

HIPPO SIGNALING PROTEIN MST1 REGULATES OSTEOCLAST DIFFERENTIATION BY INTERACTING WITH INTEGRIN LINKED KINASE (ILK) AND MODULATING ACTIN STRUCTURES

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Abstract: Hippo signaling is implicated in balancing cell proliferation, differentiation and death in multiple organs. However, its role in specific bone cell types such as osteoclasts, and its significance in maintaining overall bone tissue homeostasis remain largely unknown. In this study, we investigated the role of the Hippo pathway in osteoclast differentiation. Human primary monocyte cells were treated with receptor activator nuclear factor kappaB ligand (RANKL) and evaluated for osteoclast differentiation by marker protein analysis, tartrate-resistant acid phosphate (TRAP) and resorption assays. Our results showed that Ste20-like kinase 1 (MST1) underwent the maximum change after RANKL treatments and is negatively associated with osteoclast differentiation. Furthermore, proteomic approaches involving co-immunoprecipitation and mass spectrometry identified MST1 interaction with integrin-linked kinase (ILK) which is lost during RANKL induced differentiation. Finally, using RNAi and ectopic expression experiments we observed that MST1-ILK interaction negatively inhibits osteoclast differentiation at the level of actin ring structure formation, which is facilitated by ILK. Together, our data highlight a role for the Hippo pathway protein, MST1, in negatively regulating osteoclast differentiation through its interaction with integrin signaling. Given that integrin signaling is progressively implicated in pathological osteolysis, augmenting this pathway could have therapeutic implications.

Key words: osteoclast; Hippo signaling; Ste20-like kinase 1 (MST1); integrin linked kinase (ILK); integrin signaling; actin ring structures.

INTRODUCTION

Organ functionality in complex multicellular organisms is maintained by balancing proliferation, differentiation and cell death. Recent research studies have identified a tumor suppressor pathway called the Hippo signaling pathway that primarily regulates cell proliferation, death and differentiation [1]. Research over the last decade into Hippo signaling has used *Drosophila* genetics extensively to identify pathway components, and in recent times their mammalian homologs are also being identified and intensively investigated [2,3].

Although several tissue-specific variations are implicated, the core to Hippo signaling is a kinase

cascade, wherein mammalian serine/threonine kinase 20 (STE20)-like proteins (MST1/2) phosphorylate large tumor suppressor (LATS1/2) kinase proteins, which in turn phosphorylate the major Hippo pathway effector proteins, Yes-associated protein (YAP) and transcriptional coactivators with PDZ-binding motif proteins (TAZ) to cause its cytoplasmic sequestration. Upon inductive signals, YAP/TAZ proteins are dephosphorylated and translocate to the nucleus, interacting with the transcription enhancer factors – TEA domain family (TEAD 1-4), to induce gene expression and facilitate cell proliferation [4-7].

Bone is a dynamic tissue that undergoes active remodeling throughout life. Bone tissue mass and func-

tion are regulated by a combinatorial action of two cell types: osteoclast and osteoblast [8,9]. Osteoblasts are derived from mesenchymal cells and are primarily responsible for new bone formation, while osteoclasts are derived from monocyte/macrophage cells and are responsible for bone resorption [8,9]. Defects in the underlying signaling cascades regulating the osteoclast/osteoblast balance lead to bone disease [8-15]. In particular, increased osteoclast mass and activity is associated with diseases such as osteoporosis [8-15]. Furthermore, the inflammatory secretions present in disease conditions are also progressively implicated in osteoclast differentiation and activity, but the intramolecular events leading to the normal differentiation or pathological state are largely unknown.

Multiple studies have underscored the importance of the tumor necrosis factor (TNF) receptor (TNFR)/TNF-like proteins (i) osteoprotegerin (OPG), (ii) receptor activator of nuclear factor (NF)- κ B (RANK) and (iii) RANK ligand (RANKL) in regulating osteoclast differentiation and function. More importantly, from these studies it is now known that RANKL or TNF- α combined with macrophage colony-stimulating factor (M-CSF) alone is sufficient to induce osteoclastogenesis from bone marrow macrophage cells *in vitro* [10-15]. However, deeper understanding of how diverse physiological and pathological signals modulate the RANKL or pathway and osteoclast functions would allow intervening therapeutics to regulate bone erosion. Given that the Hippo pathway is associated with cell differentiation in most tissues, it is intriguing to hypothesize a similar role in these processes.

