OSTEOCYTE SIGNALING AND ITS EFFECTS ON THE ACTIVITES OF OSTEOBLASTS AND BREAST CANCER CELLS

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science in Biomedical Engineering

Department of Biomedical Engineering at IUPUI

Indianapolis, Indiana

May 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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ACKNOWLEDGMENTS

I would like to appreciate my supervisor, Dr. Sungsoo Na, for his guidance and support. This study would not be possible without his careful direction, expertise, experience and encouragement. I would also like to thank the members of my thesis advisory committee, Dr. Hiroki Yokota and Dr. Jiliang Li for their support throughout my project. Finally, I would like to appreciate my family and friends for their support during my graduate studies.

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2D	Two dimensional
α-SMA	Alpha- smooth muscle actin
AIP	AMPK inhibitor peptide
AMPK	AMP-activated protein kinase
CM	Condition medium
CFP	Cyan fluorescent protein
CTC	Circulating tumor cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
FA	Focal adhesion
FAK	Focal adhesion kinase
FRET	Fluorescent resonance energy transfer LKB1 Liver kinase B1
LCS	Lacuna-canalicular system
MMPs	Matrix metalloproteinases
mROS	Mitochondrial reactive oxygen species
MSN	Moesin
MSN+	Constitutively active moesin
MSN-	Moesin silencing RNA
mTOR	Mammalian target of rapamycin
OPG	Osteoprotegerin
OPN	Osteopontin
RANKL	Receptor activator of NF-κB ligand
ROCK	Rho associated protein kinase
SEM	Standard error of the mean
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein
eNOS	Endothelial nitric oxide synthase

ABSTRACT

Bone is a common location for breast cancer cell metastasis, and progression of tumor in bone can lead to bone loss and affect human health. Osteocytes have important roles in bone homeostasis and osteogenesis, and their interaction with metastasized cancer cells are known to affect progression of metastasized tumor. However, the potential role of metabolic signaling and actin-cytoskeleton-associated moesin in the interaction of osteocytes and tumor cells remain poorly understood.

In this study, we first examined the roles of metabolic signaling, specifically global AMPK modulators and mitochondria-specific AMPK inhibitor (Mito-AIP), as well as mechanical force in beta catenin signaling through interaction between osteocytes and precursor osteoblasts as well as osteocytes and breast cancer cells. We also evaluated the role of metabolic signaling in Rho GTPases including RhoA, Rac1 and Cdc42. We observed that AMPK activator (A769662) and Mito-AMPK stimulated beta catenin translocation to the nucleus, indicating the activation of Wnt signaling, while Mito-AIP did not significantly affect beta catenin activation in osteoblasts. We also observed that osteocyte conditioned medium (CM) treated with Mito-AIP substantially increased beta catenin signaling in osteoblasts, while decreasing beta catenin signaling in breast cancer cells. CM of osteocytes treated with fluid flow increased beta catenin signaling in breast cancer cells. A769662 and Mito-AIP also decreased the activities of RhoA, Rac1, and Cdc42 in cancer cells which are known to regulate cancer cell migration.

Additionally, we evaluated the roles of intracellular and extracellular moesin (MSN) protein in well-established oncogenic signaling proteins, such as FAK, Src, and RhoA as well beta catenin signaling. Constitutively active MSN (MSN+) significantly increased FAK and Src activities in cancer cells, but decreased the activity of RhoA. Surprisingly, CM of mesenchymal stem cells treated with MSN+ decreased the activities of FAK, Src, and RhoA, suggesting the inhibitory role of extracellular MSN in tumor-promoting signaling. Our results suggest the distinct role of AMPK signaling, specifically at mitochondria of osteocytes, in the activities of beta-catenin signaling in osteoblasts and breast cancer cells and the distinct role of intracellular and extracellular MSN in these two types of cell.

1. INTRODUCTION

1.1. Cell Signaling

There are multiple cell signaling pathways in the cell affecting cell function and fate. One of these important pathways is contractility pathway which gets involved in cell contraction, movement and response to mechanical force. The mechanical force gets sensed by cell surface receptor especially integrins as transmembrane proteins, and then transfers to the cytoskeleton [1]. Cytoskeleton components including actin filaments (F-actin), microtubule and intermediate filament get regulated by enzymes inside the cell especially Rho family of GTPases including RhoA, Rac1, CDC42 [Figure 1.1.]. In the force mechanotransduction, the mediator between integrin and cytoskeleton is focal adhesions. The force activates important enzymes in focal adhesions including focal adhesion kinase (FAK) and Src which could bind and make complex [2].

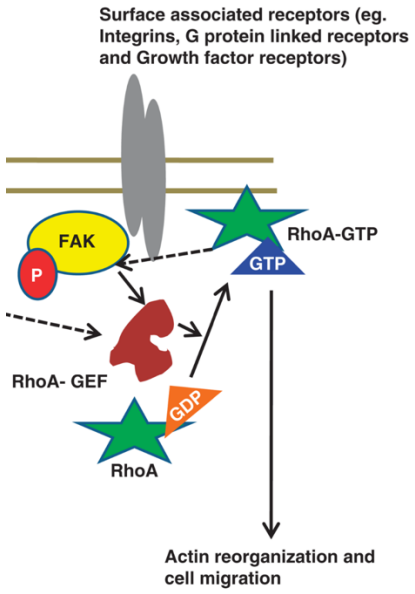

Figure 1.1. Signaling cascade from cell surface receptors such as integrin which activates FAK and RhoA [3].

1.2. Cancer Cell Growth in Bone Environment

In the ECM (extracellular matrix) of bone, OPN (osteopontin) is abundant which has a role in bone homeostasis. OPN amount in patients with breast cancer metastasized to the bone is higher than patients without this metastasis. Breast cancer and prostate cancer cells enhance expression of OPN in osteoblasts. Interaction of OPN with beta 1 integrin which is cancer cell surface receptor gives rise to activation of epidermal growth factor (EGF) which can promote proliferation of cancer cells. Therefore, OPN can prevent apoptosis and induce proliferation as well as adhesion of cancer cells [4, 5]. Moreover, transforming growth factor beta (TGFβ) can be another component in cancer cell growth. The exchange factor (TGFβ) in bone matrix getting secreted during bone resorption which influences tumor growth. TGFβ may interact with other factors in bone niche like hypoxia in bone marrow which can cause vascular endothelial growth factor (VEGF) expression leading to tumor growth [6]. When tumor metastasizes to bone, it can enhance osteoclast activity which can aggravate the growth and advancement of bone metastasis [7]. Also, calcium can be another component for cancer cells growth in the bone environment. Calcium is plentiful in the bone matrix and it can provoke breast cancer cells to inhibit apoptosis and promote proliferation [7, 8].

1.3. Cancer Cell Migration to Bone Environment

Cancer cells of origin tumor undergo different alteration to metastasize to another location in the body. In the first stage the cancer cells obtain invasive phonotype especially through epithelial to mesenchymal transition (EMT) leading to detachment of cancer cells. In the next stage, detached cancer cells invade stroma and then enter the blood flow called intravasation. Then, circulating tumor cells (CTCs) become stiffer due to resisting blood flow tension, but when they adhere to endothelial cells they become softer in order to penetrate to the target tissue called extravasation [Fig. 1.2.] [9, 10]. In the final stage, they colonize the target tissue and undergo some alteration such as mesenchymal to epithelial transition to proliferate and grow [11].

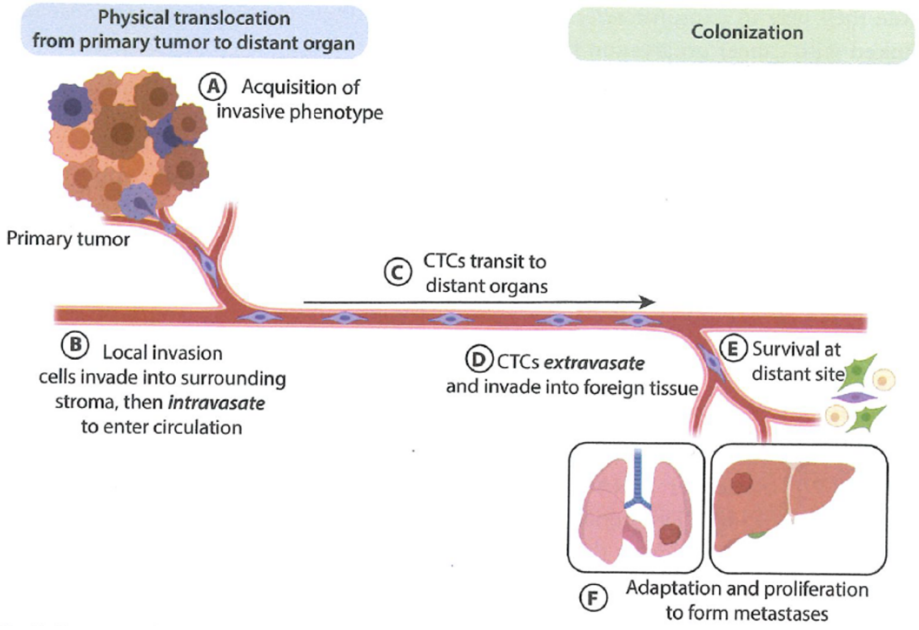

Figure 1.2. The steps of cancer metastasis including acquisition of invasive phenotype, intravasation, transmission in the blood, extravasation and colonization in the new tissue [9].

Vascularized areas of skeleton such as red marrow of long bones, pelvis, and vertebrae are the most common place for cancer cell migration leading to disrupting bone physiology, hematopoiesis and immune system [12]. As a matter of fact, breast cancer cells regularly migrate to the bone environment, and interrupts bone remodeling process, and they cause osteolysis by different factors such as matrix metalloproteinases (MMPs) [5]. Calcium in the bone matrix can be a strong chemoattractant to breast cancer cells. Breast cancer cells can express calcium sensing receptor (CASR) which can have a major role in breast cancer migration to the bone [7]. In addition, Cytokine RANKL (receptor activator of NF-κB ligand) which is osteoclast differentiation factor can be another strong agent in epithelial cancer cell migration to bone. Breast cancer cells can express RANK which can induce breast cancer migration to bone which binds RANKL [13]. Osteocytes can be another important breast cancer cells' attractant through bone matrix proteins such as collagen and proteoglycans [14]. On the other hand, osteocytes and breast cancer cells interaction can have an advantageous role in inhibiting cancer cells migration. This interaction can down-regulate Snail which is a transcription factor involved in EMT [15].

