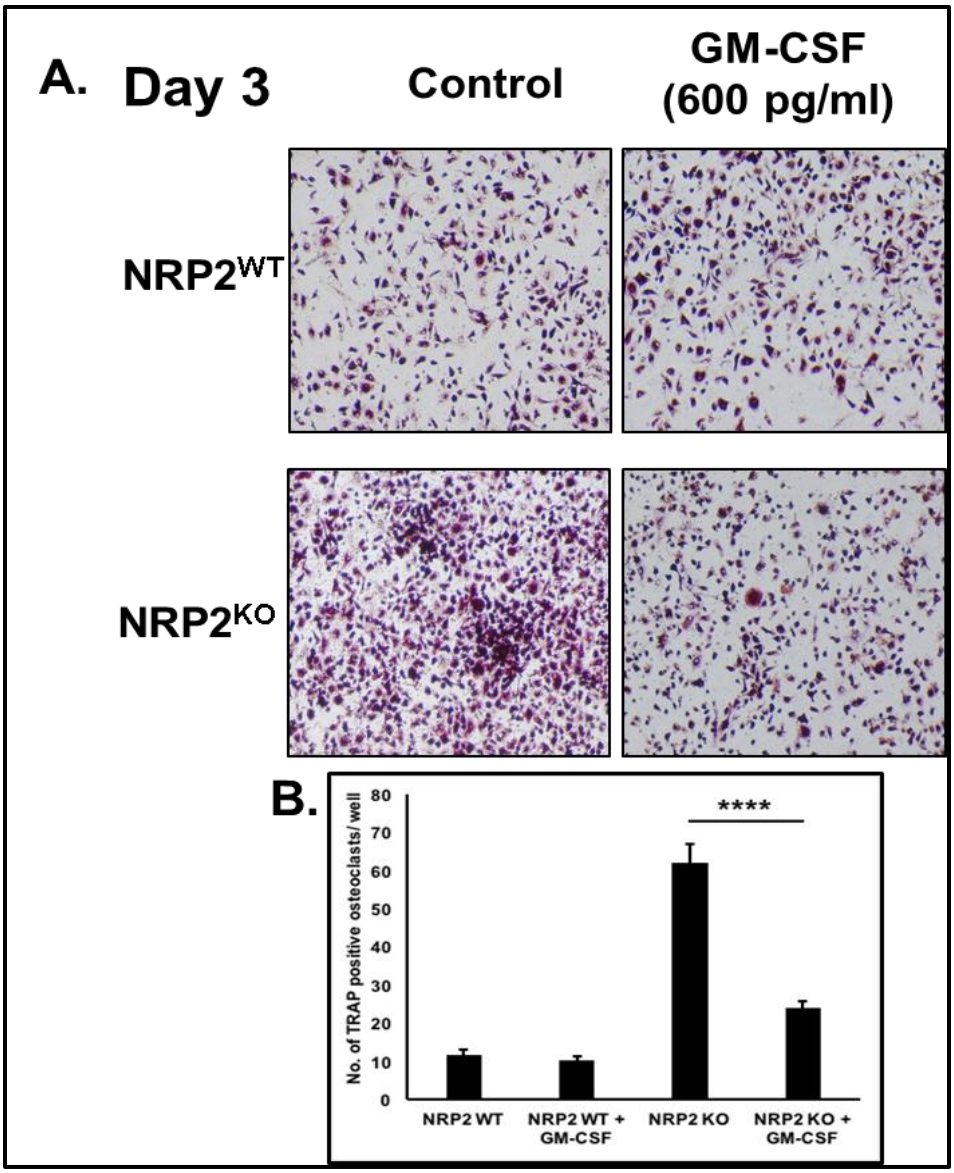


Figure **5.12**: **A.** TRAP staining of osteoclasts at day 3 in control and NRP2 depleted state in LNCaP C4-2B CM without and with GM-CSF (600 pg/mL). **B.** Graphical representation of the quantification and comparison of TRAP-positive MNCs/well in control and GM-CSF treated osteoclasts in $NRP2^{WT}$ and $NRP2^{KO}$ osteoclasts.