In this study, we evaluated the changes in Hippo pathway genes associated with osteoclast differentiation, and identified their involvement in regulating actin-ring structures associated with osteoclast differentiation. We also identified a link between the Hippo signaling pathway and integrin signaling during osteoclast differentiation.

MATERIALS AND METHODS

Osteoclast differentiation

Human primary osteoclast precursor cells (primary monocyte cells) were maintained in DMEM supplemented with bullet kit (Lonza, USA). For differentiation, cells were plated at a density of 15000 cells/cm² and supplemented with RANKL (100 ng/mL) combined with M-CSF (100 ng/mL) (Sigma-Aldrich, USA) for 7 days, with media changes after every two days.

RNAi and overexpression

siRNA sequences targeting MST1, YAP, TAZ, TEAD1, LATS2 or ILK (Santacruz Biotechnology, USA) were transfected and evaluated after 48 h. For overexpression, cells were either transiently transfected with an empty vector or as full-length constructs of MST1 or ILK (Origene, USA). RANKL treatments were induced 48 h post siRNA treatment or expression of transfected plasmid in all of the experiments. For combined siRNA and expression plasmid, both were combined as a single transfection 48 h after RANKL treatments were induced.

Tartrate-resistant acid phosphate (TRAP) staining

RANKL-treated culture systems were fixed in 10% buffered formalin and stained for TRAP (Sigma-Aldrich, USA). The presence of three or more nuclei was used as a criterion to identify osteoclasts.

Osteoclastic resorption (pit formation assay)

Osteoclast differentiation was induced in OsteoAssay plates (Corning, USA) for 1 week and the cells were removed with 10% sodium hypochlorite, washed with water and air-dried. Resorption pits were measured using Pixelink Capture SE software.

RT-PCR

Total RNA was isolated from 1-week differentiation cultures and reverse transcribed as described previ-

ously [16]. As our cells were derived from primary cultures, we evaluated osteoclast differentiation through marker proteins that represent mature osteoclasts, while also evaluating for proteins that are known to inhibit osteoblast differentiation. By this method, if we noticed a consistent trend due to a gene knockdown, it clearly indicated a role in osteoclast differentiation. Based on this rationale, a specific marker protein indicative of osteoclast differentiation (Cathepsin K) was selected to identify osteoclasts [17] while also ensuring the trend to inhibit osteoblast differentiation (Sp7) [18]. PCR was performed with a 7500 Applied Biosystems instrument using TaqMan probes with the universal PCR Master Mix (Life Technologies, USA). The following TaqMan probes were used to evaluate gene expression analysis: *Ctsk* (Cathepsin K): Mm00484039_m1; *Sp7* (Osterix): Mm00504574_m1; *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase): Mm99999915_g1 (Applied Biosystems, USA). Untreated samples were used as reference to determine the changes in gene expression.

Co-immunoprecipitation (Co-IP) and immunoblotting

Cell lysates with and without RANKL treatments (48 h) were immunoprecipitated (2 μ g of antibody per 0.5mg of lysate protein) for MST1 and ILK proteins (Cell Signaling Technology, USA) and analyzed for interacting protein by mass spectrometry or immunoblotted for specific proteins as described previously [19]. Briefly, the cells were lysed using a RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10 mg/ml aprotinin, 1 mg/ml leupeptin, 0.1 M phenylmethylsulfonyl fluoride, 0.5 M sodium fluoride and 1 mM sodium orthovanadate). Equal amounts of proteins were separated by 4-20% gel under reducing conditions, transferred to a nitrocellulose membrane (Bio-Rad, USA), and blotted using specific antibodies for proteins. (Cell Signaling Technology, USA). The membrane was then incubated with respective secondary antibodies and developed using Super Signal West Dura (Pierce Biotechnology, USA).

Mass spectrometry

The Co-IP proteins were in-gel digested and then injected for LC-MS/MS (MALDI/TOF-TOF, 4700 Proteomics Analyzer, Applied Biosystems) analysis. The protein identifications were made using the Trans-Proteomic Pipeline software running on a sorcerer platform, and Mascot (version 2.3.02) was used as the search database. The identified proteins were then analyzed using ProHits software, and peptide coverage of 2% was used as the threshold to identify interacting proteins.

Actin staining assay

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, phosphate-buffer washed and blocked with 3% BSA for 15 min. Cells were then incubated with rhodamine phalloidin (Life Technologies, USA) for 45 min. Cells were then counterstained for nuclei and images collected.

