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The Role of Pin1 in the fusion of osteoclasts and myoblasts

파골세포와 근육세포의 세포 융합 과정에서의 Pin1 의 역할

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Abstract

The Role of Pin1 in the physiological fusion of osteoclasts and myoblasts

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Cell-cell fusion is critical for the conception, development, and physiology of multicellular organisms. Although cellular fusogenic proteins and the actin cytoskeleton are implicated in cell-cell fusion, it remains unclear whether and how they coordinate to promote plasma membrane fusion. Pin1 null mice showed low bone mass and increased TRAP staining in histological sections of long bones, compared to Pin1 wild-type mice. In vitro osteoclast forming assays

with bone marrow-derived monocyte/macrophage revealed that Pin1-deficient osteoclasts were larger than wild-type osteoclasts and had higher nuclei numbers, indicating greater extent of fusion. Pin1 deficiency also highly enhanced foreign body giant cell formation both in vitro and in vivo. Among the known fusion proteins, DC-STAMP was significantly increased in Pin1−/− osteoclasts. Immunohistochemistry showed that DC-STAMP expression was also significantly increased in tibial metaphysis of Pin1 KO mice. I found that Pin1 bound and isomerized DC-STAMP and affected its expression levels and localization at the plasma membrane. Taken together, my data indicate that Pin1 controls of bone mass through the regulation of the osteoclast fusion protein DC-STAMP. The identification of Pin1 as a factor involved in cell fusion contributes to the understanding of osteoclast-associated diseases, including osteoporosis, and opens new avenues for therapeutic targets.

When the effect of Pin1 was tested in myoblast cells it was found that Pin1 inhibits myoblast fusion and inhibition of Pin1 enhanced myoblast fusion. If Pin1 is involved in both osteoclast and myoblast fusion it can be a key molecule to modulate in various musculoskeletal diseases as well as sarcopenia related to other

generalized ailments like cancer and ageing. The conformational regulation catalyzed by the PPIase, Pin1 is crucial for regulation of SMAD proteins. The inhibition of Pin1 activity enhanced muscle cell fusion while affected minimally the expression of key transcription factors of myogenesis, MyoD, MYF5, and Myogenin. Pin1 over-expressing myoblasts, however, failed to fuse. These findings reveal a specific role for Pin1 in myoblast fusion. Pin1 also modulates the TGF-ß signaling during muscle hypertrophy and in low dosage of inhibition it specifically reduced pSmad 3 only. Pin1 appear therefore to act as a mediator of the myogenic cell-cell fusion and hypertrophy mechanism underlying formation of functional muscle fibers.

With all the experimental results taken together it can be inferred that Pin1 can modulate myoblast and osteoclast fusion. This makes Pin1 a very important therapeutic target for several common human diseases like myopathy and osteoporosis.

Key Words: Cell Fusion, Myoblast, Osteoclast, Pin1. **Student Number: 2009-24043**

CONTENTS

ABBREVIATIONS

Pin1 peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

RUNX1 Runt-related transcription factor 1

DC-STAMP Dendrocyte Expressed Seven Transmembrane Protein

SMAD Mothers against decapentaplegic homolog

TGF-β transforming growth factor beta

I. General Introduction

The concept that all animal and plant tissues consist of cells, and that cells are the integral units of life, is the core of the 'cell theory'. The first description of cells is usually credited to Robert Hooke in 1665. The concept that cells are the building blocks of tissues was put forward in 1838 by Schleiden for plants and in 1839 by Schwann for all living things (Ogle et al., 2005). This proposition gave birth to the cell theory: cells are fundamental units of life (Wolpert, 1996). Schwann probably held a more vibrant view of cells. He observed that when cells of the superficial dorsal muscle of pig embryos come in contact with each other, "the walls coalesce at the points of junction and the blended septa become absorbed so that the cell cavities which were at first separated, now communicate ... [but], the nuclei, as we have seen, do not coalesce". A very large part of Schwann's monograph on microscopic observations of the structures of plant and animal tissues is devoted to the fusion of cells in muscle, nerve and bone (Ogle et al., 2005). So the history of the study of ‘cell fusion’ is as old as the history of the study of ‘cell’ itself.

I.1. Physiological cell fusion

Differentiation and changes in cell fate are vital for development. Cell fusion occurs physiologically and also pathologically. It is vital for fertilization (sperm-egg), development (forming syncytial tissues such as muscle), and normal physiology (bone resorption). Multinucleation may enhance or refashion the function of the fused cells, but the precise and common mechanisms in different cells that govern this process remain poorly understood. Although progress is being made, the responsible proteins are still mostly unknown. Much effort has been devoted towards the goal of identifying the factors involved in these different kinds of fusion, and some proteins have been shown to be common to several of the fusion processes studied. Pin1 also seems to be common in both the osteoclast and myoblast fusion but the role of Pin1 is different in these two kinds of fusion. Pin1 has a role in osteoclast, myoblast and trophoblast fusion so there must be a common target in all three kinds of cell to cell fusion also.

I.2. PPIases

PPIases were discovered in 1984 by Gunter Fischer and colleagues as enzymes that catalyze the cis/trans isomerization of peptide bonds

preceding proline (X-Pro) (Fischer et al., 1984). This reversible cis to trans and trans to cis interconversion does not require ATP (Fanghanel and Fischer, 2004). Prolyl isomerases typically accelerate the cis-trans isomerization of the peptide bonds within substrates (Kofron et al., 1991). PPIases were originally named “foldases”, help in the folding of nascent proteins. They can also induce conformational changes in mature proteins and consequently regulate their activity and interaction with other proteins (Gothel and Marahiel, 1999). The cyclophilins (CyPs), the FKBPs (FK506 binding proteins), and the parvulins are the three individual ubiquitously distributed families of PPIase (Arevalo-Rodriguez et al., 2004; Heitman et al., 1992). All of them are conserved amongst eukaryotes, prokaryotes and archaea (Galat, 2003; Unal and Steinert, 2014). Each family of PPIase has structurally unique catalytic domains and they have some differences in substrate specificity and are sensitive to different types of inhibitors (Schmidpeter et al., 2011; Zoldak et al., 2009). In eukaryotic cells, PPIases can be found in almost all cellular compartments including the cytoplasm, endoplasmic reticulum, mitochondrion, nucleus and even the nucleolus (Pemberton and Kay, 2005). Members of the cyclophilin and FKBP families are more commonly called immunophilins based on the effects of

immunosuppressive drugs cyclosporine A (CsA), FK506 and rapamycin (Handschumacher et al., 1984; Siekierka et al., 1989). These drugs bind in the respective active sites of the immunophilins and block PPIase catalytic activity. The interesting point in this mechanism of action is that, their immunosuppressive effects do not result from inhibition of PPIase activity, but instead from interactions with the immunophilin-drug complexes. Cyclophilin A-CsA and FKBP12-FK506 complexes inhibit the phosphatase activity of the protein phosphatase calcineurin resulting in decreased T-cell activation (Cardenas et al., 1995; Foor et al., 1992). The FKBP12-rapamycin complex inhibits the mTOR (target of rapamycin) kinase blocking signaling in T-cells in response to cytokine stimulation (Heitman et al., 1991; Sabatini et al., 1994). Thus it is evident that in case of PPIases it has to be considered whether the catalytic activity of the enzyme is critical to the biological process being studied and experiments must be designed efficiently by using mutational and drug-inhibition approaches (Gudavicius et al., 2013; Hanes, 2014).

I.3. Parvulin family

In yeast there is one parvulin, Ess1 (essential in yeast), and in

humans till now two parvulins, Pin1 (human ortholog of Ess1; protein interacting with NIMA), and Par14/17 which are protein isoforms encoded by the same gene have been discovered (Hanes et al., 1989; Lu et al., 1996a; Uchida et al., 1999). Parvulins, are all small proteins (parvulus is the latin word for very small). In bacteria, parvulins are comprised of a PPIase domain only (Rahfeld et al., 1994). In eukaryotes they also contain a N-terminal WW-domain (Sudol et al., 2001) followed by a short linker and a (Lu et al., 1996b) C-terminal catalytic domain (Hanes et al., 1989).

I.4. Pin1

Pin1 is a small protein (163 amino acids) with a simple structure comprising of a N-terminal WW domain, bearing two tryptophan residues 22 amino acids apart, and a C-terminal catalytic peptidyl–prolyl isomerase (PPI) domain (Hariharan and Sussman, 2014). The WW domain lets Pin1 recognize and bind to phospho-proteins with a serine/threonine adjacent to a proline residue and ensures its specificity (Lu et al., 1996b; Ranganathan et al., 1997).

Pin1 mediates conformational changes in its substrate proteins. Pin1 can also regulate the stability of substrate proteins by increasing or decreasing their ubiquitylation. Pin1 works with different

E3 ubiquitin ligases to regulate degradation of proteins (Welcker et al., 2004; Yeh et al., 2004; Yeh et al., 2006).

Without a tool to detect cis- or trans-specific protein conformation of a substrate protein in the cell, there is no direct evidence for protein cis-trans isomerization in vivo and for the isomer-specific regulation of its function (Liou et al., 2011; Lu and Zhou, 2007). It can be predicted that some of Pin1's functions could be mediated simply by binding of its WW domain to pS/T-P motifs like a scaffold without involving its catalytic activities (Lu and Hunter, 2014). Till now only isomer-specific antibodies have been reported for cis and trans Tau (Nakamura et al., 2012).

Pin1 is the only PPIase known to have a high specificity to substrates with phosphorylated serine and threonine side chains preceding proline (pSer/pThr-Pro) (Yaffe et al., 1997b). Both the WW-domain and the PPIase domain of Ess1 and its human ortholog Pin1, bind the same target sequence: phosphorylated Ser/Thr-Pro (Myers et al., 2001; Yaffe et al., 1997a). The WW domains are protein–protein interaction domains of about 40 amino acids and are characterized by two highly conserved tryptophan residues. They can fold into a three-stranded antiparallel β sheet (Macias et al., 2002;

Schiene-Fischer, 2014) .The WW-domain binds with ~10-fold higher affinity than does the PPIase domain (Verdecia et al., 2000). Pin1 is also unique because in case of conventional peptidyl-prolyl cis-trans isomerases (PPIases), phosphorylation slows down the isomerization rate and renders the substrate peptide bond resistant to them whereas (Yaffe et al., 1997a; Zhou et al., 2000) Pin1 binds to and isomerizes substrates only after phosphorylation. When a proline is preceded by a phosphorylated serine or threonine, the cis-trans conversion of the pSer/Thr-Pro bond is significantly more thermodynamically hindered than that of the Ser/Thr-Pro bond and rendered inaccessible to conventional PPIases, such as cyclophilins and FK506-binding proteins. In other words, this pSer/Thr-Pro bond can be specifically recognized by Pin1 (Brown et al., 2007; Lu and Hunter, 2014).

I.4.1. Pin1 and substrate specificity

The highest number of interactions between PPIases and transcription factors and signaling proteins has been reported for Pin1 isomerase. This is probably because binding sites for Pin1 are easily identified (pSer-Pro or pThr-Pro), and Pin1 shows little preference for

nearby residues (Liou et al., 2011). In one report GST-Pin1 pull-down/mass spectrometry experiment revealed over 600 Pin1-interacting proteins (Sartori and Steger, 2013; Steger et al., 2013). Some questions has been raised about the substrate specificity considering the high number of Pin1 substrates and how one protein can interact with such a huge number of proteins (Lippens et al., 2007).

I.4.2. Regulation of Pin1 function

Regulation of Pin1 function through phosphorylation is an important topic that has not been adequately explored. Phosphorylation of Pin1 at the Ser16 residue in the WW domain prevents its interaction with phosphorylated substrates (Lu et al., 2002), and thereby partially inactivates the function of Pin1. Pin1 is a substrate of DAP (Death Associated Protein) kinase (Lee et al., 2011a). Polo-like kinase-1-mediated phosphorylation stabilizes Pin1 by inhibiting its ubiquitination (Eckerdt et al., 2005). The WW domain itself can act as a negative regulator of enzymatic activity when multiple phosphorylations are present (Smet, Wieruszeski et al. 2005). Pin1 has a complex relationship with phosphorylation as it is regulated by phosphorylation, Pin1 binding is governed by substrate

phosphorylation state and after binding it can sometimes alter the phosphorylation state of the substrate.

Pin1 SUMOylation on Lys6/63 is also a mechanism for inhibition of its activity and function indicating a critical role for SENP1 (SUMO1/sentrin specific peptidase 1)-mediated deSUMOylation in promoting Pin1 function during tumorigenesis (Chen et al., 2013).

I.4.3. Pin1 inhibitors and rationale for using DTM

The PPIase Pin1 is involved in the development of cancer and Alzheimer's disease, so the inhibition of Pin1 is investigated for therapeutic usage (Takahashi et al., 2008). Juglone is the relatively non-specific inhibitor of the activity of parvulins Ess1 and Pin1, and has been used in a lot of mammalian cell culture experiments. However, given its rather general mechanism of inhibition (covalent modification of active-site cysteines), it can assumed that many different enzymes might also be targeted (Hennig, Christner et al. 1998; Zhang, Fussel et al. 2002). Juglone is also a potent inhibitor of RNA polymerase II by blocking formation of functional pre-initiation complexes (Chao et al., 2001). More specific inhibitors of Pin1 have been isolated and engineered, but are not widely available. These include peptidomimetics such as D-isomer and cyclic peptides, and

conformationally-locked substrates (Duncan et al., 2011; Liu et al., 2010; Wang et al., 2004; Wildemann et al., 2006; Zhang et al., 2007; Zhang et al., 2002; Zhao and Etzkorn, 2007). PiB, the Pin1 specific inhibitor, blocks cancer cell proliferation. However, low solubility of PiB in DMSO has made it difficult for experimental usage. DTM specifically inhibited peptidyl-prolyl cis/trans isomerase activity in HeLa cells. DTM competitively inhibits Pin1 activity, with a K(i) value of 0.05 microM (Tatara et al., 2009). Till now DTM seems to be a more practical option for experimental usage than some other Pin1 inhibitors.

I.4.4. The functional overlap between Pin1 and other prolyl cis/trans isomerases

Using a number of ess1 temperature-sensitive mutants, two groups unexpectedly discovered that cyclophilin A (CypA) can functionally replace Ess1 (Fujimori et al., 2001; Wu et al., 2000). It was the first evidence of crosstalk among different PPIase families. Despite different substrate recognition motifs, similar redistribution patterns for Pin1 and cyclophilin A (CypA, Cyp18) from the nucleus to the cytosol has been observed after PBOX-6-induced or paclitaxel-induced microtubule disruption in haematopoietic cells (Bane et al.,

2009; Theuerkorn et al., 2011). It was also discovered that there is a basal enzymatic activity towards phosphorylated substrates in cell lysates from Pin1–/– knockout mice (Fanghanel et al., 2006) and it indicates that there might be a functional overlap. Pin1 interacts with the pSer339-Pro340 motif on interferon regulatory factor (IRF)-3, leading ultimately to its degradation (Saitoh et al., 2006). In the homologous IRF-4, the Ser-Pro motif of IRF-3 is interrupted by a Leucine, although it is the best conserved regions between both transcription factors. Pin1 does not recognize this motif, and no IRF-4 regulation by Pin1 has been reported yet. However, IRF-4 is regulated by FKBP52, a member of the FK506-binding prolyl cis/trans isomerases. The tetratricopeptide repeats of FKBP52 mediate the interaction with IRF-4 and it might be comparable to Pin1's WW domain, whereas its catalytic domain could induce structural changes in the N-terminal proline-rich domain of IRF-4 (Mamane et al., 2000).

I.5. Pin1 in bone

Osteoclasts are derived from monocytes (Karsenty and Wagner, 2002), which are recruited to the bone surface to resorb the bone matrix. Large osteoclasts with many nuclei can be hyperactive (Jones et al., 1986). Hyperactivity of osteoclasts is characteristic of

bone destructive disorders, such as osteoporosis, rheumatoid arthritis, and bone metastasis (Helfrich, 2003), whereas hypoactivity of these cells is associated with osteopetrosis. Osteoclast precursors on the bone surface are stimulated by macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) secreted by osteoblasts, with this stimulation being essential for activation of signaling cascades that underlie osteoclastogenesis. NFATc1 (nuclear factor of activated T cells cytoplasmic 1) is a master transcription factor that is induced and activated downstream of the receptor activator of NF-kB on osteoclast precursors (Takayanagi et al., 2002).

In addition to multinucleated osteoclasts, hybrid cells which are osteoclast–cancer cell have been detected in bone metastasis lesions in individuals with myeloma (Andersen et al., 2007). Moreover, leukocyte–cancer cell fusion has been implicated in malignant cancer progression, including metastasis (Pawelek and Chakraborty, 2008). Although the biological relevance of hybrid cells from fusion of osteoclasts with cancer cells remains unknown (Kupisiewicz, 2011), it is possible that unique patterns of gene expression in such hybrid cells contribute to cancer progression (Dittmar and Zanker, 2011; Lu and Kang, 2009). Unraveling the molecular basis for cell–cell fusion

may thus provide insight into both physiological and pathological processes (Oikawa et al., 2012). Pin1 is dramatically misregulated in many types of human cancer samples (Khanal et al., 2013; Pulikkan et al., 2010).Pin1 is also involved in rheumatoid arthritis (Nagaoka et al., 2011). So the role of Pin1 might be important not just in osteoclast fusion but also in case of cancer cell fusion or osteoclast-cancer cell hybrid formation.