Discussion

Osteolytic prostate cancer is rare in occurrence but is aggressive leading to decrease in the quality of life and can cause deaths. Understanding the biology of osteolysis induced by prostate cancer can be helpful in developing a better therapy. Our study addressed the regulatory function of NRP2 in osteoclasts in physiological condition and prostate cancer bone metastasis condition. We reported in chapter 4 that NRP2 regulates the localization and thus functions of both NFATc1 and NFκB in osteoclasts differentiated by RANKL/MCSF and only NFATc1 in LNCaP C4-2B CM. Contrary to these findings, no change in NFATc1 or NFκB was found in PC3 CM after depletion of NRP2 in osteoclasts In fact, both NFATc1 and NFκB were undetected in the nucleus in our experimental condition suggesting the involvement of other transcription factors who may play a dominant role in inducing osteoclast-specific gene expression following the stimulation of PC3 CM. Currently, the identity of these transcription factors are not known, although we hypothesize that their activities are not regulated by NRP2. We checked the expression of various transcription factors and detected PU.1, AP1, ATF1, ATF2, ATF4, TAL1 and TFE3 at mRNA level in osteoclast. Interestingly, TAL1 is an inhibitor of osteoclastic fusion, and its expression is increased in osteoclasts induced by PC3 CM. TAL1 is reported to suppress osteoclast fusion gene, DC-STAMP at the transcriptional level by counteracting the function of active PU.1 and MITF. Our previous findings in PC3 CM showed that osteoclasts induced by PC3 CM promote the differentiation and activation of osteoclasts, but the fusion of osteoclasts is restricted thereby maintaining mononu-

clear phenotype. In fact, active osteoclasts are not always multinucleated but small, mononuclear osteoclasts also exist which are fully functional. The reason for retaining the osteoclasts in the mononuclear state can be related to their decreased metabolism and higher bone surface coverage. In the presence of TAL1, PC3 CM-induced osteoclasts may control fusion events to promote energy efficient osteolysis. However, the expression of these transcription factors was detected at mRNA level which does not necessarily rely into protein that can actively localize in the nucleus and transcribe genes. Hence, future studies will address the role of these transcription factors in the osteoclasts induced by PC3 CM.

The osteoclasts induced by PC3-derived factors have moderate resorptive capability, but show decreased fusion events. This indicates that PC3 releases signaling factors that regulate the differentiation and fusion of osteoclasts. Our cytokine profile analysis detected various cytokines and growth factors released by PC3 cells. Many of the secreted factors such as IL-1, IL-6, IL-8, CCL1, CXCL1, CXCL10, CXCL12 and G-CSF are known inducers of osteoclasts. However, one growth factor GM-CSF is released by PC3 at a prominent level. GM-CSF is a known inhibitor of early osteoclast differentiation. In the presence of GM-CSF, myeloid progenitors become osteoclasts in a two-step differentiation. GM-CSF signals the myeloid cells to commit to become immature dendritic cells. These immature dendritic cells upon activation of c-Fos differentiate to become TRAP-positive mononuclear osteoclast-like cells. Further, GM-CSF inhibits the expression of DC-STAMP to downregulate fusion of osteoclasts. Together, all of this

information indicates that GM-CSF secretion can be crucial for the type of osteoclasts we frequently observed when cultured in PC3 CM.

In the final part of our study, we addressed whether GM-CSF has an inhibitory effect on osteoclast differentiation either in the presence of RANKL/M-CSF or when treated with CM of LNCaP C4-2B especially when NRP2 was deleted. . Our TRAP staining suggests that, GM-CSF delayed osteoclastic differentiation, as well as fusion of osteoclasts during their early differentiation by RANKL/M-CSF. Importantly, depletion of NRP2 in the presence of GMCSF failed to promote the hyperactivation of osteoclasts. A delay in osteoclast differentiation and fusion was also observed due to addition of GM-CSF during early differentiation of osteoclasts induced by LNCaP C4-2B CM and once again depletion of NRP2 failed to hyperactivate their differentiation. These results therefore suggested that GMCSF has a distinct effect on the normal process of osteoclast differentiation, and it also uncouples the regulation of NRP2 on osteoclasts. It is understood from the previous reports, GM-CSF sways the myeloid cells towards immature dendritic cells, which are able to express bone resorbing enzymes although rarely form multinucleated structures. Because they follow a different differentiation program, it is likely that the transcription factors involved here are different from the classically known transcription factors involved in osteoclast differentiation. Our failure to detect NFATc1 and NFκB in the nucleus of the osteoclasts when treated with PC3 CM, despite being able to express osteoclastic genes supports this hypothesis and thus highlights the involvement of a different set of transcription factors, which are distinct from NFATc1 and NFκB. Although, we