Statistical analysis

All statistical computing and graphical presentations were prepared using GraphPad Prism software. The data from three independent experiments (n=3), each conducted in triplicate, were analyzed and presented as the means \pm standard deviations (SD). Differences are assessed by ANOVA and accepted as statistically significant at $P<0.05$.

RESULTS

Hippo pathway is involved in osteoclast differentiation

To evaluate whether the Hippo pathway is involved in osteoclast differentiation, we looked for changes in osteoclast differentiation after siRNA knockdown (KD) of the core Hippo pathway genes, MST1, LATS2, YAP, TAZ and TEAD1. Our results showed an increase in osteoclast differentiation, as evaluated by TRAP assay and specific marker gene expression

analysis after RANKL treatments (Fig. 1A and B). These results suggest a role for the Hippo pathway in osteoclast differentiation. To further understand the Hippo pathway-mediated regulation in osteoclast differentiation, we evaluated for changes in Hippo pathway proteins after RANKL treatments. Surprisingly, no change in most of the tested genes was observed, while a dose-dependent change in the levels of MST1 (Fig. 1C) and a marginal change in MST2 levels were observed (data not shown). These results suggest a critical role of Hippo pathway proteins MST1/2 in osteoclast differentiation. Similarly, previous observation has also shown that MST2 KD could increase osteoclast differentiation [20]. However, as MST1 showed a more prominent change than MST2 in our system, we evaluated the Hippo pathway with osteoclast differentiation through MST1.

MST1 inhibits osteoclast differentiation

To further understand the relationship between the MST1 protein and osteoclast differentiation, we over-expressed the MST1 protein, and examined for osteoclast differentiation. In contrast to MST1 knockdown, we observed a decrease in osteoclast differentiation after overexpression of full-length MST1 (Fig. 2A, B and Fig. 1A). These observations were further evaluated for functional significance through pit formation assay. In accordance with the osteoclast formation

Table 1. List of MST1 interacting protein differences identified through mass spectrometry.

Protein Name	Peptides Identified by MS/MS and MASCOT		Sequence coverage
	Sequence	Ion score	
ILK	G[45-66]R	45/0.0021	11%
	R[161-176]T	38/0.008	
	I[335-346]Q	42/0.014	
	A[393-406]A	45/0.0025	
14-3-3 ζ/δ	S[28-41]R	50/0.0011	11%
	V[61-74]R	72/3.5e-06	
Alpha-actinin	L[302-311]R	45/0.0057	2%
	V735-746]R	91/1.7e-07	

Minimum sequence coverage threshold for identifying interactors-2%; MS/MS – tandem mass spectrometry; MASCOT – search database

trends, MST1 KD cells showed increased pit formation, while overexpressed cells showed reduced pit formation (Fig. 2C) Together these data confirm that MST1 is involved in negatively regulating osteoclast differentiation.

MST1 interacts with ILK

To understand how MST1 is involved in regulating osteoclast differentiation, we identified interacting proteins by Co-IP, followed by mass spectrometry. We compared the interacting proteins in both untreated and RANKL-treated conditions, and selected the proteins that are exclusively present in untreated, and reduced or lost in RANKL treatments. Peptide

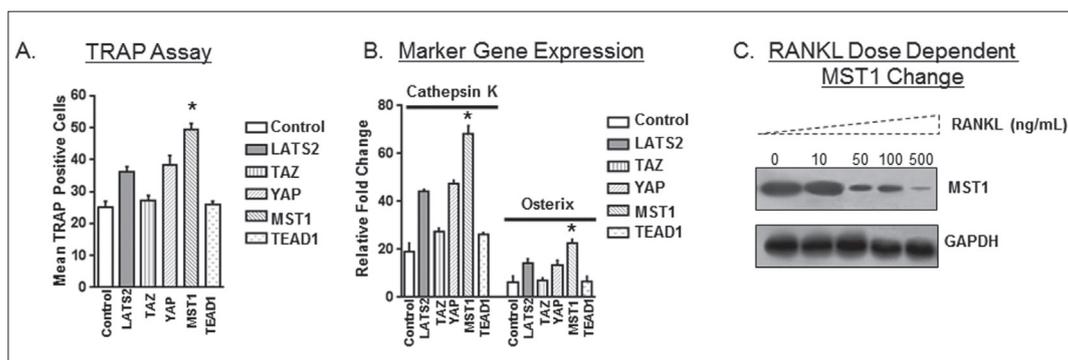


Fig. 1. Hippo pathway involvement in osteoclast differentiation. (A-B) Osteoclast differentiation upon knock-down of Hippo pathway genes, *LATS2*, *TAZ*, *YAP*, *MST1* and *TEAD1*. Non-silenced group was used as control. A – analysis by the TRAP assay. B – expression analysis of osteoclast marker proteins, Cathepsin k and Osterix, by RT-PCR. Overall, MST1 showed the maximum change in both evaluation methods, (n=3, p<0.05). C – RANKL dose-dependent change in MST1 level. A representative blot is shown (n=3).