1.4. Cytoskeleton of Osteocytes

Osteocytes are responsible for responding to mechanical force in the bone as well as bone homeostasis, and include most portion of the bone. Mechanical loading is necessary for bone homeostasis and health, and it influence osteocytes. Osteocyte's dendrites could connect to matrix through bone canaliculus, and this complex called collagen hillocks [16]. In osteocytes, focal adhesions (FAs) connect the cell membrane and matrix which is located between collagen hillocks and dendrites leading to mechanical force transfer to the cytoskeleton [Figure 1.3.]. Three major components of cytoskeleton of the cell are actin filaments (F-actin), microtubules and intermediate filaments, and F-actin plays an important role in osteocyte mechanoresponse. Therefore, inhibiting F-actin polymerization by drug affects cell contraction and morphology. On the other hand, microtubules influence calcium flow and expression of sclerostin as a bone formation inhibitor in the osteocyte. In other words, microtubules mediate opening of calcium channel by fluid flow shear stress leading to calcium flow to the cell. In addition, reactive oxygen species could be involved in sclerostin inhibition under fluid flow by microtubule network [17]. Suitable mechanical stress could prevent apoptosis and death of osteocyte leading to bone resorption due to expression of receptor activator of NF-κB ligand (RANKL) as well as osteoclastogenesis especially in old ages [18]. Wnt pathway is also important in mechanical response of osteocyte as well as bone homeostasis and mass [19]. Specifically, activation of Wnt signaling pathway and beta catenin translocation to the nucleus in osteocytes causes secretion of bone forming proteins including Osteoprotegerin (OPG) and OPN [20]. On the other hand activation of Wnt pathway can induce prostaglandin E2 (PGE2) secretion which could be stimulator of bone resorption [21].

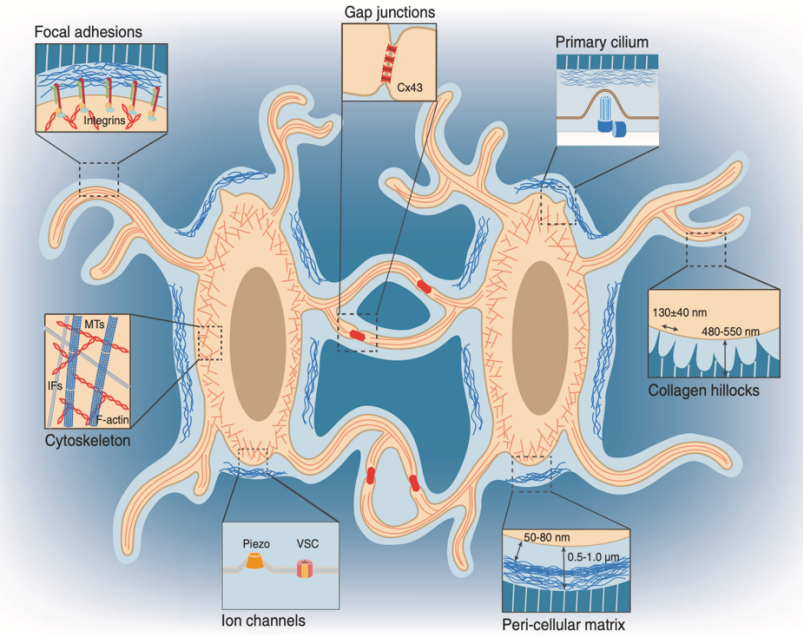

Figure 1.3. Osteocytes are in LCS of bone microenvironment, and it shows the components of osteocytes involved in mechano-response such as cytoskeleton, focal adhesions and ion channels [22].

1.5. The Role of AMPK in Cell Proliferation

Abnormal, enhanced cell proliferation is one of the hallmarks of cancer [22]. Maintaining the proliferative state is energetically costly, requiring cellular energy. AMP-activated protein kinase (AMPK) is a primary signaling node that regulates cellular energy homeostasis by sensing the AMP to ATP ratio of the cell [23, 24]. It has been reported to suppress cell proliferation by inhibiting cyclin-dependent kinases through p53-p21 axis [25] and by inhibiting protein synthesis through the mammalian target of rapamycin (mTOR) pathway [26]. mTOR pathway has an important role in regulation of cell proliferation, growth and migration, and it can be unusually regulated in cancer cells; therefore, its inhibition by AMPK activation can restrain cancer cell growth [27]. When cells face the stress such as gene mutation leading to oncogene or lack of glucose results in p53 tumor suppressor activation [28, 29]. Glucose has an important role in cell cycle during proliferation, and lack of glucose causes p53 activation induced by AMPK [30]. Energy is necessary for cell division, and lack of energy activates AMPK leading to the cell cycle stop until energy supply. On the other hand, some cancer cells have flaw in AMPK activation or

AMPK pathway during tumor cell division resulting in cancer cell proliferation with nutrient deficiency; therefore, AMPK activation can be a suitable method for cancer therapy [31, 32] .

1.6. The Connection Between AMPK and Rho Family GTPases

AMPK have an important role in cell contraction which is mediated by Rho GTPases. Rho GTPases have major role in different types of cell migration by generating traction forces. AMPK can be employed in cadherin adhesion process in order to provide energy and increase ATP for strengthening adhesion as well as cytoskeleton rearrangement leading to withstanding forces. From another angle, in the contractility pathway as well as E-cadherin adhesion, AMPK activates Abl (Abelson tyrosine kinase) which causes phosphorylation of Tyr822 vinculin culminating in RhoA GTPase activation. Therefore, AMPK is required to elevate contractility through RhoA activation when E-cadherin experiences the force [33]. In addition, AMPK can be employed in the junctions of cells in order to cell-cell adhesion. AMPK inhibition hinders phosphorylation of Abl, GTP-loading of RhoA, and myosin II; thus, AMPK acts as an upstream regulator of contractility in cell-cell adhesion [1]. In contrast, AMPK inhibition by compound C can activate Rac1 and Cdc42 Rho GTPase proteins in microglia cells which are central immune cells in nervous system leading to actin rearrangement in order to cell migration [34].

RhoA-myosin II contractility pathway employ AMPK to provide ATP by glycolysis for cell contraction. When E-cadherin experiences forces, it can be transduced to the RhoA-ROCK-myosin II pathway leading to cell contraction. Hence, this contractility pathway activates AMPK in order to providing intracellular ATP preventing cell detachment when the cell is under forces, and inhibition of myosin II reduces AMPK activation [35]. On the other hand, in the other study it is observed that inhibition of ROCK (Rho associated protein kinase) can improve metabolic disease by activating AMPK. ROCK is the effector protein of RhoA in contractility pathway which has high activity in people with metabolic diseases such as obesity; therefore, inhibition of ROCK is advantageous for improvement of these diseases. Inhibition of ROCK can up-regulate some molecular components relating to energy generation by mitochondria, fatty acid oxidation and glucose transporting which are downstream molecules of AMPK leading to higher AMPK activity [36].

1.7. The Role of AMPK in Bone Cells

Osteoblast cells have an important role in bone formation, and they control the mineral deposition such as calcium in the bone, and mineral lacking can lead to osteoporosis disease [37]. Consuming saturated fatty acids such as palmitate can reduce mineral density by prompting osteoblast apoptosis [38]. On the other hand, AMPK as a metabolic enzyme is sensitive to the cell energy level and in low energy level in the cell it causes ATP generation by fatty acid oxidation [39]. Hence, AMPK activation can prevent osteoporosis caused by palmitate through inhibition of osteoblast apoptosis as well as fatty acid oxidation [40]. In addition, it is observed that Metformin as an AMPK activator drug can cause the expression of bone morphogenetic protein-2 (BMP-2) as well as endothelial nitric oxide synthase (eNOS) in osteoblast precursor cells (MC3T3 cells) which is in favor of differentiation of MC3T3 cells to osteoblast and bone formation [41]. Moreover, AMPK activator such as Metformin can cause MC3T3 cells differentiation through another pathway including Dlx5 and Runx2 which are transcription factors for osteogenesis [42]. It is also observed that inhibition of AMPK in mice is against bone formation and it also stimulates the osteoclast activity and bone resorption [43, 44]. In addition, AMPK could be involved in osteogenesis by affecting Wnt signaling pathway. Specifically, bone development treatment such as using Naringin employs AMPK for activation of Wnt signaling and Beta catenin protein translocation to the nucleus in MC3T3 cells leading to bone formation [45].

Osteocytes have the main role in regulating bone formation and resorption by cell signaling connection with osteoblasts and osteoclasts in the bone [46]. Osteocytes as a master regulator of bone cells can make communication with osteoblasts by transmitting signals and secreting soluble factors such as fibroblast growth factor (FGF) through lacuna-canalicular system (LCS) which is porous structure enclosing osteocytes [47]. Therefore, soluble factors and biochemical molecules released from osteocytes can be transmitted to osteoblasts through LCS which can cause their proliferation and differentiation [48]. It is also observed that AMPK influences receptor activator of NF-κB ligand (RANKL) and sclerostin expressions in osteocytes which are osteoclast differentiation factor and bone formation suppressor [49-51]. Particularly, AMPK activator notably decreases RANKL expression, and also initially sclerostin expression rises under AMPK activator, but then it starts to decrease after a day; therefore, the AMPK effect on sclerostin could be insignificant [51]. In contrast, AMPK inhibition increases RANKL expression and decrease

Osteoprotegerin (OPG) expression which is osteoclastogenesis inhibitory factor in mice osteocytes [52]. In addition, AMPK activation could hinder apoptosis of osteocyte cells by high homocysteine (Hcy) in blood plasma which is an important reason for osteoporosis [53, 54].