could detect RNA expression of other transcription factors known for their ability to promote osteoclast differentiation in PC3 CM treated osteoclasts, their contribution in osteoclastic gene expression under this condition is currently unknown. It is also possible that some novel transcription factors play critical roles in inducing osteoclastic genes following their activation by PC3 CM. We detected several cytokines such as IL-6, IL-1β, IL-18, CD54 and Serpin E1, which are uniquely expressed in the CM of PC3 and are known for their ability to differentiate osteoclast. These cytokines along with GMCSF potentially regulate a transcriptional machinery specific for PC3 CM for the expression of osteoclastic genes. We also speculate that in the absence of GMCSF, the positive inducers of osteoclastic differentiation present in PC3 CM can commit the myeloid cells to become osteoclastic progenitors and thus promote a canonical differentiation program and further sensitize the osteoclasts to NRP2 regulation. These are important questions, which will be the focus in our future studies.

CHAPTER 6

Major conclusions and limitations

NRP2: A crucial regulator of osteoclasts in prostate cancer bone metastasis.

Prostate cancer bone metastasis is a complex disease. The incidence of bone metastasis in advanced-stage prostate cancer patients is very high (nearly 80%). Unlike other cancers such as breast and multiple myeloma where osteolytic lesions are predominant, the bone metastasis induced by prostate cancer cells show mixed osteoblastic and osteolytic lesions. Although radiological evidences from prostate cancer patients implicate the occurrence of osteoblastic metastases is high over osteolytic lesions, the manifestation of prostate cancer causes functional dysregulation in bone cells causing mixed lesions. Recent studies on osteoclasts in prostate cancer bone metastasis reported that the circulating prostate cancer cells establish in the bone at discrete sites where hematopoietic stem cells are present during initial stages of homing in the bone. In the hematopoietic niche, the prostate cancer cells release cytokines and growth factors to induce dysfunctional osteoclasts leading to osteolysis of bone. This function of osteolysis is important for the survival, proliferation and progression of cancer cells in the hypoxic bone environment. Therefore, therapies targeting osteoclasts such as bisphosphonates, denosumab (RANKL inhibitors) have been developed. Unfortunately, these treatment strategies are mainly palliative in nature and are used to treat skeletal related events (SREs) associated with bone metastasis. Clinically, these patients are beyond the scope of any curative treatment. Moreover, the overall complex architecture of the bone cannot be rescued with these therapies. Hence, the understanding of the biology as well as the signaling of

prostate cancer cells with osteoclasts is a prerequisite for developing an effective treatment strategy.

In this dissertation, I have examined the role of NRP2 in osteoclasts and have elucidated its function as a negative regulator of osteoclastic differentiation and activity both during physiological condition or when induced by metastatic prostate cancer cells. My studies have therefore indicated a role of NRP2 in harnessing the activity of osteoclast in an optimum level required for the physiological functions of bone and also during the metastatic progression of prostate cancer. Hyperactivity of osteoclasts is associated with osteoporotic lesions as frequently detected in Paget's disease, metastatic breast cancer, multiple myeloma and other diseases. The loss of function of NRP2 like regulators is often detected as the underlying molecular mechanism for the pathological activation of osteoclasts and therefore should be thoroughly studied. Further, I have discussed one of the intriguing findings that the key transcription factor, NFATc1 is regulated by NRP2 in osteoclasts. The detailed summary of all the findings in this study is as follows.

NRP2 expressed in osteoclasts in prostate cancer bone metastasis.