I.6. Pin1 in muscle

Myoblast fusion, a fundamental step in the differentiation of muscle in most organisms, can involve tens of thousands of myoblasts (Cossu, 2006). If the complexity of the musculature is considered, fusion must be a regulated process in which the appropriate numbers of cells fuse at the appropriate time and place. In addition to the early fusion events that occur during embryogenesis, vertebrate muscle tissue is able to regenerate in response to damage and disease (Abmayr and Pavlath, 2012). Adult skeletal muscle is a highly plastic tissue, the mass of which changes in response to environmental cues and physiological stimuli. The basic cellular building blocks of adult muscle are the multinucleated myofibers, which are able to grow during postnatal period through fusion and

hypertrophy, during regeneration after injury, and in response to a functional demand, such as exercise and external loads (Enesco and Puddy, 1964). During muscle growth in the postnatal period, large numbers of myoblasts fuse to the ends of myofibers, resulting in increased myofiber length and girth (Griffin et al., 1971).

Pin1 null mice showed an age-dependent decrease in bone mineral density and trabecular bone formation with increased osteoporotic features (Lee et al., 2009a). Peptidyl-prolyl cis-trans isomerase, Pin1, is a critical fate determinant for the post-phosphorylation modification of Runx2 (Yoon et al., 2014). Similar to Runx2 protein, the closely related other Runt-domain transcription factors, Runx1 and Runx3, also interact with Pin1 (Yoon et al., 2013b). This makes Pin1 a very important protein in bone biology. New born $Pin1^{-/-}$ mice showed features similar to cleidocranial dysplasia(Yoon et al., 2013b).

It was also reported from our lab that not only Runx2, Pin1 is also involved in the post translational modification of the Smad proteins (Yoon et al., 2015).

Pin1 is highly expressed and mainly localized to the nucleus in the neonatal heart; transition into adulthood causes a reduction in

nuclear expression and a switch to cytosolic localization in the myocardium (Hariharan and Sussman, 2014). By selectively choosing proteins among the hypertrophy signaling cascades to stabilize and modulate, Pin1 participates in cardiac hypertrophy signaling (Ranganathan et al., 1997). Post-translational modifications such as reversible protein phosphorylation form the integral portion of several complex signaling cascades governing cellular fate during cardiac pathophysiological conditions (Hariharan and Sussman, 2014). So the role of Pin1 in cardiac muscle is of no surprise.

Pin1 is known to modulate the TGFβ/BMP2 signaling (Nakano et al., 2009; Shen et al., 2012a; Ueberham et al., 2014). When directed to the nucleus by TGF-beta or BMP signals, Smad proteins undergo cyclin-dependent kinase 8/9 (CDK8/9) and glycogen synthase kinase-3 (GSK3) phosphorylations that mediate the binding of YAP and Pin1 for transcriptional action, and of ubiquitin ligases Smurf1 and Nedd4L for Smad destruction. Thus Pin1 mediates the ubiquitination process of R-SMAD proteins (Aragon et al., 2011).

SMAD proteins are implicated as regulators of muscle bulk (Goodman and Hornberger, 2014). TGF-β signaling upregulation is seen in inherited and acquired myopathies (Burks and Cohn, 2011)

and recently BMP signaling has been established as a positive regulator of muscle bulk formation (Sartori et al., 2013). Considering the role of Pin1 in SMAD signaling it is conceivable that Pin1 also has a role in regulation of the muscle bulk.

I.7. References

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II. Rationale and outline of the thesis experiments

Chapter I

Previously it was reported that runx2 Runx2 as well as other runx Runx proteins (runx1 Runx1 and runx3Runx3) bind to Pin1, and that Pin1 regulates the function of Runx2 in osteoblasts(Yoon et al., 2013a)(Yoon et al., 2013b). Because As PU.1 is the major downstream target of Runx1, I hypothesized that the Pin1-runx1 Runx1 interaction may be related to the regulation of adult hematopoiesis by PU.1 modulation at the transcriptional level. In this part, I describe a potential role of Pin1-mediated isomerization of runxRunx1 in regulating its stability and transacting activity, which, in turn, controls PU.1 transcription. Consequently, I also found that the acetylation of Runx1 in pre-monocyte cells repressed PU.1 transcription, that this effect was further enhanced by Pin1 and that this effect was also evident in early monocyte fate determination.

Chapter II

I found that Pin1-/- mice had an increased amount of the monocyte/macrophage marker F4/80 expression in spleen tissue and the total population of CD11b+/F4-80+ cells in the spleen were more than Pin1+/+ mice. Osteoclasts are derived from monocytes and old Pin1-/- mice show osteoporotic phenotype which may be caused by increased osteoclastogenesis. I observed histological sections of tibial bone from Pin1-/- mice after TRAP staining to find out if osteoclasts number and size were increased. I also did TRAP staining in vitro on primary osteoclasts derived from Pin1-/- cells and I found that total number of osteoclasts were not significantly increased but Pin1-/- osteoclasts were larger in size. It was evident that fusion was increased so I did fusion assays and found indeed fusion was enhanced in Pin1-/- osteoclasts compared to Pin1+/+. Then I tried to find the transcription factor or fusion protein responsible for this effect. I found that DC-STAMP was highly expressed in Pin1-/- osteoclasts. I also found that Pin1 binds to DC-STAMP and regulates the localization of DC-STAMP during osteoclastogenesis.

Chapter III

Similar to osteoclasts myoblasts also fuse and form heterokaryons during differentiation. As Pin1 was involved in osteoclast fusion I also desired to see if Pin1 was involved in myoblast fusion. With C2C12 cell line, the in vitro model of myoblast differentiation, I found that overexpression of Pin1 also suppressed myoblast fusion. I also observed that low dosage of Pin1 inhibitor actually caused more enhanced fusion than higher dosage. To see if this results were true in-vivo, I compared the Pin1+/+ and Pin1+/- mice muscle cross sections and found that pin+/- muscles had increased bulk. I could not find DC-STAMP expression in myoblasts. The R-SMADs SMAD 1, 2 and 3 are well known targets of Pin1 and also one of the central players in muscle bulk regulation. So I observed if the SMAD Smad protein levels were changed in muscle after Pin1 inhibition and also in Pin1+/- mice. I found that with low dose of Pin1 inhibitor treatment only pSMAD3 pSmad3 expression level was decreased and pSMAD pSmad 1 and 2 expression levels were mostly unchanged. SMAD3 Smad3 was already reported to be one of the major proteins regulating muscle bulk and my results show that Pin1 regulates muscle bulk through modulation of the SMAD Smad 3 signaling.

Chapter I

Pin1-mediated prolyl isomerization of Runx1 affects PU.1 expression in pre-monocytes

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III.1. Introduction

The transcription factor PU.1 is a hematopoietic lineage-specific ETS family member (Moreau-Gachelin et al., 1988) that is essential for normal hematopoiesis (Tenen, 2003). The expression level of PU.1 is critical for specifying the hematopoietic cell fate (Carotta et al., 2010; DeKoter and Singh, 2000), and even moderate changes in PU.1 levels can lead to leukemias and lymphomas (Mak et al., 2011; Rosenbauer et al., 2004). This crucial role of PU.1 expression level is demonstrated by the observation that either the disruption of PU.1 or the gradual reduction in its expression induces a leukemic phenotype in mice (Metcalf, 2006). The loss of expression of PU.1 in B-cells is responsible for the inactivity of immunoglobulin promoters and enhancers in HRS (Hodgkin and Reed-Sternberg cells) of classical Hodgkin disease (cHD) (Jundt et al., 2002). PU.1 prevents excessive HSC division and exhaustion by controlling the transcription of multiple cell cycle regulators. In mature cells, such as macrophages, with high PU.1 expression, PU.1 is important to establish and maintain critical levels, particularly at the earliest stage of the hematopoietic hierarchy: HSCs during commitment to a specific lineage (Staber et al., 2013).

RUNX1, also known as CBFA2 (core binding factor A2), is a

member of the Runx family of transcription factors and plays a critical role in normal fetal hematopoiesis. Homozygous deletion of RUNX1 results in embryonic lethality caused by absence of definitive hematopoiesis (Ichikawa et al., 2004a; Okuda et al., 1996; Song et al., 1999). Runx1 knockout murine embryos have no detectable definitive erythrocytes or myeloid cells in their circulation or livers and die in utero at embryonic day 12.5 (E12.5) (Okuda et al., 1996). In humans, haploinsufficiency of RUNX1 is associated with an increase in myeloid progenitors, platelet dysfunction, familial thrombocytopenia, splenomegaly and a predisposition to acute leukemia (Growney et al., 2005; Putz et al., 2006; Song et al., 1999). The development of mature blood cells from hematopoietic precursors requires cooperation between additional transcription factors and Runx1; these cooperating factors include the ETS-family member PU.1 and GATA1 (McKercher et al., 1996; Scott et al., 1997). Although many other regulatory elements exist, most aspects of PU.1 transcriptional regulation have been described by referring to just two regulatory elements, the promoter and an upstream regulatory element (URE) approximately 14 kb upstream of the transcription start site of the Sfpi1 gene in mice; these elements have cell type-based specificity for regulation of PU.1 transcription (Huang et al., 2008; Li et al., 2001).

AML1 (Runx1) binds to two conserved Runx1 binding sites in the PU.1 URE both in vitro and in vivo, and PU.1 is either downregulated or upregulated upon Runx1 binding, depending on the lineage. PU.1 also regulates its own expression through a binding site in this region (Leddin, 2011). There is still a limited understanding of the mechanisms that determine the cell-type-specific regulation of PU.1 transcription by Runx1.

The characterization of Pin1, a highly substrate specific peptidyl-prolyl *cis*/*trans* isomerase (PPIase), has led to the finding of a new post-phosphorylation regulatory mechanism that may modulate the stability of substrate proteins at the cellular level (Finn and Lu, 2008; Lu et al., 2006; Lu and Zhou, 2007; Takahashi et al., 2008; Yeh and Means, 2007). The function of Pin1 is to accelerate the intrinsically slow conformational switch between cis and trans forms of proteins, giving it the capability to function as a molecular switch (Lu et al., 2007). This function appears to be crucial during physiological events that require rapid or precisely timed responses (Lu et al., 2007; Yeh and Means, 2007); myeloid cell commitment is one such biological process. Pin1 deregulation is implicated in a number of diseases, notably ageing and age-related diseases, including cancer and Alzheimer disease (Lee et al., 2011b). Pin1 is already known to

bind to and modulate PU.1 at the post-translational level (Akiyama et al., 2011; Barberi et al., 2012), but the high increase in PU.1 protein level in the absence of Pin1 might not be solely due to post-translational modification. The possibility that there is a relationship at the transcriptional level cannot be ignored. Previously, PU.1 mRNA was also shown to be increased in Pin1-/- MEF cells (Barberi et al., 2012). At the same time, however, it was not changed in Flt3 ligand-generated bone marrow dendritic cells (Barberi et al., 2012). This suggests that there is a cell type-specific regulation of PU.1 mRNA by Pin1. Because Runx1 is already known to regulate PU.1 transcription in a cell type-specific manner, I hypothesized that PU.1 mRNA is modulated by Pin1 through Runx1.

Previously, I found that Pin1 binds to all three Runx proteins and that the interaction of Pin1 with Runx2 is critical for bone development (Yoon et al., 2013a). This raised the possibility that RUNX1 isomerization by Pin1 is significant in hematopoiesis, embryogenesis, cell proliferation and other Runx1-dependent physiological pathways. Because PU.1 is the major downstream target of Runx1, I hypothesized that the Pin1-Runx1 interaction may be related to the regulation of adult hematopoiesis by PU.1 modulation at the transcriptional level. In this study, I describe a

potential role of Pin1-mediated isomerization of Runx1 in regulating its stability and transacting activity, which, in turn, controls PU.1 transcription. Consequently, I found that the acetylation of Runx1 in pre-monocyte cells repressed PU.1 transcription, that this effect was further enhanced by Pin1 and that this effect was also evident in early monocyte fate determination.

III.2. Materials and methods

III.2.1. Animal Studies

The Pin1 +/- mice have been described before and were kindly given by Dr. Takafumi Uchida (Tohuku University). Pin1-/- mice were generated from heterozygous matings. For genotyping analysis, genomic DNA was isolated from tail biopsies and subjected to PCR. PCR products were resolved by agarose gel electrophoresis. All animal studies were reviewed and approved by the Special Committee on Animal Welfare, Seoul National University, Seoul, Republic of Korea.

III.2.2. Cell lines, recombinant proteins and plasmids

Primary mouse embryonic fibroblasts (MEFs) were isolated from E13.5 mouse embryos of the Pin1+/+, Pin1 +/- and Pin1-/-

genotypes as described previously (Fujimori et al., 1999). MEFs were grown in DMEM supplemented with 10% FBS and Passage # 2-3 cells were used for this study. The human promonocytic cell line U937 was kindly provided by Dr. Seung Hyun Han (School of Dentistry, Seoul national university). MEF and HEK293 cells were cultured in DMEM (Hyclone laboratories, Inc., Utah, USA) while U937 human leukemic cells and Jurkat cells (ATCC, Manassas, VA) were cultured in RPMI-1640; both were supplemented with 10% FBS and 1% **Penicillin** and **Streptomycin** unless otherwise mentioned.

The PU.1, Pin1 and Pin1-C115 expression vectors were engineered as previously described (Yoon et al., 2013a). The pCS4-3Myc-Runx1 expression vector was a kind gift from Dr. Suk-Chul Bae (Chungbuk National University).

III.2.3. Transient transfection and the luciferase reporter assay

Transient transfections of U937 cells with plasmid constructs were carried out using Hilymax transfection reagent (Dojindo laboratories, Kumamoto, Japan) with optimum conditions for transfection as recommended by the manufacturer. SMART pool siRNAs, a mixture of four siRNAs for rodent Pin1, were purchased from Dharmacon (Chicago, IL). Non-targeting siRNA (control siRNA; si-Scr) were also purchased from Dharmacon and used as a negative

control. These siRNAs were transfected into U937 cells by using a Neon electroporation device according to the manufacturer's guidelines. The reporter plasmid 6xOSE2-luc was described before, and the Luciferase reporter constructs containing the wild-type 0.5-kb mouse PU.1 promoter, extending from bp - 334 to +152 relative to the major PU.1 transcription start site with the URE (a 3.4-kb fragment located 14 kb 5'-upstream of the PU.1 gene) and the same vector with mutated Runx1 binding site were kindly given to us by Dr. Daniel G Tenen (Harvard Stem cell institute, Boston).

For the reporter assays, HEK293 and U937 cells were transfected with Luc reporters in defined ratios with expression plasmids. In all the samples, the pGL3 basic reporter (Promega, Madison, Wisconsin, USA) was included as negative control. Equimolar amounts of the empty vectors pCDNA 3.1 was used as controls. Twenty-four hours after the transfections, the cells were lysed and assayed for luciferase activity using the Bright-Glo Luciferase kit (Promega).

III.2.4. Pulse-chase analysis

HEK293 or MEF (Pin1+/+ and Pin1-/-) cells transfected with pCS4-3Myc-Runx1 were treated with cycloheximide (20 μg/ml) and cells were then collected at the indicated time points and subjected to

lysis and western blot.

III.2.5. Immunoprecipitation and immunoblotting analysis

Cultured cells were washed 3 times with ice-cold PBS and cell extracts were prepared in a lysis buffer of 50 mM HEPES (pH 7.5), 150 mM NaCl, 100 mM NaF, 1 mM DTT, 1 mM EDTA, 0.25% Na-deoxycholate, 0.25% CHAPS, 1% NP-40, and 10% glycerol supplemented with protease and phosphatase inhibitors. All antibodies and chemical reagents are described in the supplementary tables.

For immunoprecipitation and immunoblotting of endogenous Runx1, the cells were lysed in a denaturing lysis buffer (1% SDS, 5 mM EDTA, 10 mM dithiothreitol or beta-mercaptoethanol, Protease inhibitors, and 15 U/ml DNase1), denatured at 95 degrees for 5 min, and diluted with non-denaturing buffer (20 mM Tris HCL pH8,137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA). For immunoprecipitation, the lysates were immunoprecipitated with primary antibody for 3-12 hours (depending on the protein), incubated with pre-blocked (with BSA) Protein G Magnetic beads (Millipore) for 3-4 hours, and then immunoblotted.

III.2.6. Real-time PCR

Total RNA was extracted from the whole spleen of 6-week-old

mice (crushed in liquid nitrogen and homogenized) or cultured cells, using the easy-BLUE™ Total RNA Extraction Kit. cDNAs were synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit (Takara bio, Otsu, Shiga, Japan). Approximately 100 ng of RNA was used for each relative qPCR reaction. The sequences of the primers used for PCR are given in the supplementary methods section.

III.2.7. ChIP assay

Chromatin-immunoprecipitation (ChIP) experiments followed standard protocols as described (Okuno et al., 2005). The two primer sets used were derived from the distal and proximal homology regions (Huang et al., 2008), and the primer sequences are listed in Supplementary Table.