1.8. The Role of Mitochondrial AMPK in Overall Cell Behaviors

AMPK can mainly regulate mitochondrial biogenesis when the energy chronically has diminished [55, 56]. AMPK has a role in autophagy which is degradation process in order to provide energy when there is lacking in nutrients. Hence, in critical circumstances such as mitochondrial stress AMPK gets activated and removes selectively impaired mitochondria through autophagy. As a matter of fact, continuous cancer cell survival can be caused by inspection of impaired mitochondria as well as preventing intrinsic apoptosis owing to mitochondrial AMPK. As a result, mitochondrial AMPK can cause changes in mitochondria through ATG5 (autophagy related 5) [57]. Mitochondrial AMPK can also have a role in preserving homeostasis in cell metabolism. ROS (reactive oxygen species) has the role in cell metabolic balance, and mitochondria is the principle producer of it. Mitochondrial reactive oxygen species (mROS) can activate AMPK which decreases mROS production as a feedback and decreases cell damage induced by ROS. Moreover, many cancer cells have high amount of ROS inducing cancer cell growth; therefore, combination of mitochondrial AMPK and mROS can have a role in inhibiting tumor growth by decreasing ROS amount [58].

Mitochondria biogenesis regulated by AMPK activation can encourage cancer cell proliferation and migration. When there is lacking in glucose in cancer cells, mitochondrial biogenesis rises affected by AMPK activation. In other words, mitochondria biogenesis elevation is in favor of oxidative metabolism and preserving energy for cell metabolism as well as proliferation [59]. In addition, mitochondrial AMPK can inhibit cancer cell growth and proliferation. Mitochondrial AMPK can reduce glycolysis rate and increase oxidative phosphorylation in cancer cells. Glycolysis is the major energy supply to cancer cells, and its rate is much higher than normal cells which is beneficial for cancer cell proliferation and migration through providing acidic environment for cancer cells and activation of matrix metalloproteinases respectively. As a result, mitochondrial AMPK can inhibit cancer cell proliferation and migration by reducing glycolysis [60]. Moreover, during cancer cell migration mitochondria moves to the

leading edge of cell influenced by AMPK, and this causes local ATP accumulation in the leading edge of cell in favor of cytoskeletal dynamics and cell migration. As a result, inhibition of mitochondrial AMPK may disrupt cancer cell migration [61, 62].

1.9. The Role of Mitochondrial AMPK in Bone Cells and Osteogenesis

Mitochondria can promote AMPK activity by production of reactive oxygen species (ROS) during the environmental stress such as hypoxia [58]. On the other hand, AMPK activation can increase the activities of enzyme in the mitochondria especially mitochondrial oxidative enzyme such as mitochondrial AMPK, and biogenesis of mitochondria in rat skeletal muscle [63] [64]; therefore, AMPK elevates oxidative metabolism through mitochondrial biogenesis [59]. Mitochondria as a cell energy source can have an important role in cell differentiation as well as apoptosis [65]. Osteoblastic differentiation can be accompanied by mitochondrial biogenesis and its mass increase [66]. Also, mitochondrial-derived peptide MOTS-c treatment increases OPG/RANKL ratio in the osteocytes in favor of inhibiting osteolysis [52]. In addition, this mitochondrial peptide inhibits osteoclastogenesis as well as osteoporosis through AMPK activation, and specifically AMPK inhibition hinders the effect of MOTS-c [67].

1.10. The Role of Mechanical Stress in Osteocytes and Osteogenesis

Mechanical stress including fluid flow in bone canaliculus has a major role in bone remodeling as well as homeostasis [68]. Osteocytes are the mechanosensory bone cells that affect the bone formation and osteogenesis by controlling osteoblastic differentiation through Wnt signaling pathway [14, 69]. In other words, Wnt pathway activation causes the accumulation of beta catenin protein in the nucleus of osteoblast leading to differentiation of precursor osteoblast (pre-osteoblast) cells as well as bone formation [70]. Therefore, osteocytes under mechanical stress secrete Wnt growth factors influencing pre-osteoblast cells through LCS [71]. Besides, Wnt/Beta catenin pathway activation can prevent the osteocytes apoptosis in bone tissue. Particularly, fluid flow causes opening of gap junction channels (hemichannels) in connected osteocytes leading to ATP release and Calcium increase in osteocytes preventing their apoptosis [72]. Mechanical force can transmit to the cytoskeleton of osteocytes through connection among extracellular matrix (ECM), integrin, catalytic protein such as Src kinase and cytoskeleton leading to cytoskeleton

reorganization [73, 74]. On the other hand, eliminating mechanical force causes osteocytes hypoxia and hemostasis disruption affecting osteocyte apoptosis [75]. Also, mechanical stress on osteocytes can induce autophagy which degrades and recycles cellular components needing AMPK activation [76].

Mechanical stress affects AMPK activity and localization in the cell because of balancing energy and oxygen level of the cell [61, 77]. Also, gene ontology a bioinformatics method has revealed that AMPK is one of top canonical signaling pathway in the osteocyte under fluid flow stress [78]. Moreover, mechanical cyclic stretch of bone marrow derived mesenchymal stem cell elevates the phosphorylation of AMPK causing osteogenic differentiation, and on the other hand inhibition of AMPK abolishes the effect of mechanical stress in favor of osteogenesis [79].

1.11. Role of Moesin in Cytoskeleton and Cancer Progression

ERM protein family includes three proteins ezrin, radixin and moesin (MSN) located in cell membrane and especially adherens junction. ERM upregulation in epithelial cells causes apical microvilli formation as well as adherent junction stability, and also Rho kinase can lead to activation of ERM in most of the cell types [80]. Moreover, ERM proteins have a considerable role in actin arrangement through crosslinking between actin and membrane proteins and phospholipids. Particularly, ERM could be both upstream such as involving in stress fiber and downstream regulator of RhoA GTPase [81]. In immune cells such as T and B cells and neutrophiles MSN is the dominant protein of ERM family, and inhibits the activation of Rho GTPase, so inhibiting MSN causes upregulation of Rho GTPase , and abnormal cell spread and polarization leading to flaw in capturing microorganisms and pathogens [82]. In addition, MSN has a crucial role in microglia's cytoskeleton which is the main immune defender in central nervous system (CNS), and it affects the function, migration and morphology of microglia through interaction with Rho family of GTPases including RhoA, Rac1, Cdc42 [83]. Also, MSN could have a function in mitotic cell division due to binding to microtubules in the cytoskeleton [84]. On the other hand, MSN expression could affect epithelial to mesenchymal transition (EMT) of epithelial cells through actin reorganization and changing location of contractile components such as alpha- smooth muscle actin (α-SMA). It is also observed that inhibition of MSN disrupt cell contraction and actin organization leading to migration suppression. Therefore, MSN upregulation

alters actin organization as well as contraction components such as α-SMA inducing EMT in epithelial cells [85]. Moreover, MSN expression similar to Snail expression is related to EMT of breast cancer especially invasive ductal carcinoma which could be a biomarker in early detection of cancer [86]. In breast cancer cell, Rho association protein kinase (ROCK) activates MSN leading to expression of programmed cell death ligand (PD-L1) which is an immune checkpoint inhibitor causes evasion of cancer from immune system attack and difficulty in cancer detection. Thus, either ROCK inhibition or MSN silencing can downregulate PD-L1 expression leading to penetration of immune cells in favor of cancer treatment [87].

1.12. FRET Imaging

Fluorescent resonance energy transfer (FRET) microscopy is a method for imaging and discovering the subcellular proteins activities. FRET biosensors include donor and accepting fluorescent proteins that can change their conformation. The donor protein is cyan fluorescent protein (CFP), and the acceptor is yellow fluorescent protein (YFP). In RhoA biosensor, CFP and YFP get close to each other when the enzyme is getting activated. Hence, the biosensor emits high YFP signal and low CFP in the active state, so the FRET ratio could be calculated by the ratio of FRET YFP/FRET CFP [Fig. 1.4.]. On the other hand, in Src and FAK mechanism is reverse and FRET ratio equals to ratio of FRET CFP/FRET YFP. In addition, in the active state there are CFP excitation and YFP emission with wavelengths of 433 nm and 527-530 nm respectively[88].

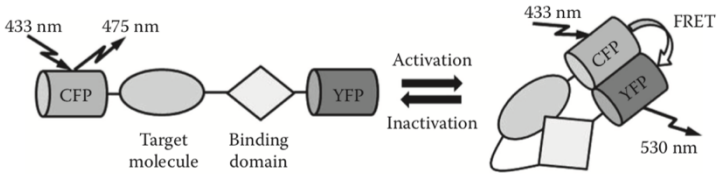

Figure 1.4. FRET signaling in activation of RhoA GTPase in which fluorescent proteins get to close proximity [88].

2. METHODS

2.1. Biosensors and Plasmids

FRET (fluorescence resonance energy transfer) biosensors including Src, FAK, Rho family of GTPases , Rac1, CDC42 and AMPK were used for transfection leading to observation of subcellular activities of the cell. Src and FAK biosensors were composed of a cyan fluorescent protein (CFP) connected to a yellow fluorescent protein (YFP) [89, 90]. Rho family of GTPases plasmids including RhoA, Rac1, CDC42 were composed of CFP and YFP in which intramolecular binding of kinase to the binding domain of an effector protein causes proximity of CFP with YFP leading to FRET elevation from CFP to YFP [91]. AMPK biosensors divided to Cyto-AMPK and Mito-AMPK included CFP, an FHA1 binding domain, an AMPK substrate peptide and YFP. Also, mitochondrial AMPK inhibitor peptide (Mito-AIP) was used for specific inhibition of Mito-AMPK [92]. In addition, MSN SiRNA and constitutively active MSN were used for inhibition and activation of MSN.

2.2. Chemical Reagents

A769662 as AMPK activator with 25 μM concentration and compound C as AMPK inhibitor with 5 μM concentration were used. Also, Y-27632 as ROCK inhibitor with concentration of 10 μM was used.