Previous reports from our group has shown that NRP2 is expressed on prostate cancer cells that are metastasized to bone and its removal causes the sensitization of cancer cells to therapeutic agents such as Docetaxol. Therefore, NRP2 axis can be a potential therapeutic target for prostate cancer bone metastasis. A therapy targeting NRP2 is unlikely be specific for cancer cells and can potentially block NRP2 axis in other cells present in cancer microenvironment. I am there-

fore interested to understand what might be the effect of inhibiting NRP2 axis in those cells and their overall effect in cancer growth. A recent study showed that osteoclasts express NRP2 in healthy bone. Therefore, I was interested to know whether expression of NRP2 is maintained in osteoclast during its induction by metastatic prostate cancer cells and its implications in prostate cancer bone metastasis. In this respect, it is also important to note that the previous study did not address the importance of NRP2 in osteoclastogenesis during physiological bone remodeling process. Here, I showed for the first time that prostate cancer cells induce the osteoclasts to express NRP2. PC3 is a prostate cancer cell line, which promotes osteolysis and LNCaP C4-2B induces mixed lesions in mice. Interestingly, the expression of NRP2 in osteoclastic precursors remained undetected and upon differentiation, NRP2 levels increase at substantial rate in the osteoclasts. Furthermore, the expression of NRP2 in healthy (RANKL/M-CSF treated) osteoclasts is much higher than in osteoclasts induced by bone metastatic prostate cancer cells. However, no difference in the expression of NRP2 was observed between the osteoclasts differentiated by LNCaP C4-2B and PC3 CM. Altogether, NRP2 is induced in osteoclasts in prostate cancer bone metastasis.

NRP2 regulates differentiation and function of osteoclasts in prostate cancer bone metastasis.

The expression of NRP2 suggests that it has a function in osteoclasts. To understand the function of NRP2, osteoclastic precursors were depleted of NRP2 and their differentiation and function was analyzed under prostate cancer CM. Intri-

guingly, NRP2 depletion in RANKL and M-CSF influenced osteoclasts showed a drastic increase in osteoclast differentiation and activation. The NRP2–depleted osteoclasts showed increased expression of osteoclastic genes as well as increased resorptive capability. In LNCaP C4-2B CM, removal of NRP2 showed a substantial increase in the differentiation and function of osteoclasts. Large, multinucleated osteoclasts in comparison to the control were observed in NRP2 depleted osteoclasts and the resorbing capacity of these cells increased exponentially from their control counterparts. Contrary to these observations, osteolytic prostate cancer such as PC3-induced osteoclasts did not show any change in differentiation and activation upon depletion of NRP2. Collectively, it can be concluded that NRP2 negatively regulates osteoclast differentiation and function under physiological condition and when induced by LNCaP C4-2B cells, which promote mixed lesions as observed in majority of prostate cancer patients with bone metastasis. Interestingly, PC3, which promotes mainly osteolytic lesions, induce osteoclasts through an NRP2-independent mechanism.

NRP2 controls NFATc1 to regulate osteoclast differentiation and activation in prostate cancer bone metastasis.

With the information that NRP2 regulates osteoclast differentiation and activation, it became imperative to address how NRP2 functions to hamper osteoclasts. In our earlier data, we observed a significant increase in the expression of many of the osteoclastic genes in the absence of NRP2, which directs our attention towards signaling molecules such as transcription factors that promote gene tran-

scription. Hence, we studied transcription factors that have been reported to be central to osteoclasts, NFATc1 and NF-κB, to evaluate whether NRP2 regulates the function of these transcription factors. Compelling evidence from protein and immunofluorescence analysis implicate that NRP2 inhibits the expression and localization of NFATc1 in RANKL and M-CSF and prostate cancer bone metastasis. Further, translocation of NF-κB into the nucleus is regulated by the signaling of NRP2 in RANKL and M-CSF condition suggesting that NF-κB cooperatively function with NFATc1 to promote osteoclastogenesis. In this scenario, it is interesting to understand whether NF-κB is activated prior to NFATc1 and that it is involved in the early induction of NFATc1. Also, we observed an increase in the global expression of NFATc1 in NRP2- depleted osteoclasts in RANKL and M-CSF and LNCaP C4-2B CM. This data entails that NFATc1 which is central to the osteoclastogenesis is inhibited by NRP2 signaling. It is well understood that NFATc1 expression is regulated by its upstream signaling which includes Ca2+ signaling. Furthermore, NFATc1 upregulates its own transcription via the autoamplification mechanism. Future studies in this direction can provide insight into the mechanism of action of NRP2 in osteoclasts in prostate cancer bone metastasis (Fig. 6.1).