III.2.8. FACS analysis

For flow-cytometric analysis, the spleen was cut into small pieces and single-cell suspensions were prepared after incubation in red blood cell lysis buffer for 5 minutes at room temperature. The suspended cells (1×10^6) were washed in PBS containing 3% FCS, blocked with anti-CD16/32 to prevent nonspecific binding to the Fc receptor, and then stained with Alexa488 or PE. The cells were treated with 7AAD (BD Pharmingen, San Diego, CA) immediately

before analysis to exclude dead cells. All steps were performed at 4ºC or on ice. FACS was performed on a BD Biosciences FACSCalibur flow cytometer, and the data were analyzed using the BD CellQuest Pro software.

III.2.9. Statistical analyses

All quantitative data are presented as the mean ± SD. Each experiment was performed at least three times, and the results from one representative experiment are shown. Significant differences were analyzed using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

III.3. Results

III.3.1. Pin1 binds to Runx1 and regulates its stability and transacting activity

My lab had had previously shown that Runx2 as well as other Runx proteins (Runx1 and Runx3) bind to Pin1 (Yoon et al., 2013a). It was also found that Pin1 regulates the function of Runx2 in osteoblasts. Because Runx1 is an indispensable transcription factor in hematopoietic cells, I treated two blood cell lines, Jurkat cells (a T lymphocyte cell line) and U937 cells, with the Pin1 inhibitor dipentamethylene thiuram monosulfide (DTM) (Tatara et al., 2009) and observed that the Runx1 protein level was decreased (Figure. 1A). In the peripheral blood of Pin1-/- mice, I also observed a decreased level of Runx1 protein (Figure. I.1C). The overexpression of Pin1 in U937 cells increased the expression level of Runx1 (Figure. I.1B). The inhibition of Pin1 also decreased the acetylation level of Runx1 (Figure. I.1D), and the overexpression of Pin1 increased the acetylation of Runx1 (Figure. I.1E), almost to the level observed when incubated with the histone deacetylase inhibitor TSA (Trichostatin A).

Because my goal was to study the role of Pin1 in Runx1 regulation in hematopoietic cells, and PU.1 is the major downstream

target of Runx1, I chose monocyte development as an ideal biological process to study this effect. To this end, we chose the cell line U937, which has the potential to serve as a model for the differentiation of monocytes and macrophages in vitro. With a pulse-chase assay, I could see that in Pin1-/- MEF cells and U937 cells, the stability of Runx1 was reduced (Figure. I.2A and I.2C) and the overexpression of Pin1 attenuated the ubiquitination level of Runx1 (Figure. I.2B), whereas the overexpression of an enzyme activity mutant of Pin1 (Pin1-C115A) had the opposite effect (Figure. I.2B). In U937 cells, the overexpression of Pin1 decreased the ubiquitination level of Runx1 (Figure. I.2D). Therefore, Pin1 stabilizes Runx1 by decreasing its ubiquitination and degradation.

The transacting activity of Runx1 is reduced by inhibition of Pin1 with the inhibitors DTM (2 μM) and Juglone (5 μM); this reduction in activity was detected by Luciferase assays with the Runx reporter pGL3-6xOSE2-luc (Figure. I.3A). DTM treatment resulted in almost a sevenfold decrease in Runx1 transacting activity. Transfection with si-Pin1 also greatly decreased Runx1 transacting activity (Fig. I.3B), whereas the overexpression of Pin1 augmented the transacting activity of Runx1 (Figure. I.3C).

III.3.2. Acetylation of Runx1 represses PU1 transcription in pre-monocytes

Runx1 is known to interact with HDAC 1 and 3 (Guo and Friedman, 2011), and I found that over expression of HDAC 1 did indeed totally abrogate the acetylation of Runx1 (Figure. I.4A), but the overexpression of HDAC 2, 4 and 5 did not have the same effect. It was previously reported that TSA treatment can cause a block in PU.1 gene expression in three macrophage-monocyte cell lines (RAW 264.7, WEHI-3, and 2052 (Laribee and Klemsz, 2001)), but TSA also stimulated the PU.1 transactivation potential in other cells, including NIH3T3 (Bai et al., 2005). I hypothesized that this differential change in PU.1 mRNA was due to the acetylation level of Runx1. In U937 cells, I found that the overexpression of HDAC1 increased the PU.1 mRNA level 1.9-fold, whereas the overexpression of P300 decreased the mRNA level of PU.1 more than 2-fold (Figure. I.4B and C). Using a reporter construct containing the PU.1 promoter and the 14 kb upstream regulatory region, I also found that TSA did indeed reduce luciferase activity. Mutating the Runx1 binding sites in the same construct caused a very high basal level of luciferase activity, most likely due to the removal of inhibition by endogenous Runx1 (Figure.

I.4E and F). The luciferase activity was further increased with Runx1 overexpression or TSA treatment, suggesting that there is a role of other Runx1-interacting proteins and TSA targets in PU.1 transcription when the repression mediated by Runx1 is removed in pre-monocytes.

III.3.3. Down-regulation of Pin1 impairs Runx1-induced repression of PU.1 expression

I observed that Pin1 stabilizes Runx1 and increases its acetylation and acetylation of Runx1 represses the transcription of PU.1 in early monocytes. Therefore, I wished to examine the role of Pin1 in PU.1 transcription. The treatment of U937 cells with the Pin1 inhibitor DTM resulted in a 2.7-fold increase in PU.1 mRNA expression (Figure. I.5A and B). I also measured the mRNA level of PU.1 in spleen tissue, which has a high and heterogeneous monocyte/macrophage lineage population. I compared the splenic PU.1 mRNA in the absence of Pin1 and compared it to PU.1 mRNA expression in similar tissue in wild type mice of same age. Pin1-/- mice showed a 2.6 fold increase in PU.1 mRNA expression compared to wild type mice but had no significant change in Runx1 expression

(Figure. I.5C and D). Similar to previously published work, I found that PU.1 protein was also increased in Pin1-/- mice spleen tissue by western blot and immunohistofluorescence (Supplementary data).

Using a ChIP assay, I also observed that in Pin1-/- spleen and bone marrow cells, Runx1 binding to the approximately 14 Kb-URE regions is reduced compared to Pin1 wild type mice (Figure. I.5E). This region has three Runx1 binding sites through which Runx1 regulates PU.1 transcription in a cell context-dependent manner (Huang et al., 2008). Consistent with my previous observations, this might indicate that Pin1 acts on PU.1 transcription through Runx1 and that a lack of Pin1 reduces the inhibitory effect of Runx1 on PU.1 transcription in monocyte lineage cells and precursors of this lineage.

To confirm this, I transfected U937 cells with the reporter construct containing the 5' ~14 Kb-URE regions and the PU.1 promoter and found that the inhibition of Pin1 with siRNA rescued the inhibition induced by Runx1 overexpression (Figure. I.5F). I also observed a similar result with DTM treatment; with the Runx1 binding site-mutated construct there was no further change in luciferase activity after DTM treatment, suggesting that Pin1 acts through Runx1 in PU.1 transcription (Figure. I.5G and H). Therefore, I can summarize from these observations that a knockdown or total lack of Pin1

increases PU.1 transcription through Runx1 in monocyte precursors and monocyte lineage cells.

III.3.4. Pin1 affects monopoiesis

PU.1 is the major transcription factor in monocyte development (Friedman, 2007). I already observed that Pin1 regulated PU.1 mRNA level through Runx1 and it is known to stabilize PU.1 by posttranslational modification. Some researchers have suggested that Runx1 is induced by high levels of PU.1 and in turn trans-represses PU.1 expression; this establishes a negative feedback loop that creates a favorable PU.1 level required for balanced fate commitment to neutrophils versus macrophages (Jin et al., 2012). In case of absence of Pin1 this feed-back loop might be altered, resulting in further increases in PU.1 and reductions in Runx1 activity, further augmenting monocyte development through PU.1 overexpression. A previous study already found that in Pin-/- mice, the common monocyte granulocyte cell precursor population is not significantly changed compared to wild type mice (Barberi et al., 2012), but the more differentiated dendritic cell population was altered. I focused on monocyte lineage cells exclusively and found that in spleen, $CD11b^{+}/F4\text{-}80^{+}$ cells were significantly increased in Pin1-/- mice (Figure. I.6A), and using immunohistofluorescence of whole

spleen tissue sections, I found an increase in the monocyte/macrophage marker protein F4/80 compared to wild type mice (Figure. I.6C). In assessing the effects of Pin1 on monopoiesis, I have used several strategies to minimize bias due to cell cycle inhibition; cells freshly isolated from spleen tissue were analyzed within three hours of extraction and dye (7AAD) for the exclusion of dead cells was added immediately prior to data acquisition. I used the data as percentage of a fixed number of cells freshly isolated rather than gating particular groups and culturing them in cytokines.

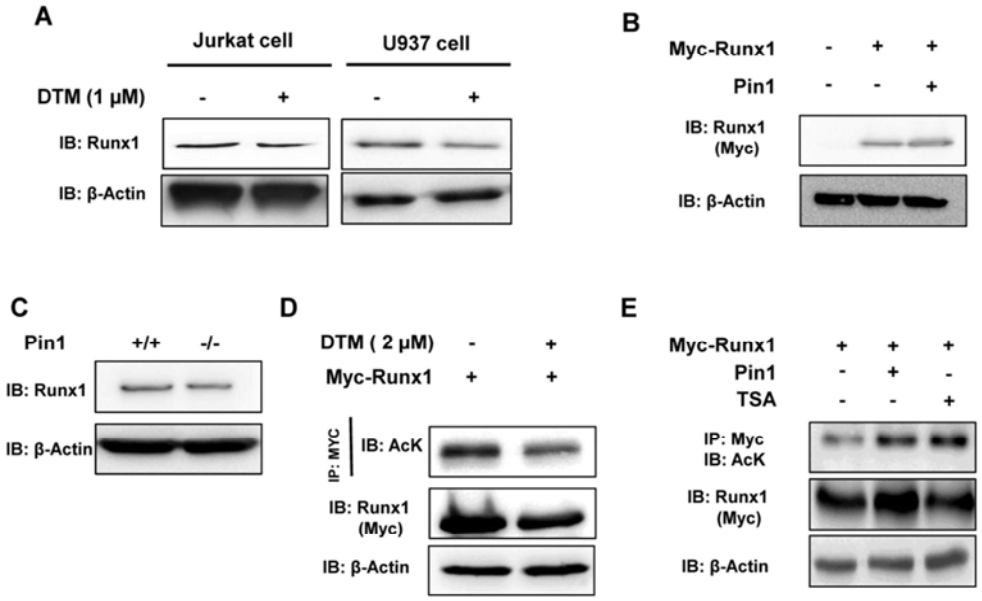

Figure I.1. Runx1 protein expression levels and acetylation are reduced in the absence of Pin1 in hematopoietic cells.

(A) U937 and Jurkat cells were treated with 1 μM DTM for 12 h. Endogenous Runx1 was detected from whole cell lysates prepared in denaturing lysis buffer by immunoblotting with anti-Runx1 antibody. (B) The peripheral blood of adult (8-week-old) Pin1+/+ and Pin-/- mice was collected and subjected to lysis followed by immunodetection of Runx1 protein (n=4). (C) U937 cells were transfected with empty vector (PCDNA3.1), PCS4-3Myc-Runx1 or Pin1 expression vector. The cells were harvested 48 h post transfection. Whole cell lysates were

prepared and immunoblotted with Anti-Myc antibody. (D) HEK 293 cells transfected with PCS4-Myc-Runx1 were treated with 2 μM of the Pin1 inhibitor DTM, immunoprecipitated with Anti-Myc antibody, and immunoblotted with an antibody against acetylated lysine. The input samples were immunoblotted to detect exogenous Runx1 protein levels, and beta actin levels were analyzed as a loading control. (E) HEK293 cells were transfected with PCS4-Myc-Runx1 and Pin1 or treated with TSA (100 nM); lysates were immunoprecipitated with anti-Myc antibody and then immunoblotted with anti-Acetylated lysine antibody or anti-Myc antibody.

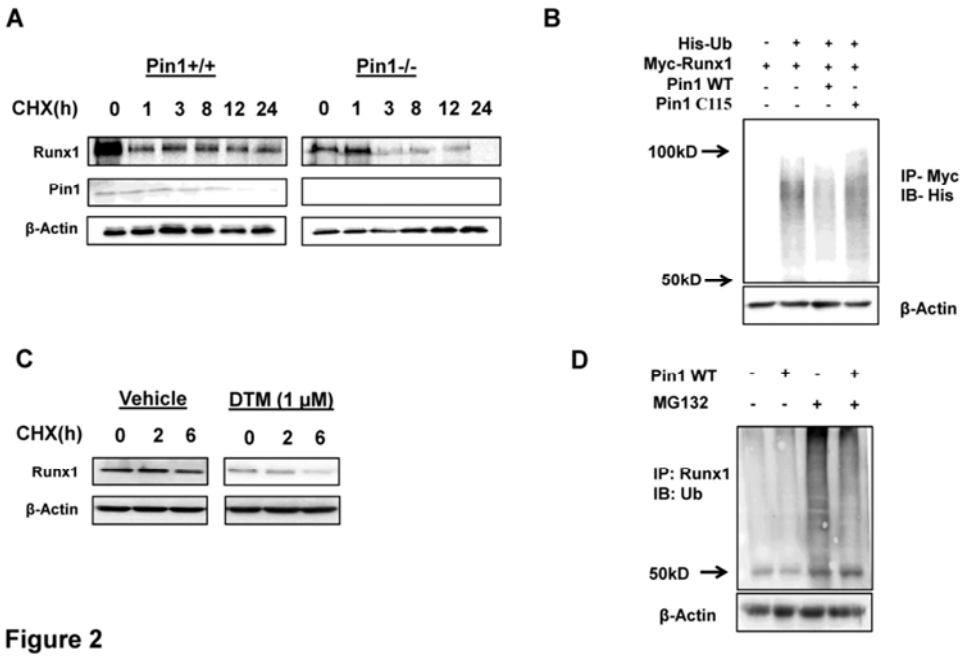

Figure 2

Figure I.2. The stability of Runx1 is reduced without Pin1 due to an increase in ubiquitination.

(A) Pin1+/+ and Pin1-/- MEF (Mouse embryonic fibroblast) cells were electroporated with PCS4-Myc-Runx1, treated with 20 μg/ml cycloheximide (CHX), and harvested at the indicated time points. Whole cell lysates were prepared and the expression of Myc-Runx1 was analyzed by western blot. (B) Ubiquitination assays were performed in HEK293 cells that were transfected with Myc-Runx1 and His-Ubiquitin with or without two Pin1 constructs (Pin1-WT and Pin1-C115A). Cells were transfected with PCDNA3.1 instead of His-

Ubiquitin as negative control. After lysis, the cells were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-His antibody. Before harvest, the cells were treated with MG132 for 2 h. (C) U937 cells transfected with PCS4-3Myc-Runx1 with or without incubation in 1 μM DTM were harvested at 0, 2, 6 and 8 h after being subjected to a pulse-chase assay with 20 μg/ml cycloheximide (CHX). Whole cell lysates were immunoblotted with anti-Myc antibody after western blotting. (D) Pin1 was overexpressed in U937 cells and incubated in the presence or absence of MG132 for 6 h before harvesting. Ubiquitination assays were performed using whole cell lysates that were immunoprecipitated with an anti-Runx1 antibody and immunoblotted with an anti-Ubiquitin antibody. The input sample lysate was used to detect beta actin levels.

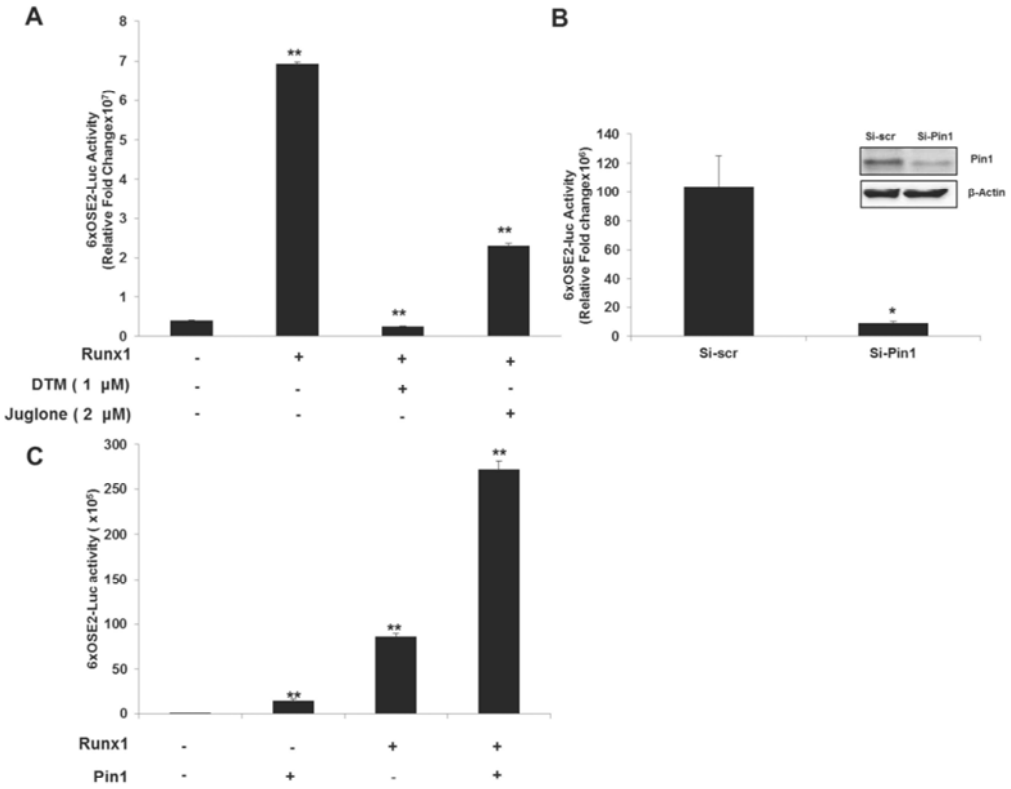

Figure 3

Figure I.3. The transacting activity and stability of Runx1 are increased by its interaction with Pin1.