2.3. Cell Culture and Transfection

Culture of four cell types including human breast cancer cell (MDA-MB-231), osteocyte (MLO-A5), precursor osteoblast (MC3T3) and mouse mesenchymal stem cell (MSC). 89% dulbecco's modified eagles medium (DMED, Lonza) plus 10% fetal bovine serum (FBS, Gibco) plus 1% penicillin (Lonza) were used to for cancer cells culture. On the other hand, 89% minimum Essential Medium α (MEM α, Gibco) plus 5% FBS (Gibco) plus 5% bovine calf serum (BCS, Gibco) plus 1% penicillin (Lonza) were used for osteocytes culture. In addition, combination of 89% minimum Essential Medium α (MEM α, Gibco) plus 10% FBS (Gibco) plus 1% penicillin (Lonza) were used for growth of pre-osteoblasts. Moreover, 89% mesenchymal stem cell medium

(Cell Biologics) plus 10% FBS (Gibco) plus 1% penicillin (Lonza) were used for MSC culture. All cells were maintained in 37 degree of Celsius and 5% CO_2 in the humid incubator. For transfection of cells two methods of chemical reagent and electroporation were used. In chemical transfection, Lipofectamine 2000 was used, and cells got incubated for at least 4 hours in OPTI-MEM (Gibco) media without serum and antibiotic. Then, transfection medium with 5% FBS was added to cells for long time incubation, and cells became transfected 24 hours before imaging. Osteocytes and pre-osteoblasts transfections were done by electroporation. In electroporation method with Neon device (Thermo Fisher), the cells became suspended in buffer R, and then cell solution in 10 μl tip was inserted into Neon tube including 3 ml buffer E. The settings of device for electric shock for bone cells were 1200 Volt, 20 ms, 1 pulse, and after shock transfected cell solution became transferred to the transfection media with 5% FBS and without antibiotics.

2.4. Condition Medium (CM) Experiments

We followed four different protocols for CM of osteocytes experiments. First protocol was for CM without any modulators. In the first day we cultured and grew of osteocytes, and in the second day we changed the osteocyte media with transfection media including 2% FBS, 99% MEM alpha (condition media). In the third day we did transfection of either cancer or pre-osteoblast cells with beta catenin and transferred condition media to the cells 3 hours after transfection. In the last day we did imaging of beta catenin activity of the cells with condition media.

Second protocol was for double transfection treatment of osteocytes like Mito-AIP. After growing the cells, in the first day we transfected osteocytes with specific DNA vector like Mito-AIP (media1). In the second day we transfected either osteocyte or cancer cells with beta catenin. In the third day we replaced CM, and after 6 hours started imaging.

Third protocol was for drug treatment of osteocytes. After growing cells, in the first day we did transfection of cancer cells with beta catenin, and add AMPK modulator drug to osteocyte dish and incubated for 24 hours. In the second day, we transferred CM to the cells, and after 6 hours started imaging.

2.5. Shear Stress Application

For mechanical stimulation of cells, μ-slide cell culture chambers (Ibidi) were used [Fig. 2.1.A]. We used cyclic flow device with frequency of 1 Hz exerting 12 dyn/cm^2 shear stress [Fig. 2.1.B]. Also, Unidirectional fluid flow imposing 10 dyn/cm^2 was used for flow induction simultaneous with imaging [Fig. 2.1.C]. On the other side, the sterile tubing was connected to the chamber and transfection media flows inside the tubing.

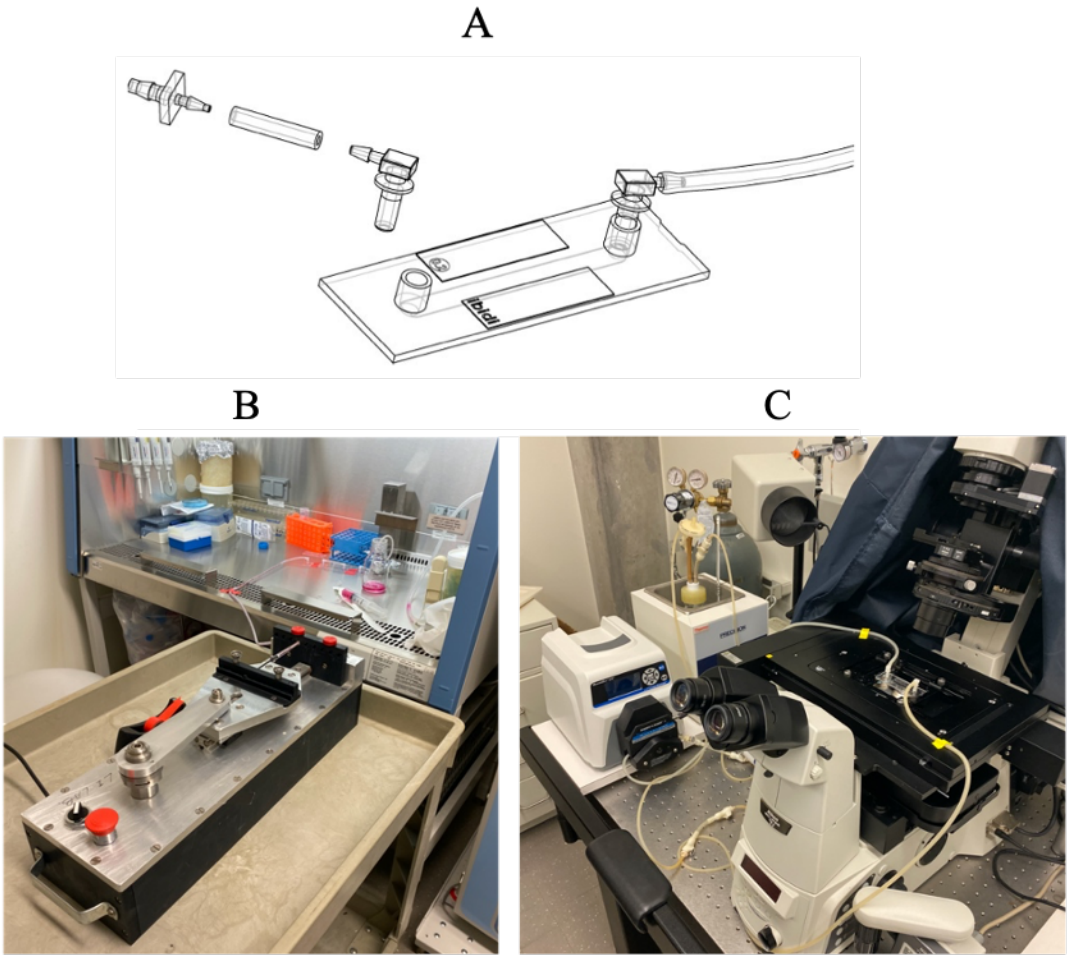

Figure 2.1. (A) Image of the μ-slide cell culture chamber connecting to the tubing. (B) The experimental set-up for cyclic flow experiment. (C) The experimental set-up for unidirectional flow while imaging under microscope.

2.6. Microscopy and Image Analysis

Nikon Ti-E inverted microscope plus EMCCD camera (Photometrics, Evolve 512) and perfect focus system (PFS, Nikon) were used for imaging. In FRET imaging the wavelengths of CFP excitation, CFP emission and YFP emission are 438, 488 and 542 respectively. In addition, the wavelength of GPF emission was 522.5 for capturing green fluorescent image. Analysis of FRET ratios of subcellular components were done by NIS-element software (Nikon), and GFP images were made by ImageJ software.

2.7. Statistical Analysis

All data were analyzed with standard error of mean (SEM), and the graphs are plotted by GraphPad Prism 5 software. Statistical significance between two groups in column bar graphs was assessed with student's t-test, and a p-value lower than 0.05 shows statistically significance. In addition, one-way-analysis of variance (ANOVA) followed by Dunnett's post hoc test was used for evaluating statistical significance between multiple groups.

3. RESULTS

3.1. Role of AMPK Modulators on Osteogenesis and Tumor Suppression

Beta catenin translocation to the nucleus was one of the important signs in bone formation and osteogenesis. Increasing beta catenin activity in the nucleus of pre-osteoblast (MC3T3) cells was an important factor for osteoblast differentiation as well as bone formation. In addition, beta catenin activity and Wnt signaling in osteocytes which were the bone mechanosensory cells can be important in bone formation. We evaluated beta catenin translocation in osteocytes and pre-osteoblast cells cultured in collagen coated glass dishes under AMPK modulators [Fig. 3.1.]. AMPK activator affected Wnt signaling pathway in osteocyte cells (MLO-A5) and increased beta catenin activity in the nucleus 10% in 2 hours although it did not increase beta catenin translocation in the first 1 hour [Fig. 3.1.A]. On the other hand AMPK inhibitor did not affect the nucleus activity of the beta catenin within 2 hours [Fig. 3.1.B]. Moreover, inhibition of mitochondrial AMPK in the osteocyte increased the beta catenin basal activity in the nucleus 80% [Fig. 3.1.C].

We conducted experiments for observing the role of AMPK modulators and mitochondrial AMPK in beta catenin activity in pre-osteoblast cells and osteocytes. Both cells transfected with Beta catenin biosensor were plated in glass dishes coated with collagen, and for inhibition of mitochondrial AMPK (Mito-AIP) the cells became double transfected with beta catenin and Mito-AIP. Using AMPK activator (A 769662) increased the beta catenin translocation to the nucleus in pre-osteoblast cells, and it slowly elevated beta catenin activity in nucleus (15% increases in 2 hours) [Fig. 3.1.D]. Using AMPK inhibitor (compound C) did not alter the beta catenin activity in the nucleus of pre-osteoblast cells within 2 hours [Fig. 3.1.E]. Unlike global AMPK inhibitor, mitochondrial AMPK inhibitor (TOM-20mchf-AIP) did not affect the beta catenin basal activity in nucleus of pre-osteoblast cells [Fig. 3.1.F].