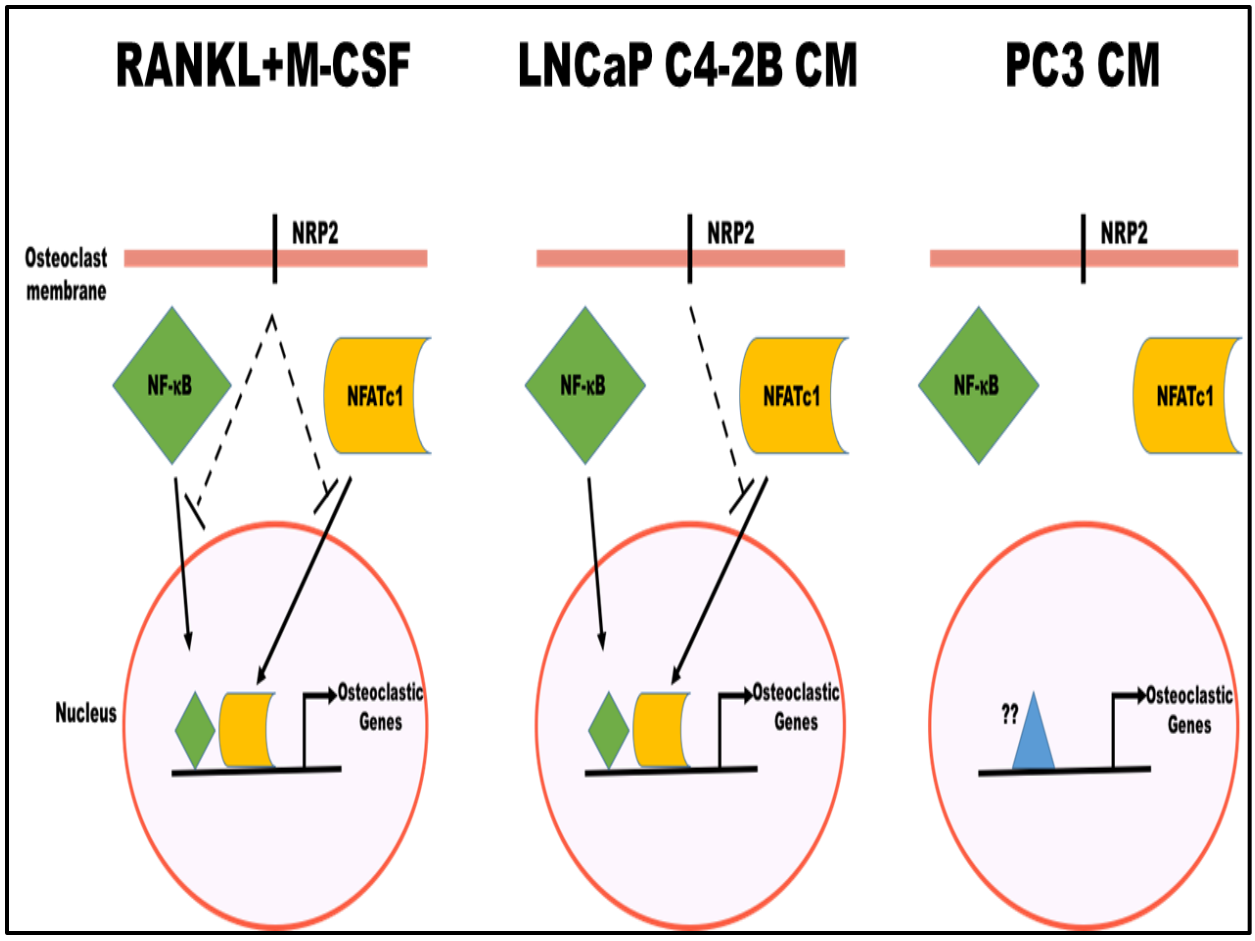


Figure **6.1**: Schematic illustration of the molecular pathways through which NRP2 regulates the gene transcription in osteoclasts. In RANKL and M-CSF, NRP2 inhibits NFATc1 and NF-κB translocation into the nucleus while only NFATc1 nuclear translocation is blocked by NRP2 in LNCaP C4-2B CM. Osteoclasts differentiated by PC3 CM do not work through NFATc1 and NF-κB but withunknown transcrition factors to promote osteoclastic gene transcription.

Osteolytic PC3 evades the regulation of NRP2 in osteoclasts.