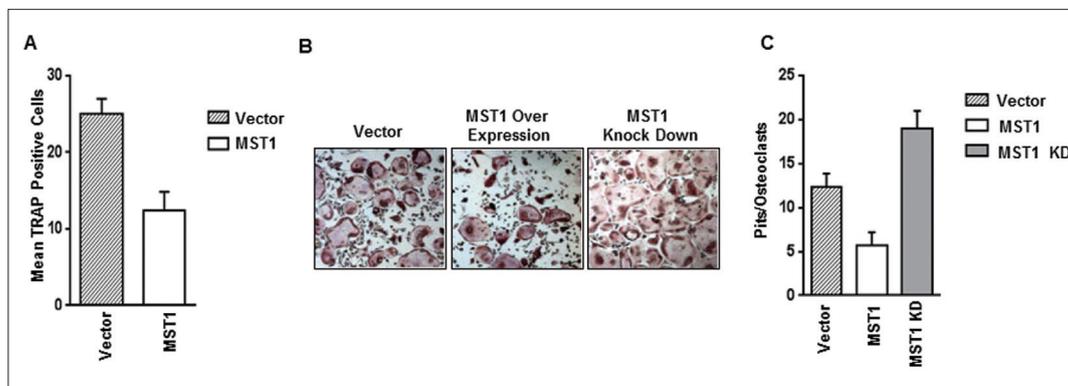


Fig. 2. MST1 negatively regulates osteoclast differentiation. Overexpression and knockdown of MST1 were performed and evaluated for changes in osteoclast differentiation by TRAP and pit formation assays. (A-B) TRAP assay. **A** – overexpression of MST1 showed a reduction in TRAP positive cells, while knockdown increased it (Fig. 1A). **B** – TRAP staining changes upon overexpression and knockdown of MST1. **C** – pit formation assay. Vector – empty vector of overexpression plasmid, MST1 – overexpression, MST1 KD – knockdown group. Statistical significance was seen between control group and KD or overexpression group ($n=3$, $p<0.05$).

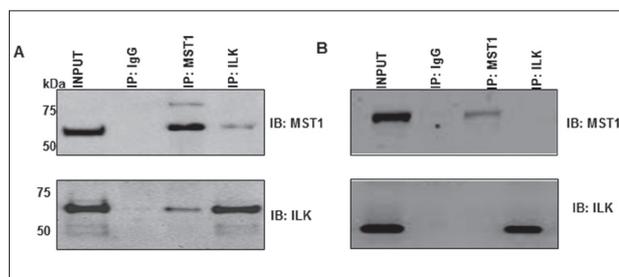


Fig. 3. MST1-ILK interaction analysis. Co-immunoprecipitated samples were prepared from both untreated and RANKL-treated conditions, and probed for MST1 and ILK interaction. **A** – without any treatment. **B** – RANKL treatment. Interaction is lost in the RANKL-treated condition.

coverage of 2% was used as the threshold and the list of proteins identified through such selection is provided in Table 1. Of them, integrin-linked kinase (ILK) was identified as a prominent interacting protein with ~11% protein coverage, and related to osteoclast differentiation [21]. To further confirm this MST1-ILK interaction, independent Co-IP experiments were performed (Fig. 3A and B). Overall, our results indicated that MST1 interacts with ILK and is lost in osteoclast differentiation after RANKL treatments. Given that MST1 levels decreased with RANKL treatment (Fig. 1), it is as question if the MST1-ILK interaction loss is either a direct loss or an effect acquired due to MST1 protein reduction after RANKL treatments. Although

speculative, the complete loss of ILK as identified through both mass spectrometry and Co-IP experiments, suggests that it could be an additive effect due to both direct interaction loss and reduction in MST1 protein levels. Future experiments on MST1 degradation and expression analysis after RANKL treatment could help in understand the mode of loss of interaction loss. Our data indicate the loss of MST1-ILK interaction after RANKL treatments.