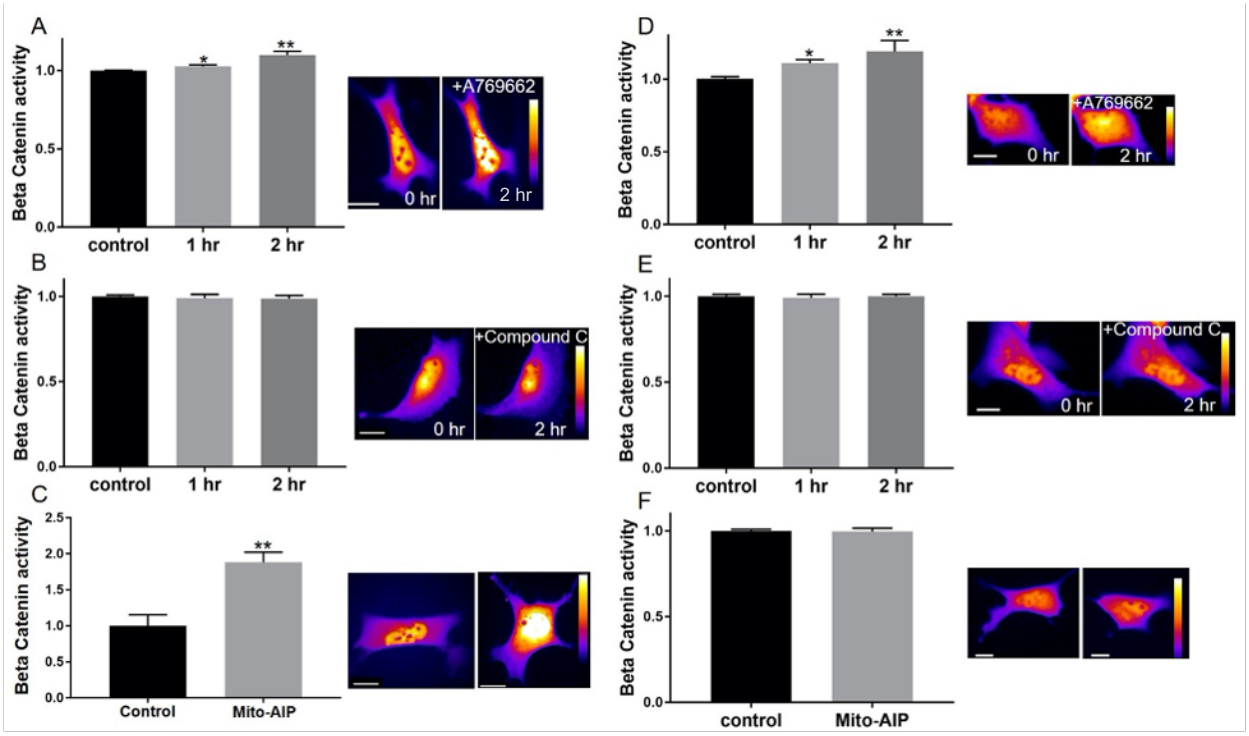

Figure 3.1. Effects of AMPK modulators on beta catenin activity of osteocytes and osteoblasts; color bar of images represents GFP signaling; scale bar=10 μm; * $p<0.05$, ** $p<0.01$. (A) The bar graphs of beta catenin activity in the nucleus of osteocytes after 1 and 2 hours treatment with AMPK activator; n=14. (B) The bar graph of beta catenin activity in the nucleus of osteocytes after 1 and 2 hours treatment with AMPK inhibitor; n=12. (C) The bar graph of beta catenin basal activity in the nucleus of osteocytes in control and transfected with Mito-AIP; n=11. (D) The bar graphs of beta catenin activity in the nucleus of pre-osteoblasts after 1 and 2 hours treatment with AMPK activator; n=16. (E) The bar graph of beta catenin activity in the nucleus of pre-osteoblasts after 1 and 2 hours treatment with AMPK inhibitor; n=12. (F) The bar graph of beta catenin basal activity in the nucleus of pre-osteoblasts in control and transfected with Mito-AIP; n=10.

We also set up experiments to evaluate the connection between osteocytes and pre-osteoblasts. Specifically, we evaluated the effect of conditioned medium (CM) of osteocytes with AMPK modulators on basal beta catenin activity in pre-osteoblast cells. At first, adding CM to pre-osteoblast cells (b) increased beta catenin basal activity in the nucleus (30% increase) comparing to normal media (a) of pre-osteoblasts. AMPK inhibition and activation in osteocytes affected the CM as well as pre-osteoblast cells. Applying AMPK activator to osteocytes CM (c) increased beta catenin basal activity in the nucleus of pre-osteoblast cells including 60% increase comparing to normal media and 30% increase comparing to CM without AMPK modulators. Also, mitochondrial AMPK inhibition of osteocytes (d) affected the CM using for pre-osteoblast cells,

and this treated CM increased beta catenin basal activity in the nucleus (70% increase) of pre-osteoblast cells.

In contrast, applying AMPK inhibitor to osteocytes (e) did not alter beta catenin basal activity comparing to CM without AMPK modulator, but its activity was higher than normal media (30% higher). Moreover, AMPK activation and inhibition in pre-osteoblasts in the presence of CM affected beta catenin basal activity on them. Applying AMPK activator to pre-osteoblasts with CM (f) increases beta catenin basal activity in the nucleus comparing to CM without AMPK modulators (20% increase) and normal media (50% increase). On the other hand, applying AMPK inhibitor to the pre-osteoblasts with CM (g) decreased beta catenin basal activity in the nucleus of pre-osteoblast (20% decrease) comparing to CM without AMPK modulators, and increased its activity (10% increase) comparing to normal media. Therefore, Inhibition of mitochondrial AMPK in osteocytes caused the highest increase in beta catenin basal activity in nucleus of pre-osteoblast cells through affecting CM of osteocytes. Conversely, AMPK inhibitor caused the highest decrease in beta catenin activity in CM comparing to CM without AMPK modulators. Also, CM improved the effects of AMPK activator and inhibitor on beta catenin translocation to the nucleus of pre-osteoblast cells, because AMPK modulators did not affect beta catenin activity in pre-osteoblasts with normal media [Fig. 3.2A].

We set up experiments to evaluate the connection between osteocytes and metastasized cancer cells. Specifically, we evaluated the effect of conditioned medium (CM) of osteocytes treated with AMPK modulators on basal beta catenin activity in cancer cells. At first, adding CM to cancer cells (b) decreased beta catenin activity in the nucleus (30% decrease) comparing to normal media (a) of pre-osteoblasts. Also, AMPK inhibition and activation in osteocytes affected the CM as well as cancer cells. Applying AMPK activator to osteocytes CM (c) slightly increased beta catenin activity in the nucleus (4% increase). Also, mitochondrial AMPK inhibition (Mito-AIP) of osteocytes (d) significantly decreased beta catenin basal activity in the nucleus (40%) in cancer cells. Applying AMPK inhibitor to osteocytes (e) reduced beta catenin activity 6% in the nucleus comparing to CM without AMPK modulator. In addition, AMPK activation and inhibition in cancer cells in the presence of CM affected beta catenin basal activity on them. Applying AMPK activator to cancer cells with CM (f) decreased beta catenin basal activity in nucleus 14%. Applying AMPK inhibitor to the pre-osteoblasts with CM (g) did not change beta catenin basal activity in nucleus comparing to CM without AMPK modulators [Fig. 3.2B].

Therefore, Inhibition of mitochondrial AMPK in osteocytes caused the highest increase in beta catenin basal activity in nucleus of pre-osteoblast cells and highest decrease in beta catenin basal activity in nucleus of cancer cells through affecting CM of osteocytes. Conversely, AMPK inhibitor caused the highest decrease in beta catenin activity in CM comparing to CM without AMPK modulators. Also, CM improved the effects of AMPK activator and inhibitor on Beta catenin translocation to the nucleus of pre-osteoblast cells, because AMPK modulators did not affect beta catenin activity in pre-osteoblasts with normal media.