Although the expression of NRP2 was observed in PC3 CM-induced osteoclasts, NRP2 depletion did not change the differentiation and function of osteoclasts. Few interesting observations in osteoclasts paved way to the further investigation of PC3 CM. We observed that PC3 CM-induced osteoclasts were able to conduct significant expression levels of osteoclastic genes and were not influenced by the status of NRP2. This hints that PC3 CM activates transcription factors, which are not regulated by NRP2 to enable the expression of osteoclastic genes. We did not observe the localization of NFATc1 and NF-κB in the nucleus in PC3 CM induced osteoclasts. This implies that these crucial factors that are central to osteoclasts are not activated by PC3 CM. Further studies into the transcription factors will enable the understanding of the signaling pathways activated by PC3 CM. Also, the osteoclasts in PC3 CM maintain 2-3 nucleated state and the resorptive function of these osteoclasts is limited to pore formations rather than large pits. This raised the question- what is present in the PC3 CM that is restricting the fusion and function. Cytokine analysis of the PC3 CM showed high concentrations of GM-CSF, which is a reported inhibitor of early differentiation and fusion in osteoclasts. Addition of GM-CSF in osteoclasts induced by RANKL and M-CSF and LNCaP C4-2B CM caused a delay in the early differentiation and fusion of the osteoclasts. In addition, treatment of NRP2-depleted osteoclasts with GM-CSF also showed a lag in the differentiation and fusion of osteoclasts in these condi-

tions. This event can be a result of uncoupling of the GM-CSF and NRP2 regulation pathways. This further confirms our findings in PC3 CM that presence of GM-CSF restrains the osteoclast differentiation and fusion in PC3 CM. It will be important to study whether removal of GM-CSF from the PC3 CM can facilitate the regulation of NRP2 in osteoclasts.

In summary, this study delineated the role of NRP2 in osteoclasts in prostate cancer bone metastasis. We report that NRP2 functions as a negative regulator of osteoclasts in prostate cancer bone metastasis but is rendered ineffective in osteolytic lesions. In future, this study can be further extrapolated to understand the mechanisms through which prostate cancer cells promote bone lesions and this insight can be helpful in developing better therapies for prostate cancer bone metastasis.

Limitations:

Although this study showed a novel mechanism of NRP2 in osteoclasts in promoting prostate cancer bone metastasis, there exists some limitations in the understanding of the exact role of osteoclasts induced by prostate cancer cells. It includes but not limited to choice of model system, the experimental setup as well as in the interpretation of results.

Choice of model system: For our study, we used conditioned media from human prostate cancer cell lines that metastasize to bone to induce osteoclasts derived from mouse bone marrow. Use of CM from prostate cancer cell lines is widely used and is effective. However, many growth factors which are known inducers of osteoclasts released by human prostate cancer cells cannot identify or bind to their receptor counterparts on mouse osteoclasts rendering them ineffec-

tive. For example, IL-8 of human origin cannot bind its receptor on mouse osteoclasts. Hence, it would be important to understand whether CM from human prostate cancer cell lines can induce osteoclasts of human origin (PBMCs). Parallel to this, use of mouse model is essential for the elucidation of mechanisms involved promoting bone metastasis. So, this study can be conducted using CM from mouse prostate cancer cell lines such as RM1 which is reported to exhibit bone metastasis. This enables the use of mouse growth factors present in the CM to differentiate osteoclasts efficiently and study mechanisms involved in prostate cancer bone metastasis. Further, these prostate cancer cells can not only be useful in in-vitro studies but also in in-vivo studies.

Experimental setup: Our study mainly focused on the use of in-vitro system to understand the role of NRP2 in osteoclasts induced by CM from prostate cancer cells. Use of CM is effective in differentiating the osteoclasts but in a bone microenviroment, osteoclasts function in co-ordination with osteoblasts and prostate cancer cells in close proximity. Monolayer culturing of osteoclasts is devoid of these interactions. Hence, co-culturing of osteoclasts, osteoblasts, and prostate cancer cells can enable better interaction as well as release of growth factors and binding of osteoblast membrane bound ligands to receptors on osteoclasts.