MST1-ILK interaction inhibits actin ring structure formation

Given that ILK is known to mediate integrin signaling and is usually found at actin-integrin connection sites, we inferred that actin cytoskeleton rearrangements associated with osteoclast were modulated due to MST1 and ILK interaction. After RANKL treatments, the control vector-infected cells differentiated normally, exhibiting osteoclastic actin ring structures, while MST1-overexpressing cells showed reduced differentiation and actin ring structure formation (Fig. 4A and B), suggesting negative regulation by MST1. Accordingly, MST1 KD cells showed a RANKL-dose-dependent increase in both actin ring structures and overall osteoclast differentiation (Fig. 4A and B). These results suggest that MST1 inhibits osteoclast

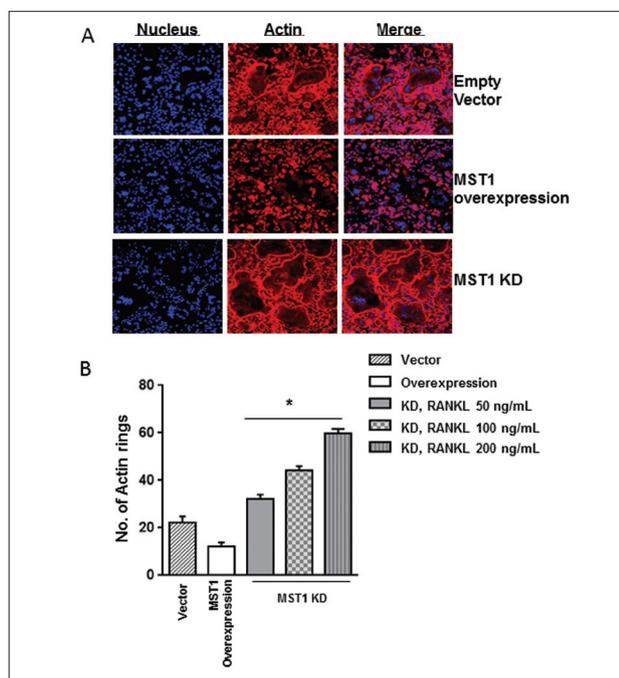


Fig. 4. MST1 inhibits actin ring structure formation. Overexpression and knockdown of MST1 were performed and evaluated for changes in actin ring structure by phalloidin staining. **A** – actin rings observed after expression and knockdown of MST1 (RANKL 100 ng/mL). **B** – quantification of actin ring structures after overexpression and knockdown of MST1 (n=3; p<0.05).

differentiation at the level of actin ring structure formation, which is important for the progression of osteoclast differentiation and maturation.

MST1-ILK interaction negatively inhibits ILK activity

Given that actin rearrangements are mediated by ILK [22], and with our observation of the negative influence of MST1, we wondered whether MST1-ILK interaction negatively inhibits ILK-mediated actin rearrangements. To that end, we overexpressed both the wild-type (WT) and kinase-deficient ILK mutant in MST1 KD cells and checked for changes in osteoclast differentiation. Our results showed a reduction in osteoclast differentiation in ILK mutant cells. Similar results were obtained in double KD of ILK and MST1 cells (data not shown). In contrast, the cells overexpressing WT ILK showed an increase in osteoclast

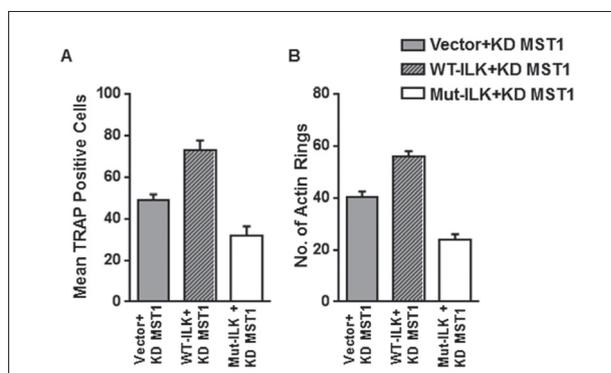


Fig. 5. MST1-ILK interaction negatively inhibits the ILK. Overexpression of wild-type (WT) or mutant (MUT) form of ILK performed along with MST1 knockdown and evaluated for changes in osteoclast differentiation. **A** – osteoclast number evaluated by the TRAP assay. **B** – actin ring structures evaluated by phalloidin staining (n=3; p<0.05).

differentiation (Fig. 5A). This change in osteoclast differentiation is also associated with actin ring structure formation (Fig. 5B). Together, these results suggest that ILK activity is required for osteoclast differentiation, and this activity is inhibited by MST1.