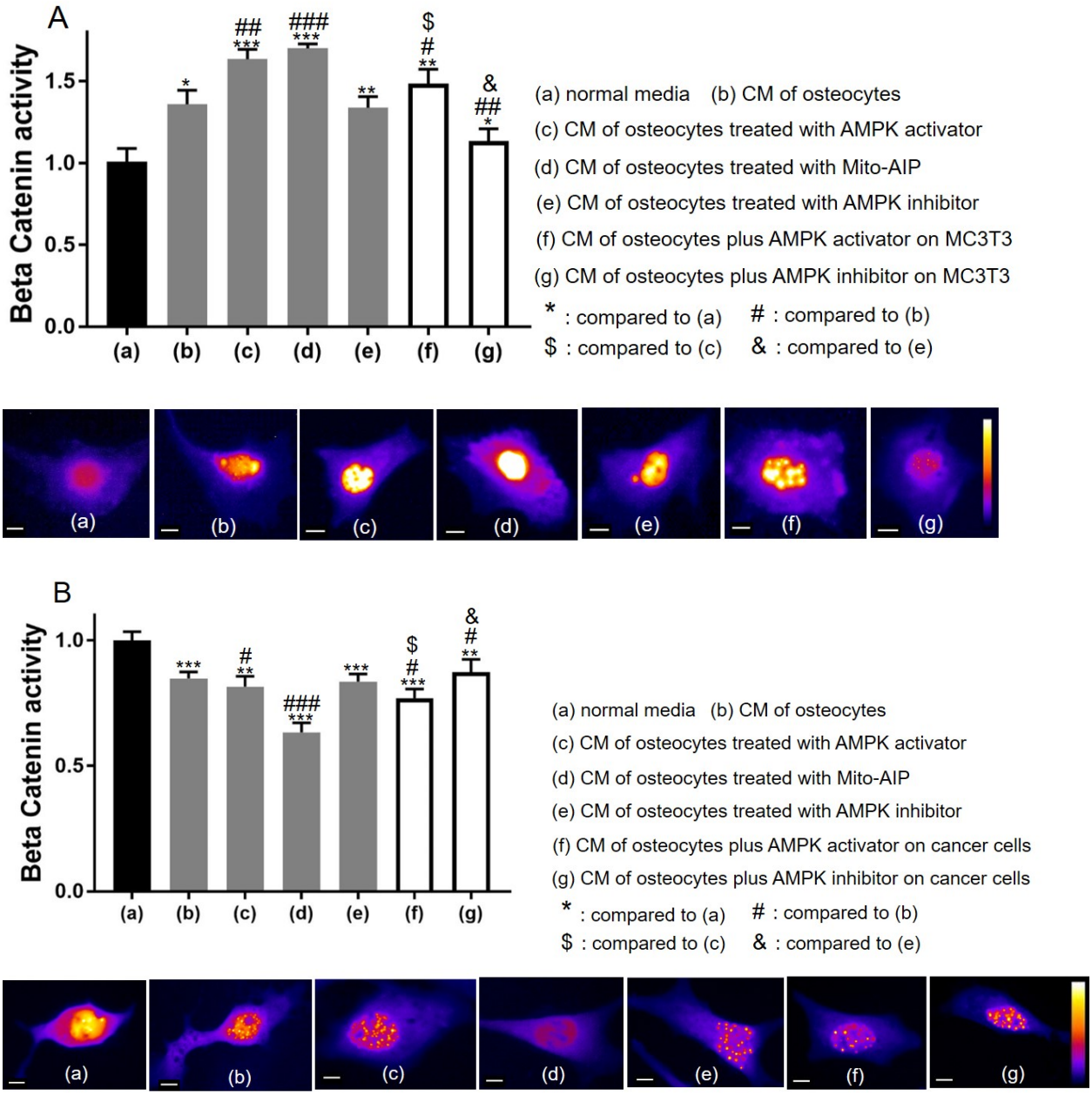

Figure 3.2. Effect of CM of osteocytes treated with AMPK modulators on beta catenin activity of cancer cells and pre-osteoblasts; *, #, $, & $p<0.05$, **, ## $p<0.01$, ***, ### $P<0.001$; scale bar=10 μm. (A) beta catenin activity in nucleus of pre-osteoblasts. (B) beta catenin activity in nucleus of cancer cells.

We also evaluated effects of AMPK modulators in Rho family GTPases including RhoA, Rac1 and Cdc42 in cancer cells, and also we did time course experiment for 1 hour for drug treatment [Fig. 3.3]. AMPK activator (A769662) reduced RhoA, Rac1 and Cdc42 activity about

8%, 30% and 11% respectively [Fig. 3.3.A-C]. On the other hand, AMPK inhibitor (Compound C) did not change the activity of these kinases in cancer cells [Fig. 3.3.D-F]. In addition, Mito-AIP treatment of cancer cells reduced basal activities of RhoA, Rac1 and Cdc42 12%, 9% and 6% respectively [Fig. 3.3.g-I]. So, inhibition of mitochondrial AMPK caused similar effect of AMPK activator in cancer cells, and both decreased activity of cytoskeleton related kinases which are relevant to migration and metastasis.

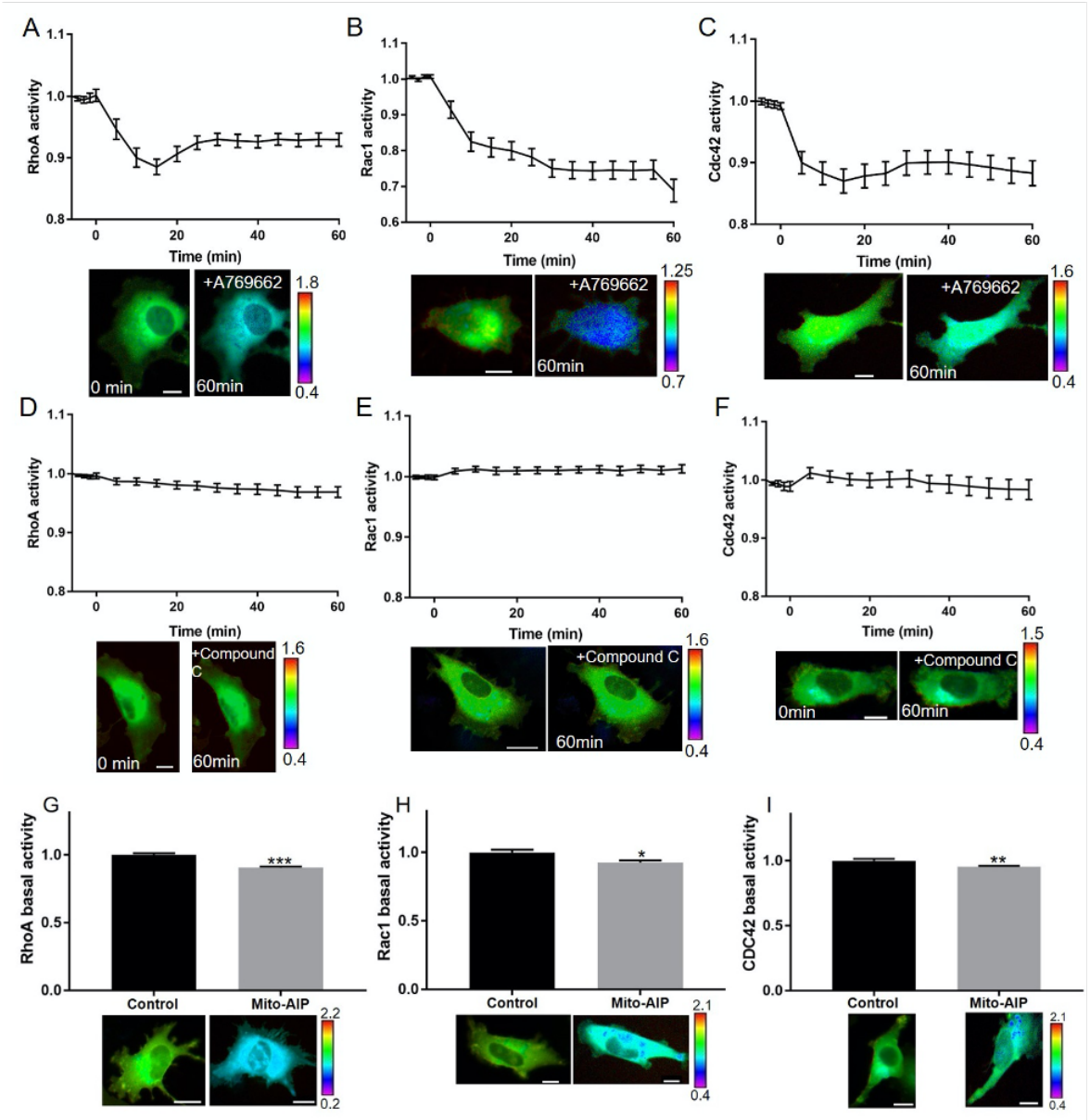

Figure 3.3. The effects of AMPK modulators on Rho family GTPases of breast cancer cells; color bar represents FRET ratio. (A) Time course graph showing FRET ratio of RhoA with 1 hour AMPK activator treatment in t=0; n= 18; scale bar= 10 µm. (B) Time course graph showing FRET ratio of Rac1 with 1 hour AMPK activator treatment in t=0; n= 14; scale bar= 10 µm. (C) Time course graph showing FRET ratio of CDC42 with AMPK activator treatment in t=0; n= 13; scale bar= 10 µm. (D) Time course graph showing FRET ratio of RhoA with AMPK inhibitor treatment in t=0; n= 11; scale bar= 10 µm. (E) Time course graph showing FRET ratio of Rac1 with AMPK inhibitor treatment in t=0; n= 11; scale bar= 20 µm. (F) Time course graph showing FRET ratio of CDC42 with AMPK inhibitor treatment in t=0; n= 13; scale bar= 10 µm. (G) The graph bar showing FRET ratio of RhoA with Mito-AIP treatment ; n= 18; scale bar= 20 µm. (H) The graph bar showing FRET ratio of Rac1 with Mito-AIP treatment ; n= 14; scale bar= 10 µm. (I) The graph bar showing FRET ratio of CDC42 with Mito-AIP treatment ; n= 17; scale bar= 10 µm; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2. Role of Fluid Flow Stress on Osteocytes and Cancer Cells

Mechanical force plays an important role in cytoskeleton components rearrangement including microtubule assembly and actin polymerization. Therefore, when the cell does not experience mechanical force, the cytoskeleton would not make contraction force needing energy consumption. We conducted the experiments to observe the role of AMPK in the cytosol and specifically in the mitochondria of osteocytes with fluid flow. Osteocyte cells (MLO-A5) transfected with cytosolic AMPK (Cyto-AMPK) biosensors were plated in two different flow chambers coated with collagen. Then, during the imaging they were exposed to continuous fluid flow imposing 10 dyne/cm^2 shear stress similar to physiological condition. During 1 hour experiment, fluid flow increased Cyto-AMPK activity 35% and Mito-AMPK activity 12% in osteocytes [Fig. 3.4 A,B]. Moreover, we evaluated the connection between AMPK and cytoskeleton in osteocytes. Specifically, We treated osteocytes with the inhibitor of Rho-associated protein kinase (ROCK) with 1 hour incubation before imaging. In the experiment, fluid flow did not increase Cyto-AMPK activity, so ROCK could be an upstream regulator of AMPK [Fig. 3.4 C]. On the other hand, we evaluated the effect of CM of osteocytes treated with cyclic flow on beta catenin activity of metastasized cancer cells [Fig. 3.4D]. In other words, we exposed cyclic flow with frequency of 1 Hz causing 12 dyne/cm^2 shear stress on osteocytes, and then transferred the treated CM to cancer cells 12 hours before imaging. We observed that flow treated CM significantly increased beta catenin translocation to the nucleus of cancer cells (35% increase) which shows the negative effect secreted soluble factors of osteocytes under flow on metastasized cancer cells.