Interpretation of results: As our model system has limitations in respect to human and mouse model, the cytokine/ growth factors detected in the cytokine analysis needs further validation. Analysis of CM from mouse prostate cancer cell lines can address the discrepancies between the model systems. Further, due to variance in the status of the prostate cancer cells, we observed a variance

in the quality of CM obtained from these cells. Rigorous standardization is required to successfully conduct an experiment. Other factors such as osteoclastic precursors, growth factors and fetal bovine serum affected the isolation, differentiation and culturing of osteoclasts.

CHAPTER 7

Future Directions

Directions for future work in this study:

1. *What are the upstream signaling modulators of NFATc1 controlled by NRP2?*

Our study has shown that NFATc1, a crucial transcription factor in osteoclasts is regulated by NRP2 signaling. Further, the expression of NFATc1 is also enhanced due to removal of NRP2. Previous studies have reported that NFATc1 is activated by the function of various signaling molecules. These are Ca2+/ calcineurin pathway, c-Fos and autoamplification by NFATc1. Our future studies will address which signaling pathway leading to NFATc1 expression and translocation is prominently regulated by NRP2.

2. *Understand how NRP2 function is bypassed by the osteoclasts induced by PC3 CM.*

It is clear from our data that NRP2 regulation is avoided by the PC3 CM induced osteoclasts. In addition, high GM-SCF secreted by PC3 causes a delay in the differentiation and fusion of osteoclasts as suggested by our results. The question arises whether removal of GM-CSF from the CM of PC3 can sensitize the osteoclasts to the regulation of NRP2. To accomplish this, we will transfect siRNA against GM-CSF in PC3 cells and collect the conditioned media which is devoid of GM-CSF and treat osteoclastic precursors in presence or absence of NRP2.

3. *Elucidation of the signaling factors that promote osteoclasts in PC3 CM.*

From earlier results, it is quite evident that PC3 CM downregulates the translocation of major transcription factors, NFATc1 and NF-κB irrespective of the status of NRP2. Also, the expression of osteoclastic genes such as TRAP and cathepsin K suggest that there are transcription factors involved in the osteoclastic gene transcription. Further, mRNA studies on these osteoclasts hint the expression of transcription factors such as PU.1, AP1, TFE3 and TAL1. Hence, we will evalu-

ate which transcription factors are functional in the expression of the osteoclastic genes in the PC3 CM.

4. *In-Vivo model to address prostate cancer bone metastasis.*

Prostate cancer bone metastasis exhibits mixed osteoblastic/osteolytic lesions which makes it clinically untreatable. Recent reports in prostate cancer bone metastasis suggested that prostate cancer cells in the bone localize to the hematopoietic niche. In order to localize to the bone, the prostate cancer cells release growth factors to induce osteoclastic differentiation. The active osteoclasts resorb bone to release growth factors necessary for the survival of prostate cancer cells as well as make space for the cancer cells to settle and grow. Therefore, osteoclasts are the first cells in the bone to be activated. However, the prostate cancer cells proceed to induce osteoblastic thereby promoting mixed lesions. Our previous reports indicate that depletion of NRP2 from bone metastatic prostate cancer cells causes the cells to sensitize to therapeutic agents thereby killing the cells. Further, our data in this study suggest that NRP2 controls the osteoclast differentiation and function. Altogether, our findings imply that NRP2 can be a therapeutic target in prostate cancer bone metastasis. The disease can be targeted with the administration of inhibitors specific for NRP2. However, information is missing as to the effect of NRP2 inhibitors on the prostate cancer cells and the cells in the bone microenvironment. In order to propose NRP2 inhibitors for therapy, it is essential to address the functions of NRP2 in bone microenvironment. *In vivo* studies will be conducted by using RM1 cells (developed using the MPR model and syngeneic to C57BL/6 mice) as a model for studying the interactions between prostate cancer cells and bone stroma specifically osteoclasts. By injecting

the RM1 cells having stably expressing inducible NRP2 shRNA construct intratibially into CSF1R-Cre; NRP2flox/flox mice, we will monitor the progression of prostate cancer in bone and how osteoclasts play a role in the development of bone metastasis. Under these circumstances, we will also evaluate the consequences of removal of NRP2 in the prostate cancer cells by addition of doxycycline as well as in the osteoclasts by administering Tamoxifen. Overall, these in vivo studies will address the critical question of the function of NRP2 in prostate cancer cells as well as osteoclasts and whether NRP2 can be a therapeutic target in prostate cancer bone metastasis.

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