DISCUSSION

RANKL-based *in vitro* osteoclast differentiation systems have been widely used to dissect the molecular mechanisms associated with osteoclasts differentiation and function [23-25]. In this study, using human precursor cells and RANKL treatments, we have provided evidence that the Hippo signaling pathway protein, MST1, negatively regulates osteoclast differentiation. Furthermore, we have identified MST1-ILK interaction and its significance in regulating osteoclast actin structures, based on which we have proposed a model for MST1-ILK mediated osteoclast differentiation.

Previous studies on MST1 using mice models have largely implicated its function in T-cell development and trafficking, tumor suppression and organ size regulation [1-6]. However, its relation with bone tissue function and the underlying molecular mechanisms are not known. Our data as reported through knockdown and overexpression experiments suggest that MST1 negatively regulates osteoclast differentia-

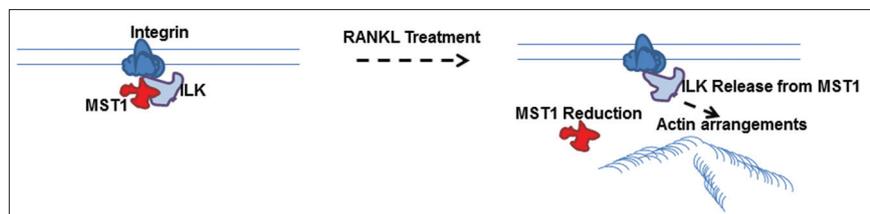


Fig. 6. A model for MST1-ILK-mediated osteoclast differentiation. Hippo pathway protein MST1 interacts with ILK and inhibits its activity to maintain undifferentiated conditions. After exposure to signals that induce differentiation, MST1 interaction with ILK is lost, causing ILK to become active, which in turn mediates the formation of the actin ring structure and osteoclast differentiation.

tion. Similar results were also obtained from MST2-based studies [20], suggesting that Hippo signaling in general might act in negatively regulating osteoclast differentiation, and could be exerted by the action of MST1/2.

Multiple studies have highlighted the significance of integrin signaling in promoting osteoclast differentiation [26-28]. However, molecular crosstalk between signaling pathways makes it difficult to segregate the components associated with osteoclast differentiation. Our results using a combination of Co-IP and mass spectrometry have identified an interaction between the Hippo pathway protein MST1 and the integrin signaling pathway protein ILK. Previous studies on ILK have also shown it to function as a cytoplasmic effector for integrin receptors and to modulate multiple cellular functions [29]. More importantly, osteoclast-specific ablation of ILK in mice showed reduced bone resorption function [21]. Consistent with this observation, our results also show that ILK inhibition as mediated by MST1 shows reduced osteoclast differentiation and maturation, which would directly impact its overall functions.

Specific actin arrangements such as podosome- and actin-ring structures are known to be associated with the state and function of osteoclasts, and are effectors in ILK and integrin signaling [30-32]. Our results on ILK inhibition and MST1 overexpression showed defects in these structure formations, suggesting that Hippo pathway negatively regulates this structure formation at the level of its formation.

The results presented herein are summarized in a model for MST1-ILK-mediated osteoclast differentiation (Fig. 6). Our data indicate that MST1 interacts with ILK in undifferentiating conditions, and upon exposure to stimuli that induce differentiation, such as RANKL, when the MST1-ILK interaction is lost, causing ILK to be active, which in turn mediates actin ring structure formation and osteoclast differentiation. Nevertheless, it is also possible that there could be additional regulators that respond to stimuli and add to the complexity of the pathway.

CONCLUSION

Our study highlights a previously unidentified link between the Hippo and integrin signaling pathways. Although it is unclear whether this is a direct interaction, given the role of integrin signaling in osteoclast differentiation, the interaction implicated here is significant and could have a regulatory function. We report that this interaction regulates downstream actin organization. However, it is worth pointing out that the model is partly derived from overexpression and knockdown experiments, and further studies are required to confirm its relevance *in vivo* systems.

Authors' contribution: Xiao-han Huang and Pan Su contributed equally to this work. Xiao-han Huang, Pan Su, Wu-Yin Li conceived and devised the experimental plans, Xiao-han Huang, Pan Su performed the experiments and Xiao-han Huang, Pan Su, Wu-Yin Li wrote the manuscript.

Conflict of interest disclosure: None.

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