(A) HEK 293 cells were transfected with pCS4-3Myc-Runx1 and the 6xOSE2 reporter vector and treated with either vehicle (DMSO) or Pin1 inhibitors DTM (2 μM) and Juglone (5 μM) for 12 h after transfection. Luminescence was observed with the Bright-Glo luciferase system. ** indicates a p-value < 0.001. (B) U937 cells were transfected with si-scr and si-Pin1 and whole cell lysates were used

for western blot and immunoblotted with anti-Pin1 antibody. U937 cells transfected with si-scr and si-Pin1 were co-transfected with the 6xOSE2-luc Luciferase reporter vector and luminescence was observed using the Bright-Glo luciferase assay system (Promega) after 48 h. Pin1 protein level after 48 h of si-scr and si-Pin1 transfection was observed with western blot. * indicates p-value < 0.05. (C) Pin1 was overexpressed in U937 cells with and without PCS4-3Myc-Runx1 and the relative luciferase activity was observed with the 6xOSE2-luc Luciferase reporter. The PCDNA3.1 vector was used as an empty vector negative control. ** indicates p-value < 0.001.

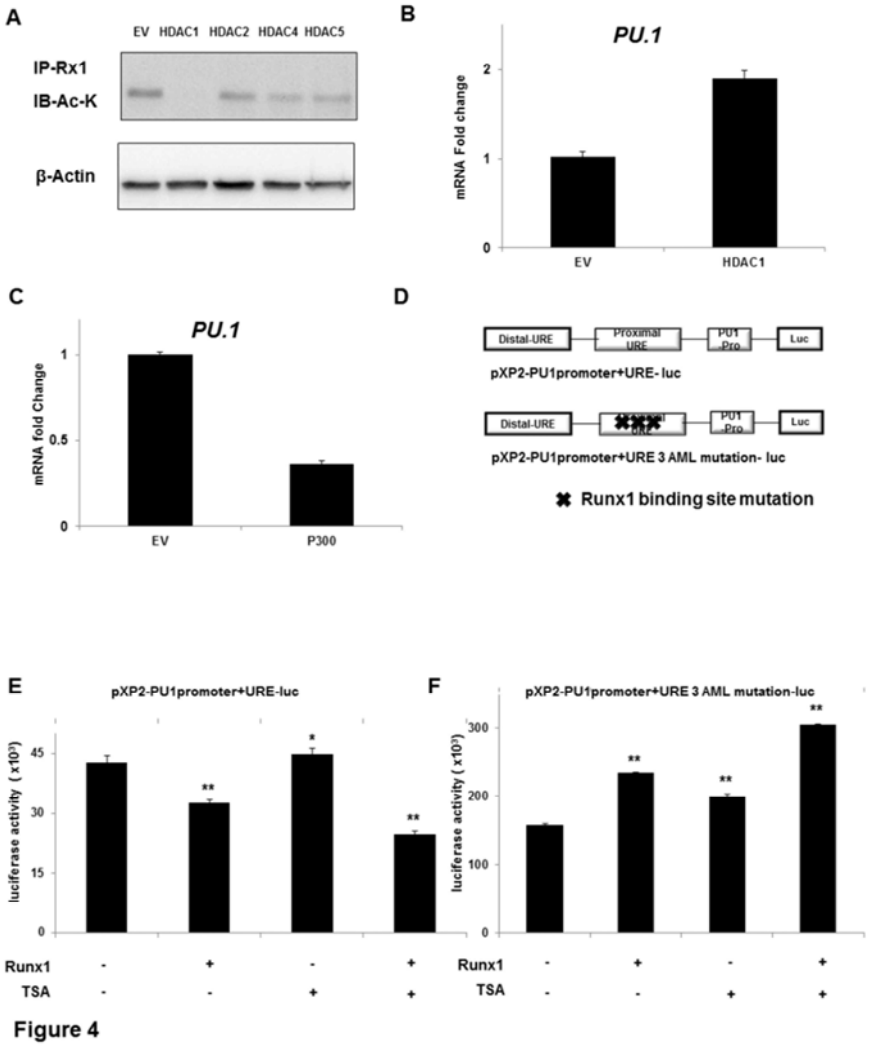

Figure 4

Figure I.4. The acetylation of Runx1 represses Pu-1 transcription in pre-monocytes.

(A) U-937 cells were transfected with constructs for HDAC 1, 2, 4 and 5 and harvested 24 h post transfection. Whole-cell lysates were

prepared, immunoprecipitated with an anti-Runx1 antibody, and immunoblotted with an anti-acetylated lysine antibody. (B) U937 cells were transfected with an HDAC1 expression vector and total RNA was extracted from the cells 24 h after transfection. Real time PCR (qPCR) was performed, and the relative expression level of PU.1 mRNA was detected in the $2^{-\Delta\Delta}$CT method. (C) In a similar manner U937 cells were transfected with P300 expression vector and qPCR was performed to detect the level of PU.1 mRNA. (D) Diagrammatic representations of the reporter vectors containing the PU.1 promoter and the 14Kb URE enhancer region of PU.1, with or without the mutations in the three AML1 (Runx1) binding sites in the proximal URE region. (E) U937 cells were transfected with a Luciferase reporter containing the PU.1 promoter and the 14Kb URE enhancer region of PU.1, along with a Runx1 expression vector. The cells were incubated either in TSA (100 nM) or in vehicle alone. Twenty four hours after transfection, the cells were subjected to lysis and luciferase reporter assays with the Bright-Glo luciferase assay system. (F) U937 cells were transfected with the same reporter vector bearing mutations in the three AML1 (Runx1) binding sites in the proximal URE region and incubated either in TSA (100 nM) or in vehicle alone. Twenty four hours after transfection, the cells were subjected to lysis

and luciferase reporter assays. * and ** indicate p-values < 0.05 and < 0.001, respectively.

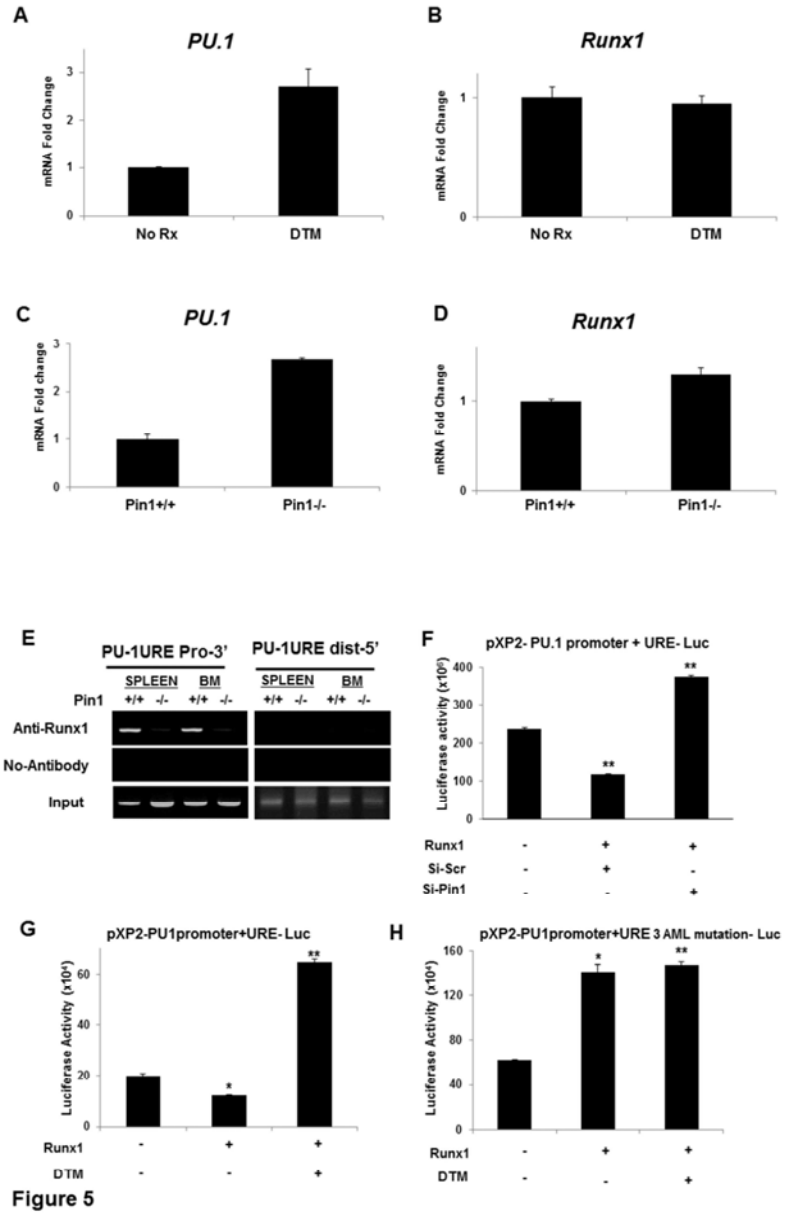

Figure 5

Figure I.5. A lack of Pin1 diminishes Runx1-mediated repression of PU.1 transcription.

(A and B) U937 cells were incubated in 1 μM DTM for 12 h, total RNA was extracted, and qPCR was performed to detect (A) PU.1 and (B) Runx1 mRNA expression level. (C and D) Spleens were extracted from adult (8-week-old) Pin1+/+ and Pin1-/- mice and snap frozen in liquid nitrogen (n=3). Total RNA was extracted from whole tissue and qPCR was performed to measure the expression level of (C) PU.1 and (D) Runx1 mRNA using the $2^{-\Delta\Delta CT}$ method. (E) Spleen and Bone marrow tissues were collected from adult Pin1+/+ and Pin1-/- mice. Thirty milligrams of tissue from each sample were freshly lysed after crosslinking with formaldehyde for 15 m and chromatin was sheared with sonication followed by Immunoprecipitation with anti-Runx1 antibody and Rabbit IgG. PCR was carried out using primers for the PU.1 URE proximal region (Runx1 binding region) and the PU.1 URE distal region (has no Runx1 binding site/negative control). (F) Pin1 was knocked down using si-Pin1 in U937 cells and co-transfected with PCS4-reporter vector containing the PU.1 promoter and the URE region. Luminescence was observed after 24 h. ** indicates p-value < 0.001. (G) U937 cells were transfected with the reporter vector containing PU.1 promoter and URE region followed by treatment with 1 μM DTM for 24 h post transfection. Luminescence was detected using the Bright-Glo luciferase assay system. * and ** indicate p-

values < 0.05 and < 0.001, respectively. (H) A reporter vector containing the PU.1 promoter and the URE region with mutations in the Runx1 binding sites was transfected into U937 cells followed by incubation in 1 μM DTM for 24 h after transfection. The cells were subjected to luciferase assays with the Bright-Glo luciferase assay system. * and ** indicate p-values < 0.05 and < 0.001, respectively.

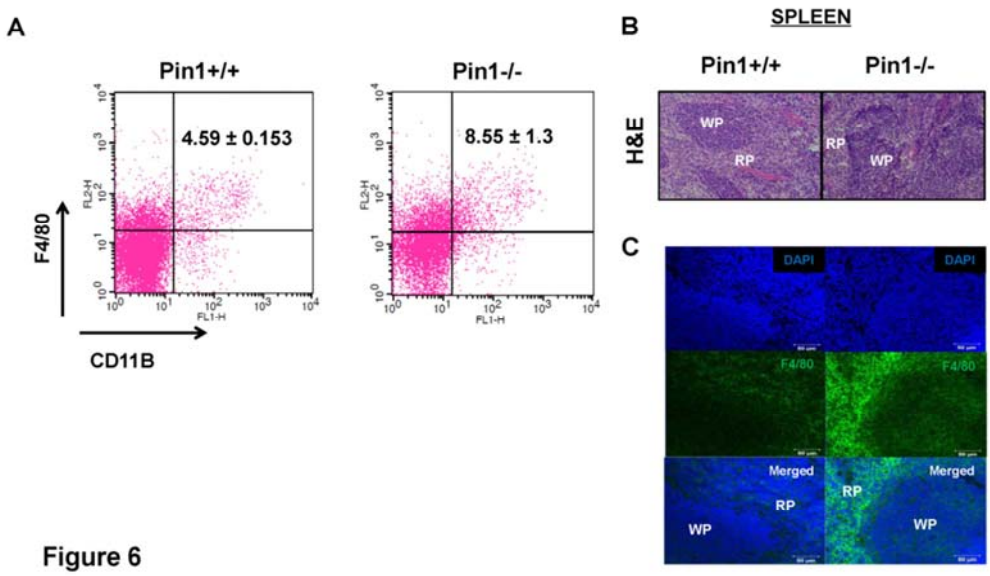

Figure 6

Figure I.6. Lack of Pin1 induces monopoiesis.

(A) Single-cell suspensions of spleen cells prepared from freshly extracted spleens of Pin1+/+ and Pin1-/- mice were stained with a PE-conjugated anti-F4/80 antibody and a FITC-conjugated anti-CD11b antibody to detect the population of $CD11b^{+}$ $F4/80^{+}$ cells after blocking the Fc receptor with antibody for CD16/32 and excluding erythrocytes and dead cells with 7AAD. Data acquisition was performed within 3 h of cell extraction using FACSCalibur and data analysis was carried out using BD CellQuest pro software. (p-value < 0.05). (B) Spleens were extracted from Pin1+/+ and Pin1-/- mice, instantly frozen in liquid nitrogen, cryosectioned, and the resulting slides were post-fixed in 4% PFA. H & E staining was performed to identify the white pulp and red pulp regions in Pin1+/+ and Pin1-/- spleen sections as a control step for immunohistofluorescence experiments. (WP=White Pulp, RP=Red Pulp). (C) Immunohistofluorescence staining of F4/80 protein with an Alexa488-conjugated antibody was performed in splenic tissue sections to observe the F4/80 protein expression in the red pulp region of Pin1+/+ and Pin1-/- spleen tissue of 8-week-old adult mice (n=3).

Discussion

A key observation in this study is that Pin1 has the capacity to modulate the process of monocyte development from myeloid progenitors by suppressing PU.1 transcription at an early time point via a Runx1 conformational change.

Runx1 modulates PU.1 transcription

Endogenous RUNX1 interacts with endogenous HDAC1 or HDAC3, and in proliferating hematopoietic cells, Cdks modify RUNX1 on Ser-48, Ser-303, and Ser-424 to displace HDAC1 and HDAC3 to favor the trans-activation of genes (Guo and Friedman, 2011). Lysine acetyltransferases associate with RUNX1, increase its DNA binding activity and assist in promoting gene expression (Wang et al., 2009). In previous reports, it was noted that HDAC inhibition totally diminished PU.1 transcription in macrophage and B-lymphocyte cell lines (Laribee and Klemsz, 2001) but that TSA treatment resulted in a very dramatic increase in PU.1 transcriptional activity in NIH3T3 fibroblast cell line (Bai et al., 2005). This cell lineage specificity suggests that Runx1, which is differentially regulated in a cell type-specific manner, may be involved.

The murine PU.1 kb −14 Kb upstream regulatory region confers myeloid- and B-lymphocyte lineage-specific expression in

transgenic mice (Okuno et al., 2005). Transcription factors do not act on their own; rather, they interact with epigenetic regulatory complexes that modify and remodel chromatin structure and thus create a chromatin environment that is permissive for active transcription (Li et al., 2007). The expression of the PU.1 gene is tightly regulated and maintained during myelopoiesis and B lymphopoiesis but is down-regulated in erythroid cells and T cells. PU.1 is not required for HSC formation but is involved in regulating cell fate decisions in the downstream committed precursor cell groups (Dakic et al., 2005). The initiation of PU.1 expression in HSCs depends on Runx1, which unfolds the chromatin structure of the Sfpi1 gene and primes it for expression. Runx1 also regulates the growth and survival of macrophage cells (Himes et al., 2005). The overexpression of PU.1 in transgenic mice leads to erythroleukemia (MoreauGachelin et al., 1996). Precursor cells, macrophages, and B cells adopt different chromatin architectures and use different sets of transcription factors to drive PU.1 expression, and the URE also has promoter activity and drives the transcription of noncoding RNAs (ncRNAs) (Hoogenkamp et al., 2007). A Runx1 input into the URE was proposed to mediate silencing as well as activation in a cell-context dependent manner (Huang et al., 2008). The proximal URE

enhancer has three conserved Runx1 sites that are able to bind Runx1. The URE Runx1 sites are maintained in an open state of accessibility, and the Runx1 sites are apparently occupied, in PU.1-expressing myeloid and B cells and PU.1-negative T-lineage cells alike (Hoogenkamp et al., 2007). The deletion of the URE (UREΔ) does not fully block the activation of PU.1 expression within hematopoietic cells (Rosenbauer et al., 2006). This suggests that the post-translational modifications of Runx1 may play an important role in the differential regulation of PU.1, and more so in this URE region. I observed that with HDAC1 overexpression and P300 overexpression, PU.1 mRNA in U937 cells was decreased and increased, respectively.