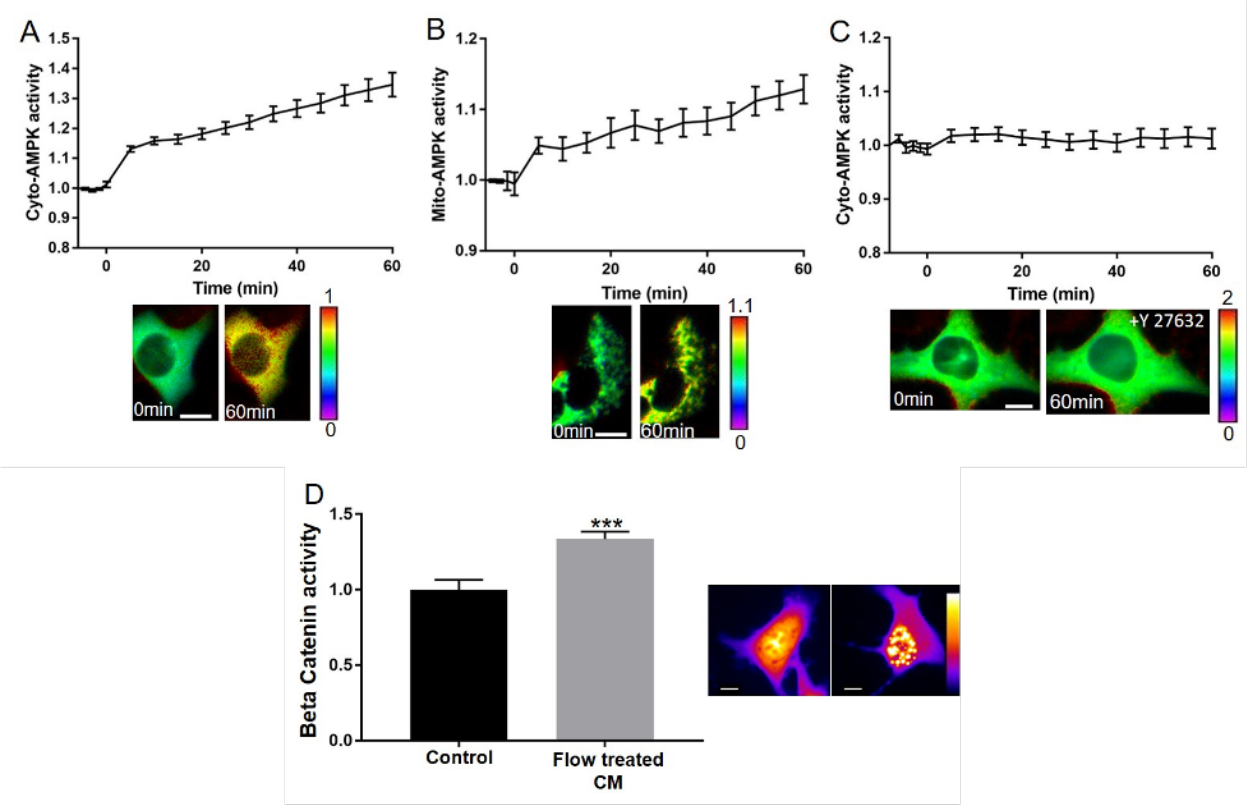

Figure 3.4. Fluid flow effects; scale bar= 10 μm. (A) The effect of 1 hour fluid flow on FRET ratio of Cyto-AMPK in osteocytes; color bar represents FRET ratio (FRET YFP/FRET CFP) (B) The effect of 1 hour fluid flow on FRET ratio of Mito-AMPK in osteocytes; color bar represents FRET ratio (FRET YFP/FRET CFP). (C) The effect of 1 hour fluid flow on FRET ratio of Cyto-AMPK in osteocytes treated with Y-27632; color bar represents FRET ratio (FRET YFP/FRET CFP). (D) Beta catenin basal activity in cancer cells in CM of osteocytes treated with cyclic flow; color bar represents GFP signal; *** $p<0.0001$.

3.3. Role of MSN Modulators on Osteocytes and Cancer Cells

We evaluated the roles of MSN SiRNA (MSN-) and constitutively active MSN (MSN+) in contractility pathway of osteocytes and metastatic breast cancer cells to the bone including focal adhesion kinase (FAK), RhoA GTPase and Src (Proto-oncogene tyrosine-protein kinase). We observed different responses of these kinases to MSN treatment in osteocytes and breast cancer cells [Fig. 3.5.]. MSN silencing transfection in osteocytes reduced RhoA and Src basal activities 9% and 3% respectively, but conversely it increased FAK basal activity 6%. On the other hand, transfection of osteocytes with MSN+ plasmid increased RhoA and Src activity 10% and 32% respectively, and decreased FAK activity 5% [Fig. 3.5.A-C]. Therefore, in osteocytes RhoA and

Src had similar behavior to intracellular MSN modulators. In the breast cancer cells (MDA-MB 231), transfection silencing of MSN decreased FAK and Src basal activity 5% and 7% respectively, and it did not change RhoA activity. On the other side, transfection of cancer cells with MSN+ increased FAK activity 30% and Src activity 50%, and decreased RhoA activity 10% [Fig. 3.5.D-F]. Therefore, in cancer cells FAK and Src had similar behavior to intracellular MSN modulators. In addition, in cancer cells and osteocytes FAK and RhoA had opposite response to intracellular MSN modulators, but Src had the same behavior. Therefore, MSN acts differently in activities of Src and RhoA in osteocytes and cancer cells.

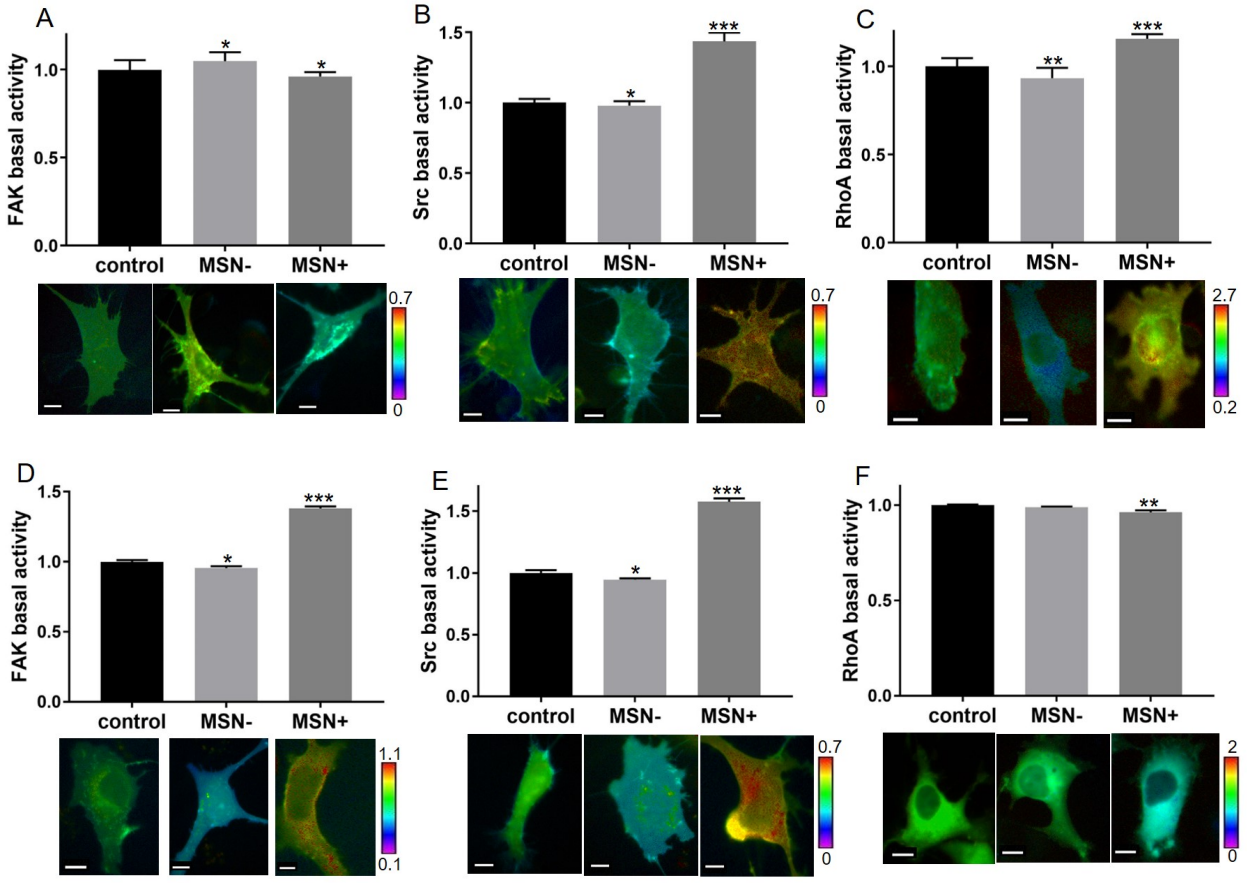

Figure 3.5. Role of intracellular MSN in osteocytes and cancer cells; color bar represent FRET ratio; scale bar= 10 µm. (A) Effect of MSN modulators on FAK activity of osteocytes; n=14. (B) Effect of MSN modulators on Src activity of osteocytes; n= 16. (C) Effect of MSN modulators on RhoA activity of osteocytes; n=16. (D) Effect of MSN modulators on FAK activity of cancer cells; n= 18. (E) Effect of MSN modulators on Src activity of cancer cells; n= 16. (F) Effect of MSN modulators on RhoA activity of cancer cells; n=18; * $p<0.05$, ** $p<0.01$, *** $P<0.001$.

We also evaluated the extracellular role of MSN modulators in these kinases activities in cancer cells [Fig. 3.6.]. We transfected mesenchymal stem cells (MSCs) with MSN modulators and then transferred condition medium (CM) of transfected MSN to cancer cells. MSN modulators became present in extracellular environment of cancer cells due to secretion of exosomes and soluble factors related to MSN by treated MSCs. CM of MSCs treated with MSN- increased Src basal activity in cancer cells 10%, but it did not affect FAK and RhoA activities. On the other hand, CM of MSCs treated with MSN+ decreased basal activities of FAK, RhoA and Src 5%, 10%, 6% respectively comparing to normal media condition. Therefore, extracellular MSN+ and intracellular Si MSN could suppress migration cancer cells.