Pin1 modulates PU.1 mRNA expression

I found that Pin1 binds to Runx1 (Yoon et al., 2013a) and is a major modulator of Runx1 (Figure. I.1 and Figure. I.2) protein. Similar to previous reports (Huang et al., 2008), I also found that this binding of Runx1 at the URE region modulates the transcription level of PU.1 (Figure. I.5). Furthermore, this binding process is influenced by the modification of Runx1 by Pin1. The lack of Pin1 increased the PU.1 transcription level both in vitro and in vivo (Figure. I.5).

Pin1 enhances monocyte differentiation

In adult myelopoiesis, all granulocytes and

monocytes/macrophages are derived from the hierarchical transformation of multipotential hematopoietic stem cells (HSCs) into lineage-restricted progenitors and the subsequent maturation of lineage-restricted progenitors (Iwasaki and Akashi, 2007). A key indispensable step that is shared by the different waves of myelopoiesis is the specification of the granulocyte or monocyte/macrophage fate from an initially almost equal pool of myeloid progenitors. PU.1 can direct the myeloid development of multipotential cell lines (Dakic et al., 2005; Dakic et al., 2007; Friedman, 2007; Nerlov and Graf, 1998). The choice of granulocyte versus monocyte/macrophage fate is thought to be primarily dictated by the interplay between various transcription factors and their dosage at appropriate developmental time points; PU.1 is one of the major players in this process (Friedman, 2007; Rosenbauer and Tenen, 2007). The in vitro reconstitution of PU.1 expression in PU.1-/- progenitors showed that granulocytes developed from progenitors that were supplied with low PU.1 expression, whereas macrophages developed from progenitors that were supplied with high PU.1 expression (Dahl et al., 2003; Laslo et al., 2006). It was reported in a zebrafish model that Runx1 is a key embryonic myeloid fate determinant and favors the neutrophil fate over the macrophage fate

and that a PU.1-Runx1 regulatory loop governs the equilibrium between distinct myeloid fates by guaranteeing an appropriate PU.1 dosage (Jin et al., 2012). The level of PU.1 was further maintained by Runx1, and this allowed neutrophil commitment by a feedback loop repressing PU.1 expression (Jin et al., 2012). Previously, it was also observed that the inhibition of Runx1 favored monopoiesis over granulopoiesis (Guo et al., 2012). In my study, PU.1 mRNA levels were measured in pre-monocytes (U937 cells) and total spleen tissue (Figure. I.5). PU.1 mRNA was increased in $Pin1^{-/-}$ cells and spleen tissue, but Runx1 mRNA levels were unaffected. I found that initially in pre-monocytes, Runx1 represses PU.1 transcription and this repression is alleviated by lack of Pin1 mediated isomerization of Runx1 (Figure. I.5F). In $Pin1^{-/-}$ cells, the stability of Runx1 protein was reduced and thus the Runx1 expression level was also reduced (Figure. I.1 and Figure. I.2). This may raise the question of why monopoiesis is increased even though Runx1 level is low.

It is already known that chromatin activation at Csf1r, the main receptor for monopoiesis, follows a clear hierarchy that is defined by a differential transcription factor dependency (Hoogenkamp et al., 2009). Although RUNX1 binds to both PU.1 and Csf1r activation loci, it is sufficient to initiate chromatin remodeling at PU.1 at the onset of

hematopoietic development, but not at the Csf1r locus. It was previously demonstrated that at the Csf1r locus, chromatin unfolding is crucially dependent on the expression of high levels of PU.1 (Krysinska et al., 2007). As in Pin1-/- mice, Runx1 is still present, so its function at the Csfr1 activation loci maybe present along with the presence of a high level of PU.1. In addition to the effects on Csf1r regulation, it was also observed that the ectopic expression of PU.1 alone can trans-differentiate neural stem cells to monocytes (Forsberg et al., 2010). Finally, I found that the lack of Pin1 in vivo increases the expression of the monocyte/macrophage marker F4/80 in spleen tissue and the total population of $CD11b^{+}/F4\text{-}80^{+}$ cells in the spleen.

Is the Runx1–Pin1 interaction of general relevance?

In the myeloid lineage, Runx1 is not required for terminal differentiation (Ichikawa et al., 2004b) but it is essential for the formation of the definitive hematopoietic system and the differentiation of lymphoid cells and platelets. According to my results, Pin1 affects the level of PU.1 via Runx1 (Figure. I.5). It can be inferred from this observation that, in the case of biological processes where PU.1 transcription is important, the Runx1–Pin1 interaction is crucial; however, we cannot ignore a more general relevance of this

interaction in the activity of Runx1 for hematopoiesis and as an oncogene. Pin1 can regulate the protein level of Runx1, so it must have a variety of physiological roles in hematopoiesis.

Significance of Pin1 mediated regulation of PU-1 in leukemia

It has been shown that the expression of PU.1 is downregulated in human acute myeloid leukemia (AML) patients (Metcalf, 2006; Steidl et al., 2006; Zhu et al., 2012), and PU.1 mutations were found in approximately 7% of 126 AML patients in a prior study (Mueller et al., 2003). It is also known that a reduction in PU.1 activity leads to AML in mice (Metcalf et al., 2006; Walter et al., 2005). Some groups have suggested that restoring PU.1 expression may be a possible therapeutic modality leading to the differentiation of AML cells (Mueller et al., 2006). Recently, a model has been proposed where PU.1 binding is necessary in HSCs to establish an active chromosomal conformation for proper PU.1 transcription to balance cell-cycle activators and inhibitors (Staber et al., 2013). From previous reports and my data, I suggest that Pin1 may be a good target to modify PU.1 regulation in AML. However, in order for that possibility to become a reality, we need further insight into how the Runx1-Pin1 interaction modulates PU.1 in cells with different genetic abnormalities, such as mutations and translocations of Runx1.

Runx1 and PU.1 have emerged as key regulators of hematopoiesis, and their expression is exquisitely and dynamically controlled throughout the various hematopoietic differentiation pathways. I can conclude from my results that Pin1 is an important modulator of PU.1 transcription through Runx1 and may have various implications in different stages of hematopoiesis, especially during myeloid cell differentiation. Therefore, Pin1 may be a very useful drug target in AML and particularly in AML varieties that have altered PU.1 expression.

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CHAPTER II

Pin1 regulates osteoclast fusion through the suppression of the master regulator of cell fusion DC-STAMP

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IV.1. Introduction

In multicellular organisms the ordered fusion of intercellular membranes is essential for basic cellular functions, and the temporally and spatially regulated fusion of membranes is required during a wide range of biological processes, including fertilization (Primakoff and Myles, 2002); the development of muscle (Chen and Olson, 2004; Horsley and Pavlath, 2004), bone, and placenta (Potgens et al., 2002); the immune response (Johansson et al., 2008; Nygren et al., 2008); tumorigenesis (Chen and Olson, 2005); and also during some aspects of stem cell-mediated tissue regeneration (Pomerantz and Blau, 2004; Wagers and Weissman, 2004). Interest in cell to cell fusion was initially stimulated many years ago by the discovery that somatic cells can be induced to fuse by viruses in vitro (Okada, 1962). But now it is evident that cell to cell fusion is fundamental to the development and physiology of multicellular organisms (Chen and Olson, 2005). Different cell-cell fusion events may share common mechanisms as sequence of fusion events are similar (Chen and Olson, 2005).

Osteoclasts are the multinucleated cells (MNCs) generated from mononuclear osteoclast precursor cells of the

monocyte/macrophage lineage upon osteoclastogenic stimulations (Boyle et al., 2003; Teitelbaum and Ross, 2003). However, the molecular mechanisms regulating the polykaryon formation still remain mostly unknown (Vignery, 2005), although several cell surface molecules are considered to play a vital role, like CD47 (Han et al., 2000), E-cadherin (Mbalaviele et al., 1995), DC-STAMP (Kukita et al., 2004), OC-STAMP (Miyamoto et al., 2012), Atp6v0d2 (Lee et al., 2006), etc. Recent evidence suggests zipper-like actin superstructures might also be involved in osteoclast fusion (Takito et al., 2012). Molecules can contribute to cell fusion by either directly participating in membrane fusion or by affecting earlier steps in the process (Oren-Suissa and Podbilewicz, 2007; Primakoff and Myles, 2007). The signaling pathways involved in cell to cell fusion have mostly been characterized in yeasts and invertebrates; much less is known about the regulation of fusion by mammalian cells. For example, EFF-1 and AFF-1 act as direct membrane fusogens during Caenorhabditis elegans development (Mohler et al., 2002; Sapir et al., 2007), but the mechanisms by which proteins such as DC-STAMP, the d2 isoform v-ATPase, and CD200 contribute to macrophage fusion remain largely undefined (Cui et al., 2007; Kukita et al., 2004; Lee et al., 2006; Mensah et al., 2010a). Several diseases of the adult

skeleton are related to disturbances in cell fusion either through increased activity (bone metastasis, osteoporosis, Paget's disease) or decreased activity (osteopetrosis). Osteoporosis is a serious disease among postmenopausal women and elderly people in developed countries. In the elderly population, spine and hip fractures are chronic diseases characterized by an abnormal balance in the activities of osteoclasts and osteoblasts, where osteoclastic bone resorption has a more critical role as therapeutic target (Khosla, 2009; Teitelbaum, 2000a). In many osteoclast-related diseases, hyperactive osteoclasts are observed, which are often larger in size (Helfrich, 2003). Osteoclast-like multinucleated giant cells have been found in multiple myeloma, metastatic breast cancer, and prostate cancer. In addition, giant cell tumors of the bone are a rare disease, which is initially benign but turns into aggressive disease in 50% of cases (Anract et al., 1998; Campanacci et al., 1987).

In recent years, it has been discovered that Pin1 [protein interacting with the NIMA (never in mitosis A)-1](Lu et al., 1996b), a peptidylprolyl isomerase catalyzing cis-to-trans conformational switches of target proteins presenting the Ser/Thr-Pro motif, regulates the function of multiple proteins, altering their catalytic activity, stability

or subcellular localization (Lu and Zhou, 2007). The striking substrate specificity of Pin1 toward certain pSer/Thr-Pro bonds results from its N-terminal WW domain and C-terminal PPIase domain, together which form a double-check mechanism. The WW domain of Pin1 binds only to specific pSer/Thr-Pro motifs, targeting the Pin1 catalytic domain close to its substrates, whereas the PPIase domain isomerizes specific pSer/Thr-Pro motifs and induces conformational changes in proteins. Its deregulation contributes to some pathological conditions, notably aging, cancer, and Alzheimer's disease (Lu and Zhou, 2007). A previous study reported that adult Pin1-/- mice exhibited decreased bone radio-density and reduced spine angle (Lee et al., 2009b). My lab has previously found a role of Pin1 in regulating the bone formation through Runx2 (Yoon et al., 2013a) and the transcription of PU-1 through Runx1 (Islam et al., 2014b). Bone remodeling is a coupled process with bone formation and bone resorption occurring sequentially. With advanced age bone resorption has the more prominent role and $Pin1^{-/-}$ mice showed significant bone loss with ageing. So, I hypothesized that Pin1 might have a critical role in osteoclastogenesis. The aim of this study was to investigate the role of Pin1 in osteoclast physiology. As without Pin1 large osteoclasts were formed, I studied the role of Pin1 in the cell fusion

process of osteoclasts.

IV.2. Materials and Methods

IV.2.1. Animal Studies

The Pin1$^{+/-}$ mice have been described before (Fujimori et al., 1999). Pin1$^{-/-}$ mice were generated from heterozygous mating. For genotype analysis, genomic DNA was isolated from tail biopsies and subjected to PCR. PCR products were resolved by agarose gel electrophoresis. All animal studies were reviewed and approved by the Special Committee on Animal Welfare, Seoul National University, Seoul, Republic of Korea. Male mice were used for all experiments.

IV.2.2. Cell lines, recombinant proteins and plasmids

Primary bone marrow macrophages were collected by flushing long bones of 8 week old mice followed by RBC lysis and overnight culture in DMEM supplemented with 10% FBS and 1% Penicillin and Streptomycin. Supernatant was further cultured for 2 days in M-CSF (10 ng/ml). After that the adherent cells were separated as bone marrow macrophage and further differentiated to osteoclasts with incubation in M-CSF (30 ng/ml) and RANKL (80 ng/ml) or foreign

body giant cells by incubating in IL4 (50 ng/ml) and M-CSF (10 ng/ml).

The Pin1 expression vector was engineered as previously described (Yoon et al., 2013a). The retroviral vector expressing full length DC-STAMP was kindly gifted by Professor Nacksung Kim (Kim et al., 2008). Recombinant GST and GST-Pin1 were made as described previously (Yoon et al., 2013a). Recombinant RANKL and M-CSF were acquired from PeproTech Ltd (Rocky Hill, NJ, USA).

IV.2.3. Resorption Assay

For the pit formation assay, osteoclast progenitor cells were cultured on dentine slices in the presence of M-CSF and RANKL as described. Resorbing lacunae were visualized by toluidine blue staining. The dentine slices were kindly provided by Professor Je-Tae Woo (Chubu University, Japan).

IV.2.4. TRAP Staining

For in vitro culture TRAP staining, commercial kit was used as recommended by the manufacturer (Sigma Aldrich, St. Louis, MO, USA). TRAP staining of histological sections was done according to the BD Biosciences TRAP staining protocol no. 445. Briefly, TRAP buffer, pH 5.0, was prepared fresh for use by mixing 50 ml of acetate

buffer (35.2 ml of 0.2 M sodium acetate and 14.8 ml of 0.2 M acetic acid), 10 ml of 0.3 M sodium tartrate, 1 ml of 10 mg/ml naphthol AS-MX phosphate disodium salt (Sigma, catalog no. N-5000), 0.1 ml of Triton X-100 and 38.9 ml of distilled water. TRAP stain was prepared fresh for use by dissolving 0.3 mg of Fast Red Violet LB salt (Sigma, catalog no. F-3381) per ml of TRAP buffer at 37°C. For TRAP staining, sections were deparaffinized and washed with PBS. Cells were incubated in TRAP stain for 5–10 min at 37°C. TRAP stain was aspirated, and sections were washed with Ca^{2+}/Mg^{2+} free PBS and were dehydrated and cleared in quick progression followed by mounting. Photomicrography was done with Nikon eclipse 50i microscope.

IV.2.5. Giant-Cell Formation

Giant-cell (GC) formation in vivo was induced as described (Gonzalo et al., 2010; Mariano and Spector, 1974). Glass coverslips (12 mm diameter) were implanted subcutaneously in the backs of mice, and after 4 days the coverslips were removed and stained with hematoxylin and eosin (H&E). GC formation was also induced in vitro (Gonzalo et al., 2010; Helming and Gordon, 2007). Bone marrow (BM) progenitors were cultured in the presence of 10 ng/ml M-CSF. After 3

days, cells were plated at $1.5x10^5$ cells/well in 96-well plates with 50 ng/ml IL4 (PeproTech) and cultured for a further 4 days. Cells were stained with H&E. The number of nuclei per multinucleated cell was counted by two blinded observers; at least 20 cells were counted per condition.

IV.2.6. Extraction of total RNAs, reverse transcription polymerase chain reaction (RT-PCR) and Real time PCR (qPCR)

Quantitative real-time RT-PCR was performed to evaluate the expression of transcripts. The total RNAs were isolated using the QIAzol (Qiagen, AMBION Inc., Austin, Texas, USA) RNA extraction reagent. The concentration and purity of the RNA preparations were determined by measuring the absorbance of RNA at 230, 260 and 280 nm. Then, 10 µl of first-strand cDNA was synthesized from 500 ng of the total RNAs using a PrimeScript™ RT reagent kit according to the manufacturer's (Takara Bio, Otsu, Shiga, Japan) instructions. 2 microliters of cDNA was used for PCR amplification. All reactions were performed with SYBR Premix Ex Taq real-PCR reagent (Takara Bio) according to the manufacturer's instructions by using 7500 Real-Time PCR System thermal cycler (Applied Biosystems, Carlsbad, CA). The denaturation was performed at 95°C for 5 s, and the

annealing/extension was done at 60°C for 34 s. The level of the target gene transcripts was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ΔCT). The fold change result was acquired by $2^{-\Delta\Delta CT}$ method.

IV.2.7. GST Pull-Down Assay, Immunoprecipitation and Western Blot Analysis

For GST pull-down assay cell extracts were prepared in following lysis buffer; 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 1 mM DTT, 1 mM EDTA, 0.25 % Na-deoxycholate, 0.25% CHAPS, 1% NP-40, 10% glycerol supplemented with protease inhibitors and phosphotase inhibitors including Sodium orthovanadate (Na_3VO_4). Recombinant GST or GST-Pin1 proteins (2 μg) were incubated with cell lysates (500 μg) at 4°C and glutathione agarose beads were then added, followed by further incubation for 2 h. The precipitated proteins were analyzed by immunoblot detection with anti-DC-STAMP antibody (Millipore, Cat. # MABF39).

For Co-immunoprecipitation, DC-STAMP was retrovirally expressed along with transiently expressed HA-Pin1 in Raw264.7 cells. Cell lysates were incubated with anti-HA antibody for 3 h and agarose beads were added, followed by further incubation for 1 h. Co-

purified proteins were subjected to immunoblot with anti-DC-STAMP antibody.

For western blot cell lysates were prepared in RIPA buffer (Radio Immuno Precipitation Assay buffer) containing 150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris (pH 8.0) with protease inhibitors and subjected to western blot. Proteins were analyzed by immunoblot detection with specific antibodies [anti GAPDH (Santa Cruz-32233), V-ATPase D2 Antibody (Santa Cruz-69111) etc.].

IV.2.8. Immunofluorescence and immunohistochemistry

For the detection of DC-STAMP and Pin1, I found that the antigen retrieval process was more efficient for detection both in immunohistofluorescence and immunohistochemistry. Briefly, for antigen retrieval, tissue sections were boiled in Tris-EDTA buffer (pH 9.2) for 20 min at 60°C. Then tissue sections were blocked and immunohistofluorescence and immunohistochemistry was done using general protocol. For detection of DC-STAMP and Pin1, the antibodies from Millipore (Catalog# MABF39)(Chiu et al., 2012) and Novus Bio (NBP1-05424) were used respectively. For immunocytochemistry, Triton-X (1%) permeabilization was used

before blocking and immunoprobing. For immunohistochemistry using mouse monoclonal antibody, an additional step of blocking was performed with Biotinylated Mouse on Mouse (M.O.M.) Anti-Mouse Ig Reagent (Vector laboratories, Cat# MKB-2225).

IV.2.9. Statistical analyses

All quantitative data are presented as the mean ± SD. Each experiment was performed at least three times, and results from one representative experiment are shown. Statistical differences were analyzed by Student's t test. A value of $p < 0.05$ was considered statistically significant. Data which did not follow a normal distribution were analyzed by 2-tailed Mann-Whitney U Test using IBM SPSS Statistics 21.0 software.

IV.3. Results

IV.3.1. Lack of Pin1 demonstrates larger osteoclasts both in vitro and in vivo

Previously it was seen that $Pin1^{-/-}$ mice at advanced age (18 months) showed distinct osteoporotic phenotypes like decreased bone radio-density and reduced spine angle (Lee et al., 2009a). My lab had recently reported reduced bone formation in younger mice with the absence of Pin1 (Yoon et al., 2013a). On TRAP staining of proximal tibial sections of 12 week old male mice I found larger areas of TRAP staining in sections from $Pin1^{-/-}$ mice (Figure. II.1A and C) without significant difference of total TRAP positive osteoclast cell number (Figure. II.1B). So I focused on the size of osteoclasts. In vitro culture of $Pin1^{+/+}$ and $Pin1^{-/-}$ cells from the same number of bone marrow macrophages showed larger TRAP stained osteoclasts with higher number of nuclei containing more than 30 nuclei in cultures compared to $Pin1^{-/-}$ cells (Figure II.1.D and F). But the total number of TRAP positive multinucleated cells were not significantly changed (Figure II.1.E). $Pin1^{-/-}$ osteoclasts also showed larger resorption areas with resorption assay on dentin disk (Figure II.1.G andH).

IV.3.2. Increased cell to cell fusion in Pin1$^{-/-}$ derived osteoclasts and foreign body giant cells

Individual nuclei number in a polykaryon indicates the fusion extent. In case of osteoclasts resorption area is related to the nuclei number in each cell (Boissy et al., 2002; Piper et al., 1992). Usually if an osteoclast polykaryon formation is affected by some condition, foreign body giant cell (FBGC) formation is also altered as they have a common progenitor. To evaluate the generality of the requirement for Pin1 in monocyte/macrophage fusion, I investigated the effect of Pin1 deficiency on FBGC formation both in vivo and in vitro. Formation of multinucleated GC in vitro was induced by stimulating BM progenitors with IL-4 50 ng/ml (Helming and Gordon, 2007). In case of in vitro osteoclast cultures the number of nuclei in Pin1$^{-/-}$ cultures had a higher nuclei number with some having an extremely high nuclei number compared to Pin1$^{+/+}$ cultures (Figure. II.2B). As the distribution of nuclei in observed cells did not follow a normal distribution pattern statistical significance was confirmed with the Mann–Whitney U test. FBGC from Pin1+/+ and Pin1-/- mice also showed a similar pattern, of increased nuclei number with both in vitro and in vivo experiments (Figure. II.2C, D and F). Fusion assay was

performed using molecular probes DiO and DiI (Humtsoe et al., 2003) as illustrated in the schematic diagram (Figure II.2.G) to evaluate early fusion events. Osteoclast precursors treated with Pin1 inhibitor DTM (Dipentamethylene thiuram monosulfide) (Tatara et al., 2009) showed a higher fusion rate which is indicated by higher number of fused cells which were stained with both DiO and DiI (Figure II.2.H). From these data I found that the lack of Pin1 accelerated fusion in osteoclasts and FBGC precursor monocyte /macrophage cells.

IV.3.3. Absence of Pin1 increases the protein level of the master regulator of osteoclasts fusion DC-STAMP

To identify the target of Pin1 responsible for this change in fusion rate I first investigated the known transcription factors involved in fusion (Miyamoto, 2011; Xing et al., 2012). With Real time PCR I found that neither NFATc1 nor c-Fos mRNA level was significantly changed in Pin1-/- osteoclast precursors (Figure II.3.A and B). Then I checked the expression of the known major proteins related to osteoclast fusion Atp6v0d2 and DC-STAMP and found that only DC-STAMP was highly increased in Pin1-/- osteoclasts (Figure II.3.C). With qPCR quantification of the mRNA level of TM7SF4 (DC-STAMP) showed a very small amount of the increase (less than 2 fold) in Pin1-/-

compared to Pin1+/+ (Figure II.3.D) whereas the protein level showed an increase of more than 5 fold after quantification of the western blot data (Figure II.3.C) with image J software (Data not shown). Overexpression of Pin1 in RAW 264.7 cells showed a decrease in the expression level of DC-STAMP (Figure II.3.E). Immunohistochemistry showed a significantly higher expression level of DC-STAMP in upper tibial sections of Pin1-/- mice than Pin1+/+ mice (Figure II.3.F). Upon closer inspection I could observe that in Pin1-/- sections the DC-STAMP was located more in the cytosol than on the surface of the cell and many mononuclear cells also showed high expression of DC-STAMP. I also quantified the mRNA expression level of genes previously shown to be changed with DC-STAMP signaling that is ACP5 (TRAP) and CD9 (Mensah et al., 2010b; Yagi et al., 2005) (Figure II3.G and H). Compared to Pin1+/+, in Pin1-/- osteoclasts the CD9 mRNA increased after differentiation and fusion had begun (Figure II.3.G). In case of ACP5 the mRNA level increased in later stages of differentiation (Figure II.3.H).

IV.3.4. Pin1 binds to and regulates the protein level of the master regulator of osteoclasts fusion DC-STAMP

Pin1 was seen to bind with DC-STAMP by GST pull down assay both

before and after 1day of RANKL stimulation and also by Immunoprecipitation (Figure II.4.A and B). This data correlates to the previous report where it was shown that DC-STAMP was immunoprecipitated with a high amount of protein below the 20 kDa size (Chiu et al., 2012). In vitro also I found that after one day of RANKL stimulation Pin1 co-localized with DC-STAMP within the cytoplasm in an area consistent with the location of the endoplasmic reticulum or perinuclear space in bone marrow macrophage cells (Figure II.4.C). Pin1 was also co-localized with DC-STAMP on the surface of multinucleated giant cells in the metaphysis of wild type bone sections (Figure II.4.D) whereas in adjacent cells of different lineage within the same field, Pin1 could clearly be seen to be localized within the nucleus. In osteoblasts previously I found a significantly higher localization of Pin1 inside the nucleus (Yoon et al., 2013a).

IV.3.5.The cellular localization of DC-STAMP during osteoclastogenesis is regulated by Pin1

From my observations I deduced that Pin1 might affect the localization of DC-STAMP as well as its expression level. I incubated primary bone marrow derived macrophages in RANKL and M-CSF

and then immune stained for DC-STAMP and endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI) to observe the localization of DC-STAMP with reference to the ER where it is known to interact with several other proteins(Eleveld-Trancikova et al., 2010; Eleveld-Trancikova et al., 2005; Jansen et al., 2009). In cells treated with Pin1 inhibitor DTM for 2 days it was observed that DC-STAMP was higher in amount. In the DTM treated cells DC-STAMP strongly co-localized with the protein PDI compared to non-treated cells where DC-STAMP was localized separately from PDI (Figure II.5.A). After 4 days I could observe a clear surface localization of DC-STAMP in the non-treated cells whereas DTM treated cells showed a strong co-localization of DC-STAMP with PDI (Figure II.5.B). The DTM treated cells also had higher amount of DC-STAMP within the cytosol with only a few cells showing surface expression of DC-STAMP. Another interesting observation was that in DTM treated cells PDI staining was of higher intensity compared to non-treated cells probably because of the difference in pH (Wang and Narayan, 2008).

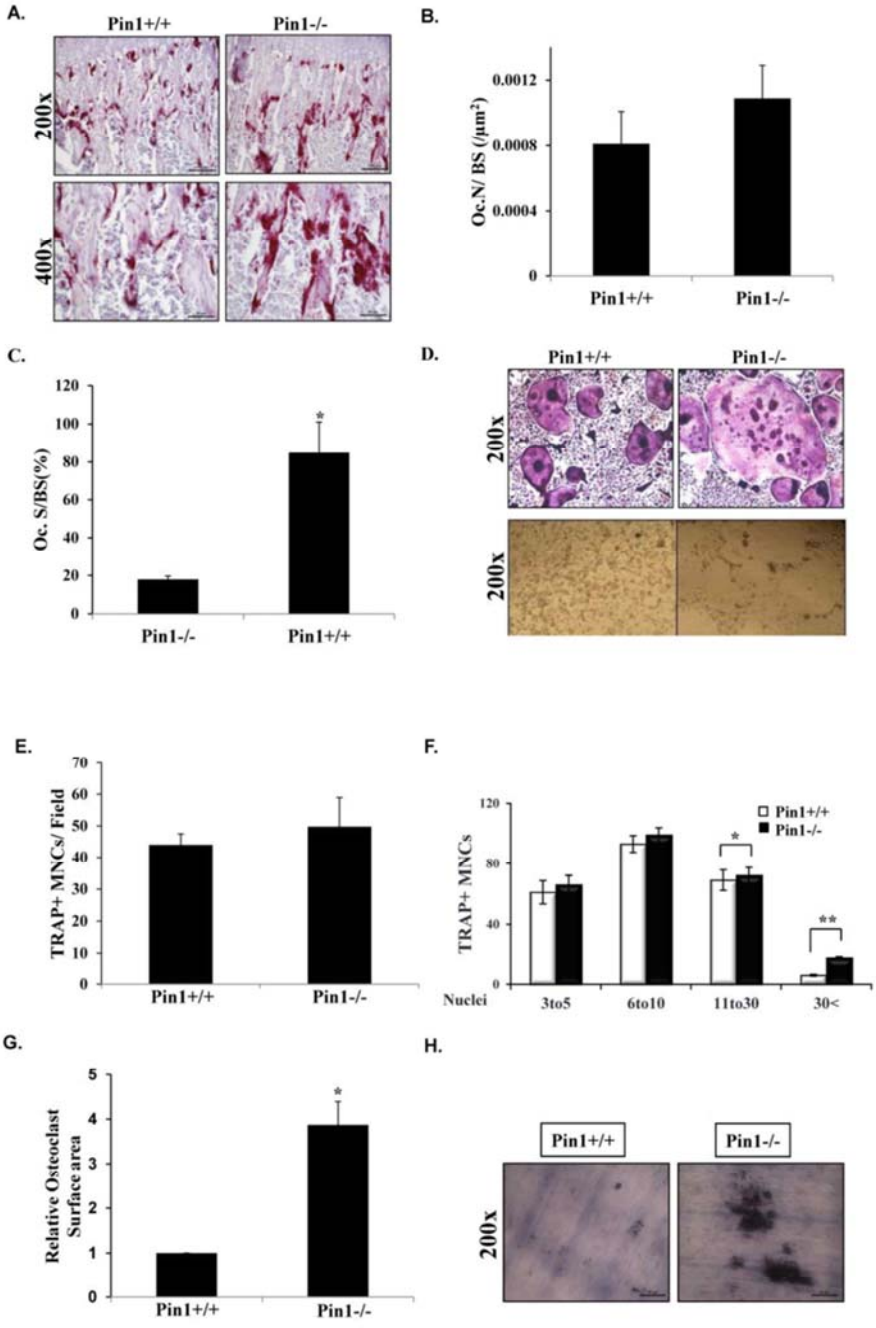
A.
Pin1+/+
Pin1-/-
200x
400x
B.
Oc.N/ BS (/μm²)
0.0012
0.0008
0.0004
0
Pin1+/+
Pin1-/-
C.
Oc. S/BS(%)
120
100
80
60
40
20
0
*
Pin1-/-
Pin1+/+
D.
Pin1+/+
Pin1-/-
200x
200x
E.
TRAP+ MNCs/ Field
70
60
50
40
30
20
10
0
Pin1+/+
Pin1-/-
F.
TRAP+ MNCs
120
80
40
0
Pin1+/+
Pin1-/-
*
**
Nuclei
3to5
6to10
11to30
30<
G.
Relative Osteoclast Surface area
5
4
3
2
1
0
*
Pin1+/+
Pin1-/-
H.
Pin1+/+
Pin1-/-
200x

Figure II.1. Lack of Pin1 demonstrates larger osteoclasts both *in vitro* and *in vivo*.

(A) TRAP staining of proximal tibial section from 12 week old Pin1+/+ and Pin1−/− mice (scale bar: 100 μm in 200x and 50 μm in 400x, n=5). (B) Comparison of the Oc.S/BS in the proximal tibial sections of Pin1+/+ and Pin1-/- mice. (C) Osteoclast number/ Bone surface area (/μm^2) were obtained from TRAP stained sections by bone histomorphometry. (D) Osteoclast precursor cells derived from the bone marrow of adult Pin1+/+ and Pin1-/- mice were differentiated into mature multinucleated osteoclasts by incubating with RANKL (80 ng/ml) and M-CSF (30 ng/ml) for 6 days on plastic dishes and stained for the osteoclast marker protein TRAP. Images under phase contrast microscope of similar cultures are shown below with the same magnification. (E) Total number of TRAP-positive multinucleated cells in each field of in vitro Osteoclast cultures from Pin1+/+ and Pin1-/- bone marrow precursor cells. (*indicates $p<0.05$). (F) TRAP-positive multinucleated cell number in these cultures on day 6. Under microscopy, the TRAP-positive cells containing three or more nuclei were counted as osteoclast-like cells and the distribution of the number of nuclei in multinuclear TRAP-positive cells were counted. (*indicates $p<0.05$ and ** indicates $p<0.001$). (G) Relative

osteoclast surface area of cells from Pin1+/+ and Pin1-/- cells in vitro. (*indicates $p<0.05$). (H)Bone marrow macrophages from Pin1+/+ and Pin1-/- mice were differentiated on dentine slices in the presence of RANKL (80 ng/ml) and M-CSF (30 ng/ml) for 7 days. After removal of cells pits were stained with toluidine blue. Representative microphotographs of bone resorption pits are shown (scale bar: 50 μm).