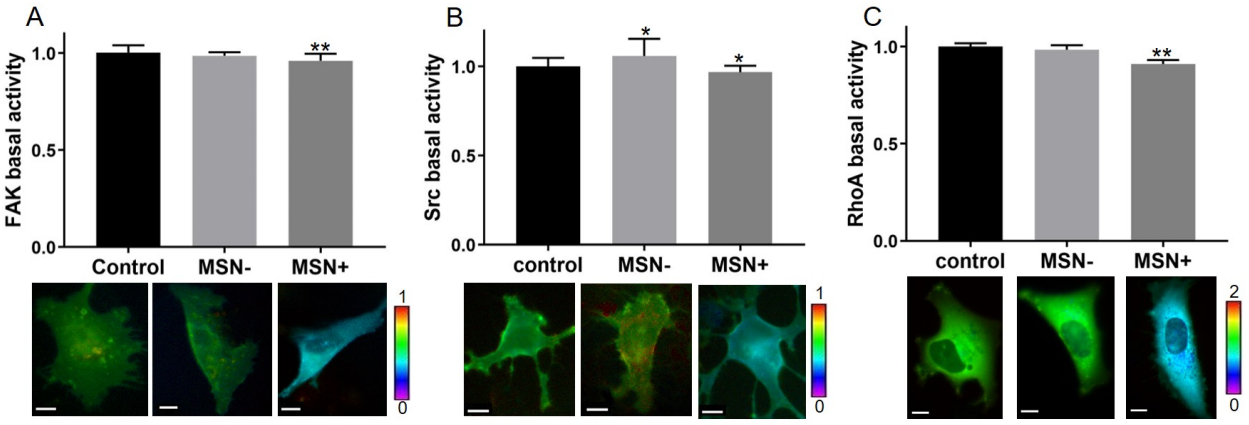

Figure 3.6. Role of extracellular MSN in cancer cells; color bar represent FRET ratio; scale bar= 10 μm. (A) Effect of MSN modulators on FAK activity of cancer cells; FRET ratio= FRET CFP/FRET YFP; n=20. (B) Effect of MSN modulators on Src activity of cancer cells; FRET ratio= FRET CFP/FRET YFP; n=17. (C) Effect of MSN modulators on FAK activity of cancer cells; FRET ratio= FRET YFP/FRET CFP; n=22; * $p<0.05$, ** $p<0.01$.

On the other side, we evaluated the role of MSN modulators in interaction between osteocytes and breast cancer cells [Fig. 3.7]. As we already observed in our experiments, CM of osteocytes (b) reduced beta catenin activity in the nucleus of metastasized breast cancer cells 30% comparing to normal media. In this experiment, CM of osteocytes treated with MSN- (c) did not change beta catenin translocation to the nucleus comparing to CM without modulators although it still reduced beta catenin activity comparing to normal media. In addition, CM of osteocytes treated with MSN+ reduced beta catenin activity in cancer cells 10% comparing to both CM and CM with MSN-. Therefore, MSN+ in extracellular matrix of cancer cells could suppress progression of tumor.

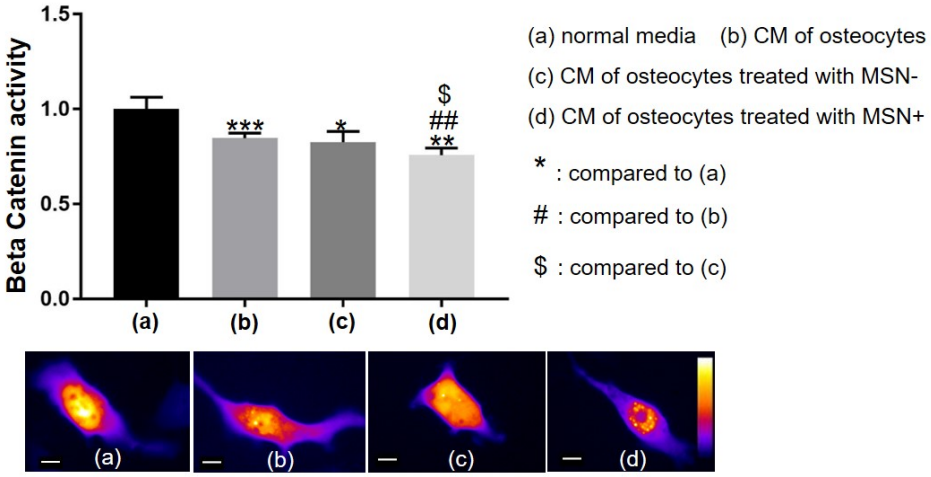

Figure 3.7. Effect of CM of osteocytes treated with MSN modulators on beta catenin activity of cancer cells; *, # ,$ $p<0.05$, **, ## $p<0.01$, *** $p<0.001$; scale bar=10 μm; n=18. color bar represents GFP signal.

4. DISCUSSION

In this study we evaluated the role of AMPK in osteogenesis and tumor suppression as well as the role of MSN in tumor suppression. Using FRET-based biosensors and a fluorescence intensity-based biosensor, we quantified the signaling activities of AMPK, FAK, Src, and RhoA as well as beta catenin translocation into the nucleus. We examined the role of global AMPK and mitochondria-specific AMPK in the signaling activities of osteocytes and pre-osteoblasts, and their interaction through osteocyte-based CM. Using CM for studying the interaction of osteocytes and breast cancer cells has been done in the other study [14]. An AMPK activator increased beta catenin translocation to the nucleus in both osteocytes and pre-osteoblasts although an AMPK inhibitor did not affect its activity. Interestingly, inhibition of Mito-AMPK increased beta catenin activity of osteocytes significantly which may cause secretion of soluble factors in favor of bone homeostasis and formation. However, inhibition of Mito-AMPK did not affect beta catenin activity of pre-osteoblast cells. Previous studies show the positive role of AMPK activation in osteogenesis. It is observed that Metformin as an AMPK activator drug can elevate the expression of BMP-2 as well as endothelial nitric oxide synthase (eNOS) in pre-osteoblasts causing osteoblastic differentiation and bone formation [41]. Moreover, an AMPK activator such as Metformin can cause pre-osteoblast cell differentiation through another pathway including Dlx5 and Runx2 which are transcription factors for osteogenesis [42]. In addition, bone development treatment using Naringin employs AMPK for activation of Wnt signaling and beta catenin protein translocation to the nucleus in pre-osteoblast cells, leading to bone formation [45]. In addition, AMPK activation could hinder apoptosis of osteocyte cells by high homocysteine (Hcy) in blood plasma which is an important reason for osteoporosis [53, 54]. Our studies suggest that soluble factors secreted by osteocytes increased the beta catenin activity of pre-osteoblasts. Particularly, global AMPK activation and inhibition of Mito-AMPK could significantly increase the beta catenin activity of pre-osteoblast leading to osteoblast differentiation and osteogenesis. Activity of osteocytes could also affect the metastasized breast cancer in the bone; therefore, we evaluated the role of AMPK in cancer cells. Interestingly, osteocyte-derived CM reduced the beta catenin activity in cancer cell, and inhibition of Mito-AMPK in osteocytes further reduced the beta catenin activity in cancer cells. Therefore, inhibition of Mito-AMPK may not only suppress cancer progression, but also stimulate osteogenesis.

Mechanical stress on bone is known to affect osteocytes and metastasized cancer progression. It has been reported that fluid flow stimulates cancer cell migration, but inhibition of Mito-AMPK suppresses the flow-induced cancer cell migration [61]. We observed that mechanical force increases AMPK activity and ROCK could act as an upstream of AMPK in osteocytes. Our observation is consistent with a published report that AMPK acts as an upstream kinase of RhoA in human breast cells when E-cadherin was experiencing the mechanical force [33]. Also, a study based on bioinformatics approach has revealed that AMPK is one of canonical signaling pathways in osteocytes under fluid flow stress [78]. It is also shown that ROCK inhibition in skeletal muscle cells activates AMPK and relieves the metabolic disorder [36].

We show that CM derived from fluid flow-treated osteocytes increases beta-catenin activity in breast cancer cells. This may cause stimulation of tumor growth. Fluid flow treatment of osteocytes increased both global and mitochondrial AMPK activities in osteocyte, and we observed that CM of osteocytes treated with global AMPK activation or mitochondria-specific AMPK inhibition reduced beta catenin activity in cancer cells. Therefore, it is possible that Mito-AMPK inhibition in osteocytes may be able to decrease flow-induced elevation of beta catenin activity in cancer cells. In addition, in cancer cells global AMPK activation or Mito-AMPK inhibition decreased the activities of Rho family GTPases.

We also examined the role of MSN in the activities of tumor-promoting proteins such as FAK, Src, and RhoA. In osteocytes partial silencing of MSN (MSN-) reduced RhoA and Src activities and increased FAK activity, while it reduced FAK and Src activities in cancer cells and did not significantly alter RhoA activity. Previous study showed that ROCK activated MSN in breast cancer cells leading to expression of programmed cell death ligand (PD-L1); thus, either ROCK inhibition or MSN silencing downregulated PD-L1 expression leading to penetration of immune cells in favor of cancer treatment [87].

Moreover, we evaluated the role of extracellular MSN by using the CM of MSN-activated cells in cancer cells. CM of MSCs treated with MSN+ reduced FAK, Src and RhoA activities in cancer cells. We reasoned that that MSCs treated with MSN modulators could secrete soluble factors and exosomes including MSN. Our studies show that extracellular and intracellular MSN may have opposite effects on the activities of FAK and Src in cancer cells. In addition, we observed that CM of osteocytes treated with MSN+ significantly decreased the beta catenin activity in cancer cells

causing suppression of tumor progression. Therefore, extracellular overexpression of MSN may have a positive role in preventing tumor progression by suppressing oncogenic proteins.

In summary, our results suggest that inhibition of mitochondria-specific AMPK and/or activation of MSN in osteocytes may be used as a new therapeutic approach for cancer treatment.

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