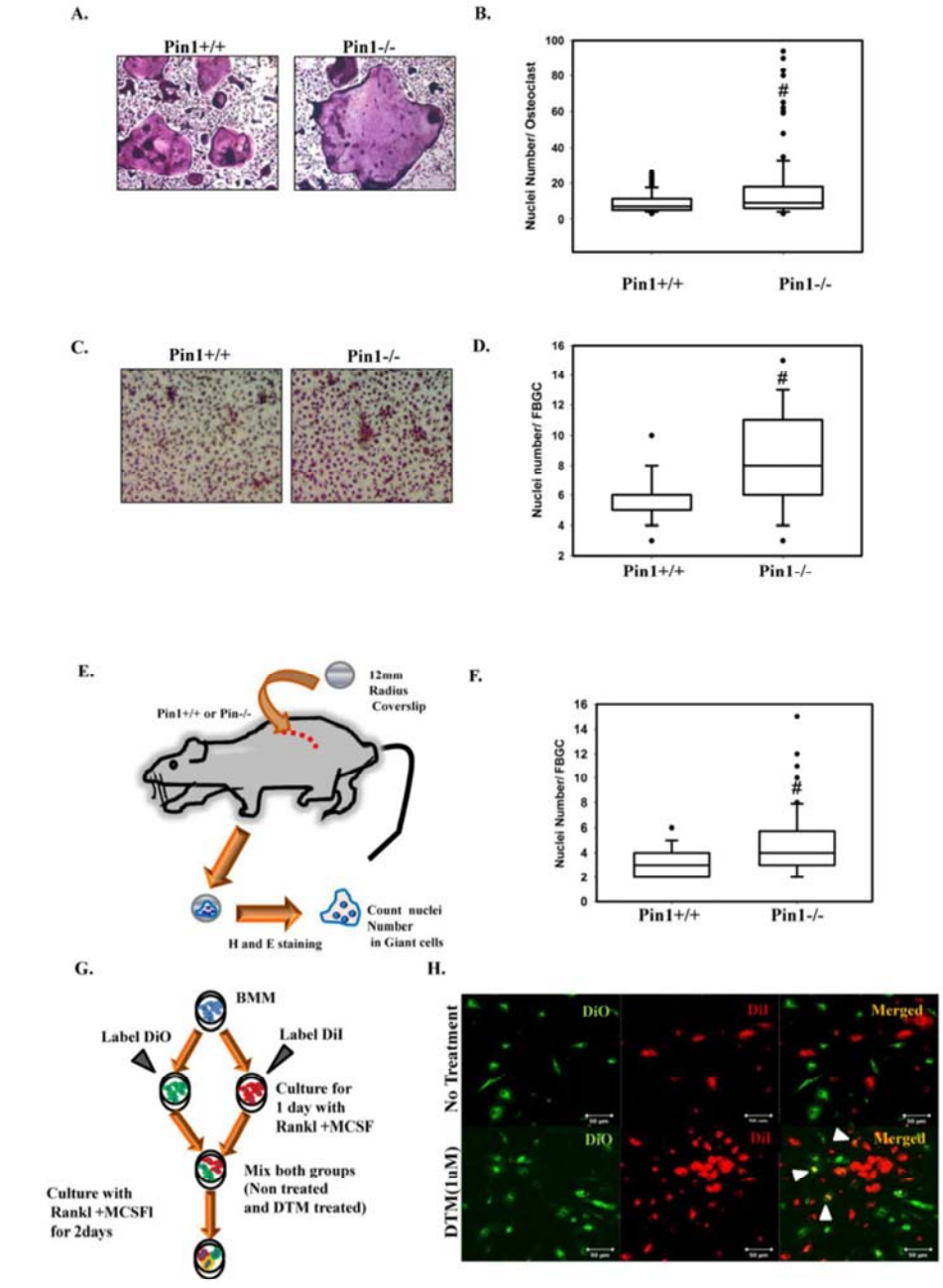

Figure 2

Figure II.2. Increased cell to cell fusion in Pin-/- derived

osteoclasts and foreign body giant cells.

(A)Trap stained osteoclasts after 6 days of differentiation from Pin1+/+ and Pin1-/- mice. (B)The distribution of nuclei in each multinuclear TRAP-positive cell is shown in a box whisker plot with SigmaPlot 10.0 (Systat Software Inc.). The graph shows the distribution and arithmetic means of numbers of nuclei per OC. Boxes show the median, 25th and 75th percentiles, the highest and lowest values are specified by the whiskers and filled circles represent statistical outliers excluded from the analyses (n=105 in each group, # indicates $p<0.05$). Statistical significance calculated with 2- tailed Mann-Whitney U Test in IBM SPSS Statistics 21.0. (C) Foreign body giant cell (FBGC) formation by adult BM progenitors induced in vitro by IL4 (50 ng/ml) with M-CSF (10 ng/ml). Representative Images of the FBGC obtained are shown. (D) The graph shows the distribution and arithmetic means of numbers of nuclei per Foreign body giant cell (n=55 in each group, # indicates $p<0.05$). (E) Foreign-body reaction was induced in Pin1+/+ and Pin1-/- adult mice (as shown in the diagram). (F) The graph shows the distribution and arithmetic means of numbers of nuclei per giant cell in cover slips implanted under the skin of 8 week old mice (at least, 11 cells in 3 fields at 200 x magnification were counted per mouse, n=3, # indicates $p<0.05$). (G)

Diagrammatic explanation of Fusion assay performed with primary bone marrow macrophage cells (BMM) differentiated in the presence and absence of Pin1 inhibitor DTM (1 μM). (H) Confocal images of cells stained with DiO and DiI from the fusion assay experiment as shown in the diagram. White arrowheads indicate fused cells with co-localized DiO and DiI (scale bar: 50 μm).

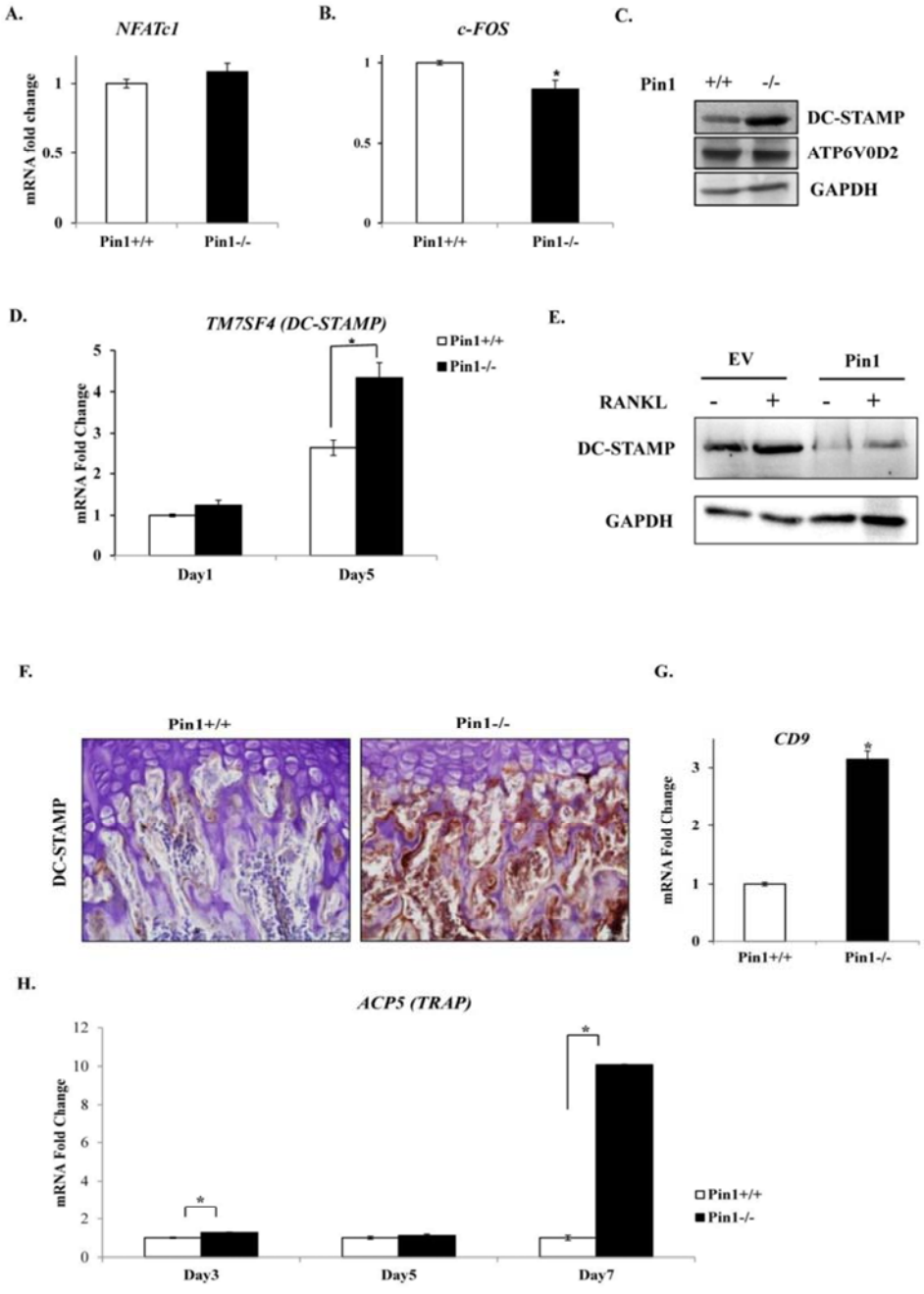
A.
NFATc1
mRNA fold change
1
0.5
0
Pin1+/+
Pin1-/-
B.
c-FOS
1
0.5
0
*
Pin1+/+
Pin1-/-
C.
Pin1
+/+
-/-
DC-STAMP
ATP6V0D2
GAPDH
D.
TM7SF4 (DC-STAMP)
Pin1+/+
Pin1-/-
mRNA Fold Change
5
4
3
2
1
0
*
Day1
Day5
E.
EV
Pin1
RANKL
-
+
-
+
DC-STAMP
GAPDH
F.
Pin1+/+
Pin1-/-
DC-STAMP
G.
CD9
mRNA Fold Change
3
2
1
0
*
Pin1+/+
Pin1-/-
H.
ACP5 (TRAP)
mRNA Fold Change
12
10
8
6
4
2
0
*
*
Day3
Day5
Day7
Pin1+/+
Pin1-/-

Figure 3

Figure II.3. Absence of Pin1 increases the protein level of the master regulator of osteoclasts fusion, DC-STAMP.

(A) Relative qPCR was done to observe the level of NFATC1 the master transcription factor of osteoclastogenesis in Pin1+/+ and Pin1-/- Bone marrow macrophage cells at the second day of osteoclastogenesis with incubation in RANKL (80 ng/ml) and M-CSF (30 ng/ml).(B) mRNA level of c-FOS another transcription factor was measured by qPCR in osteoclasts cultured for 2 days in the presence of RANKL and M-CSF (*indicates $p<0.05$). (C) The protein level of fusion related proteins ATP6V0D2 and DC-STAMP were measured in whole cell lysates in osteoclasts from Pin1+/+ and Pin1-/- osteoclasts at fourth day of osteoclastogenesis. (D) TM7SF4 (DC-STAMP) mRNA level was compared between Pin1+/+ and Pin1-/- osteoclasts after 1 and 5 days of differentiation in M-CSF and RANKL (*indicates $p<0.05$). (E) Level of DC-STAMP protein in Raw264.7 cells with overexpression of Pin1 in presence and absence of RANKL (80 ng/ml). Western blot was done with whole cell lysates. (F) Immunohistochemistry for DC-STAMP protein in proximal tibial section of Pin1 +/+ and Pin1-/- mice at 12 weeks of age (scale bar: 20 μm). DC-STAMP protein expression is seen as brown staining and hematoxylin was used as counterstain. (G) The mRNA level of

another fusion related protein CD9 was also measured in Pin-/- osteoclasts (4 days in RANKL and M-CSF) compared to Pin+/+ osteoclasts at the same stage (*indicates $p<0.05$). (H) mRNA level of ACP5 (TRAP) a marker of osteoclast maturation in osteoclasts were measured after 3, 5 and 7 days of differentiation in Pin1-/- osteoclasts and compared with Pin1+/+ osteoclasts (*indicates $p<0.05$).

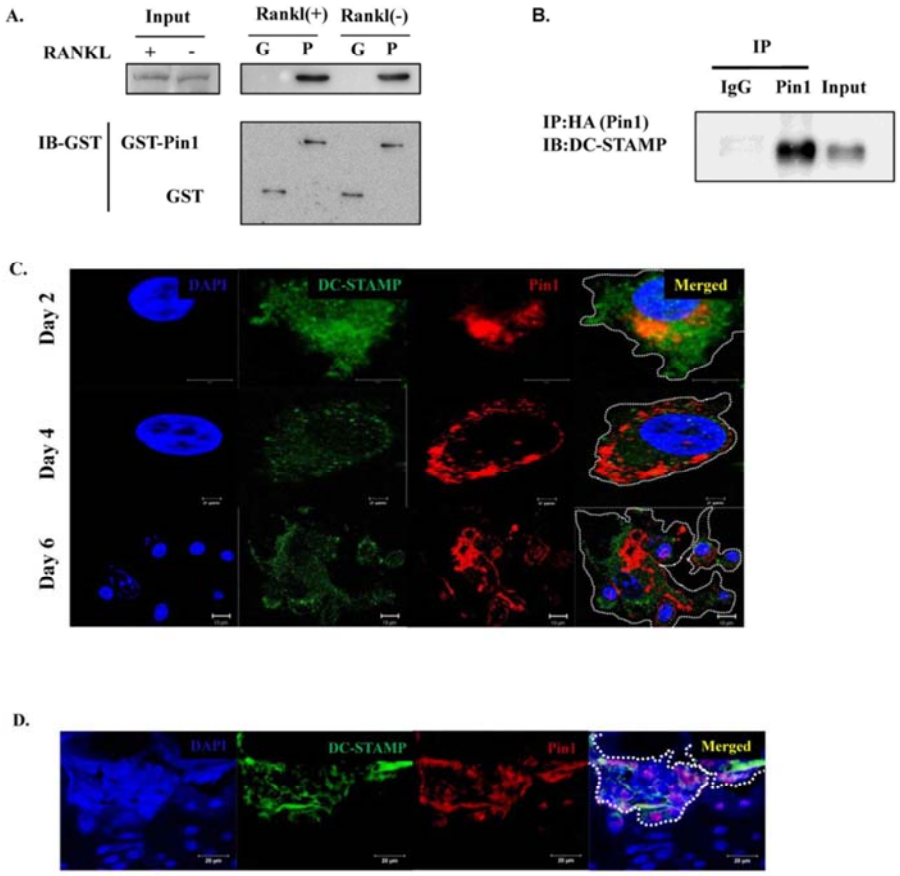

Figure 4

Figure II.4. Pin1 binds to the master regulator of osteoclasts fusion DC-STAMP.

(A) Precipitation of endogenous DC-STAMP protein from primary bone marrow macrophage cells stimulated with or without RANKL (80 ng/ml) stimulation for 24 h through pull down assay with GST-Pin1. GST was used as negative control. (B) Co-Immunoprecipitation (Co-

IP) of DC-STAMP retrovirally expressed in Raw264.7 cells with Pin1. DC-STAMP was overexpressed in PLAT-E cells and supernatant collected after 24 hours of media change and used for retroviral over expression. Pin1 was overexpressed with HA conjugation using the PCDNA3.1 vector containing full length Pin1 DNA. IP was done with anti HA antibody or Rabbit IgG for 4 h followed by immunoblotting with antibody against DC-STAMP. (C) Pin1 immunostaining (Alexa555-red) was performed with mouse osteoclasts cultured on glass and costained with DC-STAMP (Alexa488-green) at day 2, 4 and 6 of differentiation. Cells are outlined with white dotted line (scale bar: 5, 2 and10 μm in day 2, 4 and 6 samples respectively). (D) Confocal immunofluorescence of Pin1 (stained with Alexa555-red) and DC-STAMP (stained with Alexa488-green) protein on the cell surface of multinucleated bone cell in sections of tibia (scale bar: 20 μm).

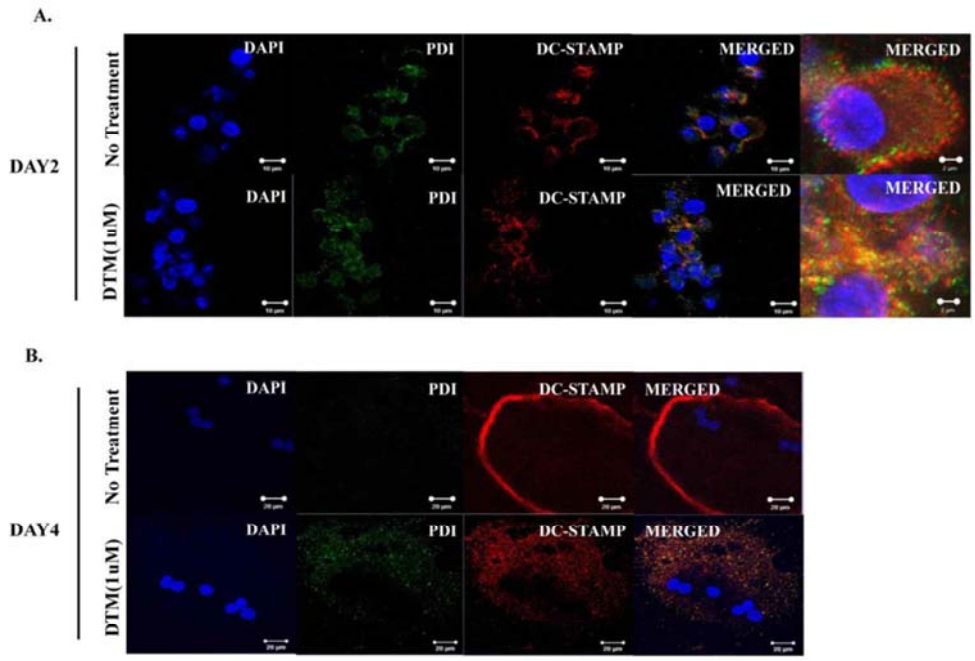

Figure 5

Figure II.5. The cellular localization of DC-STAMP during osteoclastogenesis is regulated by Pin1.

A) Primary bone marrow macrophages were treated with and without DTM (2 μM) while undergoing osteoclastogenesis after incubation in presence of RANKL(80 ng/ml) and M-CSF (30 ng/ml). DC-STAMP (stained with Alexa568-red) was localized in comparison with ER (endoplasmic reticulum) marker PDI (stained with Alexa488-green) at day 2 by confocal laser scanning microscopy (scale bar: 10 μm). (B) Again DC-STAMP localization was examined in a similar manner at day 4 after starting RANKL incubation in the presence of M-CSF(scale bar: 20 μm) .

IV.4. Discussion

In this study, I found that with deficiency of Pin1 osteoclast size is altered without significant change in osteoclast number. I found that protein expression level and localization of DC-STAMP, which is implicated in the process of multinucleation and cell fusion, changed dramatically in osteoclasts in response to Pin1 dosage. A recent report also stated that there was no change in osteoclast number between Pin1+/+ and Pin1-/- mice but there was no detail analysis of the size of osteoclasts (Shen et al., 2013). I found there is a wide variation in the size of osteoclasts in Pin1-/- with a very high number of large osteoclasts.

It was previously reported by us and other groups that PU.1 was highly increased in absence of Pin1 (Akiyama et al., 2011; Barberi et al., 2012). DC-STAMP is already known to be regulated by PU.1 (Yagi et al., 2007). So we cannot rule out the possibility that the increase in DC-STAMP expression might be indirectly caused by the increase in PU.1 expression level. But, PU.1 is known to bind DC-STAMP promoter only with GM-CSF or IL4 stimulation not with RANKL (Yagi et al., 2007). I believe that the increase in fusion might be mediated by the surface expression of DC-STAMP as well as the

total expression level, as previously suggested by another group of researchers(Mensah et al., 2010b). According to this point of view we can clearly infer from my data that lack of Pin1 increases the number of osteoclasts with low surface expression of DC-STAMP designated as the ‘master fusogens’ and this along with the increase in the total amount of DC-STAMP is resulting in higher fusion rates in the Pin1-/- osteoclasts. I also observed that the overall expression level of DC-STAMP protein was increased in osteoclasts as well as in mononuclear cells within the bone marrow of Pin1-/- mice (Figure 3.F). It was previously reported that among human cells the majority of DC-STAMP expressing cells are monocytes (Chiu et al., 2012) and in mice DC-STAMP mRNA was expressed in macrophages(Eleveld-Trancikova et al., 2005). I found that lack of Pin1 highly increased the expression of DC-STAMP protein in mononuclear cells within the bone marrow. This can indicate a role of Pin1 for giant cell formation in inflammation or bone tumor development.

From my data I found that in preosteoclasts and mature osteoclasts, Pin1 has a higher localization on the surface or in the cytosol compared to its more usual distribution of higher amounts in the nucleus. And Pin1 and DC-STAMP is co-localized both in vitro and

in vivo in multinucleated bone cells (Figure II.4.C and D). In preosteoclasts I found that DTM treatment altered the distribution pattern of DC-STAMP (Figure II.5). Pin1 mediated DC-STAMP internalization or ER modification may be responsible for this expression pattern. From these observations I could also speculate that Pin1 might have a role in DC-STAMP protein modification within the endoplasmic reticulum where DC-STAMP is known to interact with OS9 and Cyclic AMP-responsive element-binding protein 3(CREB3/LUMAN) (Eleveld-Trancikova et al., 2010; Jansen et al., 2009).

Another incidental finding from my data was that PDI intensity in osteoclasts was higher in Pin1-/- cells compared to $Pin1^{+/+}$ cells (Figure II.5). The function of PDI is related to cytosolic pH(Wang and Narayan, 2008) and this change in intensity might indicate a change in intracellular pH of osteoclasts with Pin1 deficiency. Intracellular pH is an important determinant of bone resorption (Teitelbaum, 2000b) so it is possible that Pin1 has other roles in osteoclast physiology and bone resorption through regulation of intracellular pH.

Furthermore, it has been reported that cellular fusion process is important in some tissues such as the fertilization of the egg and

also muscle formation. Thus, my observations clearly indicate the importance of Pin1 in osteoclast fusion and also suggest the need to identify the importance of Pin1 in other cells which fuse physiologically and pathologically. Previously, it was shown that DC-STAMP transgenic mice did not display any significant muscle fusion related phenotype although it was highly expressed in the muscle of transgenic mice (as shown by RT-PCR)(Iwasaki et al., 2008). But, the observation might have resulted from the ample presence of adequate Pin1 to regulate the rate of fusion. Similar to my data in osteoclasts I think Pin1 might have some rate limiting role in the regulation of DC-STAMP mediated fusion of some other cell types also.

In conclusion, my results suggest that Pin1 is a negative regulator of osteoclast fusion, and that Pin1 exerts a negative effect on the activity of DC-STAMP by modulating its protein expression level and surface localization. Additional studies of the detailed mechanism of the ER modification of DC-STAMP and how it interacts with Pin1, will pave the path to a clearer understanding of the true nature of the role of Pin1 in osteoclast differentiation. my findings point to Pin1 as a potential therapeutic target for the treatment of

human diseases caused by excessive activity of osteoclasts with increased size, such as osteoporosis and autoimmune arthritis. It can also be a good therapeutic target for conditions with giant osteoclasts like breast cancer, long term bisphosphonate therapy and Paget's disease.

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Chapter III

Pin1 modulates myoblast fusion and hypertrophy

V.1. Introduction

The regulation of skeletal muscle formation, termed myogenesis, is prerequisite for normal development as well as the repair of damaged muscle fibers in postembryonic life. The main cellular component of the skeletal muscle is the multinucleated myocyte, which is formed by the fusion of mononucleated myoblasts. Skeletal muscle differentiation is a multistep process, in which mononucleated myoblasts withdraw from the cell cycle, initiate the expression of muscle genes, and fuse with each other to form the multinucleated myotubes (Abmayr and Pavlath, 2012). This process is mostly orchestrated by the MyoD family (MyoD, Myf5, myogenin, and MRF4) and MEF2 family (MEF2A-D) transcription factors (Molkentin and Olson, 1996; Weintraub et al., 1989). Further fusion of differentiated myoblasts with nascent myotubes gives rise to mature myotubes with many nuclei. A wide variety of cell surface and intracellular molecules act to finely coordinate the cellular and molecular events that influence the ability of the mammalian myoblasts to fuse (Jansen and Pavlath, 2008). During embryo stage, skeletal myofibers are derived from the fusion and differentiation of undifferentiated mononucleated myoblasts. After birth, in response to exercise and injury and during

degenerative diseases, quiescent satellite cells become activated and fuse with the new or pre-existing myofibers (Charge and Rudnicki, 2004). Therefore, maintainance of muscle mass is mediated by satellite cell recruitment, myoblast fusion, and myotube hypertrophy in both physiological and pathological conditions.

The TGF-β superfamily directly activates the Smad signaling pathway, in addition to other Smad-independent pathways. Eight mammalian Smad proteins have been identified to date and include Smad1–Smad8. The Smad family of proteins can be divided into three functional groups: the receptor-activated Smads (R-Smads), common mediator Smads (Co-Smads), and the inhibitory Smads (I-Smads). The R-Smads Smad1, Smad2, Smad3, Smad5, and Smad8 have activated type I receptors on their C-terminal and are directly phosphorylated at this C-terminal tail. TGF-β and activin can lead to the phosphorylation of Smad2 and Smad3, whereas Smad1, Smad5, and Smad8 are phosphorylated in response to BMP ligands. This C-terminal phosphorylation allows R-Smad to bind with Co-Smads and translocate to the nucleus where they can recruit transcriptional co-activators or co-repressors and regulate TGF-β/BMP target genes Myostatin is a highly conserved Tgf- β family protein that functions as

an endogenous inhibitor of muscle growth in a wide variety of species (McPherron et al., 1997). Smad3 has been found to be necessary for myostatin-induced increases in atrogin-1 expression and atrophy of cultured myotubes (Lokireddy et al., 2011; Trendelenburg et al., 2009; Welle, 2009).

The PPIase domain of Pin1 catalyses the isomerisation of the specific pSer/Thr-Pro motifs which are present in Smads (Kamato et al., 2013). The functional consequences of the target being in a cis or trans conformation is altered and in this case the activity of Smad as a transcription factor is regulated. It was previously reported from my group that Pin1 is a critical molecular switch in the determination of Smad1 fate Pin 1 has been implicated in several disease states including cancer Pin1 interacts with Smad2/3 in a TGF-β1-dependent manner and Pin1 is actively involved in the TGFβ1-stimulated migration and invasion of PC3 prostate cancer cells (Matsuura et al., 2010). Both Smad-dependent gene transcription and phosphorylation of Smad2/3 in Pin1-null MEF cells were lower than those in Pin1 wild-type MEF cells (Yang et al., 2014). CDK8/9 mediated phosphorylation of pSmad3 creates binding sites for Pin 1, leading to transcriptional activation (Aragon et al., 2011; Gao et al., 2009). It was observed in

primary fibroblasts that, Pin1 was required for TGF-β-induced nuclear translocation and transcriptional activity of Smad3. Inhibitory Smad6 was found in the cytoplasm of $Pin1^{-/-}$ cells and absent from the nucleus. After knock down of Smad 6 in $Pin1^{-/-}$ fibroblasts TGF-β-induced Smad3 activation, translocation, and target gene expression was restored (Brown et al., 2007; Shen et al., 2012b).

Previously in I have reported that the loss of Pin1 enhanced osteoclast fusion (Islam et al., 2014a). In this study the goal was to find if Pin1 also modulates the myoblast fusion and if it can also influence the pathways in muscle hypertrophy.

V.2. Materials and methods

V.2.1. Cell culture

C2C12 myoblasts were grown and differentiated for 4 days as described in differentiating medium that is DMEM supplemented with 2% FBS or 5% horse serum and 1 % antibiotics.

V.2.2. Quantitative real-time PCR (qPCR)

Total RNA was extracted from either mouse tissue or cultured cells

with TRIZOL (Invitrogen) and cDNA synthesized using Superscript III reverse transcriptase with random hexamer primers (Invitrogen). Gene expression was assessed using standard qPCR approaches with either Power SYBR Green or Taqman Master Mix (Applied Biosystems). I used a 7500 Fast Real-Time PCR machine (Applied Biosystems) with the following Sybr primers: Myod1, ACATAGACTTGACAGGCCCCGA and AGACCTTCGATGTAGCGGATGG; Myog, TACGTCCATCGTGGACAGCAT and TCAGCTAAATTCCCTCGCTGG; Myf5, AGGAAAAGAAGCCCTGAAGC and GCAAAAAGAACAGGCAGAGG. Taqman probes for myogenin, MyoD, Ckm and Myh4 were purchased from Applied Biosystems. Expression amounts were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and represented as fold change.

V.2.3. Cytotoxicity assay

The cell cytotoxicity was measured using the EZ-Cytox Cell viability assay kit (Daeillab Service, Seoul, Korea). Briefly, 100 µl of C2C12 cell suspension (3000 cells per well) was added to each well of a 96-well plate. After the required incubation with Pin1 inhibitor in fusogenic media (DMEM with 2%FBS) for 48 hrs, 10 µl of EZ-Cytox solution

was added to each well of the plate and incubated at 37°C for 2–4 hrs. The absorbance was measured by spectrophotometry at 450 nm.

V.2.4. Histology and immunohistochemistry

Skeletal muscle or limbs were dissected and tissue was fixed in 10% neutral buffered formalin and processed for routine paraffin histology. Frozen and paraffin sections were cut and stained with H&E using routine procedures. Immunohistochemistry was performed by fixation with 1% PFA/PBS, permeabilization with 0.2% Triton X-100 in PBS, blocking with PBS/1% BSA, 1% heat inactivated rabbit serum, 0.025% Tween20, incubation with primary antibody for at least 2 h, incubation with secondary Alexa-Fluor antibodies (Invitrogen) for 1 h, and mounting with VectaShield containing DAPI (Vector Laboratories).

V.2.5. Subcellular fractionation and western blot analysis

To fractionate C2C12 cells into cytosol and membrane fractions, we first washed a 10 cm dish with cold PBS and lysed the cells by dounce homogenation in hypotonic buffer (10 mM Tris pH 8.0, 1 mM EDTA) supplemented with 1 mM PMSF and protease inhibitor mix. The homgenate was centrifuged at 15000 RPM for 5 min to pellet nuclei and cell debris. The supernatant was centrifuged at 12000

RPM for 20 min to pellet membrane structures. The supernatant from this step was the cytosol fraction and the membrane fraction was solubilized in an equal volume of hypotonic buffer + 1% n-dodecyl β-d-maltoside (DDM, Sigma) for further analyses by immunoblotting. For analysis of whole-cell extracts, RIPA buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA , 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate , caution0.1% SDS , 140 mM NaCl)] supplemented with 1 mM PMSF and protease inhibitor mix was used for cell lysate preparation. For immunoblotting, equal protein amounts were separated on a 12% SDS–PAGE, transferred to a PVDF membrane (Millipore), blocked in 5% milk in TBS-tween and incubated with primary antibodies.

V.3. Results

V.3.1. Pin1$^{+/-}$ mice show more enhanced primary and secondary fusion than Pin1$^{+/+}$

Cross sectional area (CSA) of muscle is an indicator of enhanced primary (embryonic) fusion or hypertrophy in muscle. Pin1$^{+/-}$ mice shows more fusion as measured by individual muscle circumference from section of the soleus and tongue muscle compared to sections form Pin1$^{+/+}$ mice (Figure III.1.A and B).

I also found that Pin1$^{+/-}$ mice showed less inflammatory exudates and more regenerating myofibers 7days after cardiotoxin injury compared to Pin1$^{+/+}$ mice (FigureIII.2.A). The percentage of centrally nucleated myofibers is considered an indicator of the number of regenerating myofibers (Shavlakadze et al., 2004). I observed that Pin1$^{+/-}$ mice showed a higher percentage of newly formed fibers (FigureIII.2.B) and the number of newly formed fiber in each surface area was also higher compared to Pin1$^{+/+}$ mice (FigureIII.2.C).

V.3.2. Inhibition of Pin1 enhanced fusion more so with lower concentration of inhibitor

Next to see the the range of effect of Pin1 inhibitor DTM treatment on cell fusion I treated very low (.001 μM) to very high (10 μM) of DTM to myoblast cells (C2C12) and let them differentiate. Treatment of Pin1 inhibitor in lower doses causes more enhanced fusion (Figure III. 3. A). Lower dosage of inhibition of Pin1 has a more profound impact on muscle fusion in-vitro and high dosage of Pin1 inhibition shows no significant change in muscle fusion which I observed after calculating fusion index (FigureIII.3.B).

Moreover treatment with Pin1 inhibitor not only increased muscle fusion as indicated by fusion index, it also increased myotube size. Myotube width and length indicates muscle hypertrophy or hyperplasia. Both myotube width and length was increased with DTM treatment (Figure III.4. A,B and C).

C2C12 Cells stably overexpressing Pin1 did not fuse and form myotubes after 3days of differentiation (Figure III.4.D).

V.3.3. Change of Pin1 does not affect muscle differentiation

MYO-D, myogenin and MYF 5 are important markers of muscle differentiation. Inhibition of Pin1 during myogenesis does not change the mRNA level of myogenesis markers like myogenin, Myo-D or

MYF5 (Figure III.5. A, B and C).

V.3.4. Muscle does not express DC-STAMP in vitro and in vivo

Pin1 increases osteoclast fusion partly because of the action it exerts on DC-STAMP localization and expression level. But in case of muscle DC-STAMP was not sufficiently expressed in vivo or in vitro to have any effect on muscle fusion (Figure III.6.A and B).

V.3.5. Minimal inhibition of Pin1 inhibits Smad3 signaling

Over expressed Smad3 in vivo demonstrated that this was sufficient to activate the atrogin-1 promoter, inhibit mTORC1 signaling and protein synthesis, and ultimately induce muscle fiber atrophy (Goodman et al., 2013b). It is already known that sufficient inhibition of Pin1 can inhibit the expression level of Smad 1/5 (Yoon et al., 2015). With lower concentration of Pin1 inhibitor treatment the level pSmad 3 was decreased more significantly than pSMAD2 (Figure III.7. A, B and C) and Smad 1/5 signaling was totally unaffected (Figure III.7. A). With TGFβ treatment it was seen that within 1 hour Pin1 inhibitor treatment could repress the protein expression level of pSmad 3. Phosphorylated Smad1 and Smad 5 were not repressed by low dose of inhibitor treatment along with TGFβ (Data not shown).

Nuclear localization which is required for Smad protein transcriptional activity was also observed. And again I found that Smad 2 was not changed but Smad 3 nuclear localization was highly reduced as quantified by densitometry (Figure III.7.C). In vivo the level of pSmad 3 was also decreased in muscles from $Pin1^{+/-}$ mice compared to $Pin1^{+/+}$ mice (Figure III.7. D).

Overexpression of Smad 3 showed reduced myofiber fusion (Figure III.8.A) but treatment with DTM rescued this effect as observed by the fusion index calculations (Figure III.8.B).

Muscle mass is maintained by a balance between protein synthesis and protein degradation systems. Atrogin-1 and MuRF-1 (muscle RING-finger protein-1) are unique E3 ubiquitin ligases that are expressed in skeletal muscle and direct the polyubiquitination of proteins (Bodine et al., 2001). It was discovered that FoxO3 and Smad3 coordinately and directly regulate transcription of MuRF-1 (Bollinger et al., 2014). Smad 3 is also deemed necessary for myostatin-induced atrogin-1 expression and atrophy (Goodman et al., 2013a). I observed that both atrogin-1 and MuRF-1 mRNA expression was reduced with DTM treatment in the presence of Tgf-β (Figure.III.9.A and B).

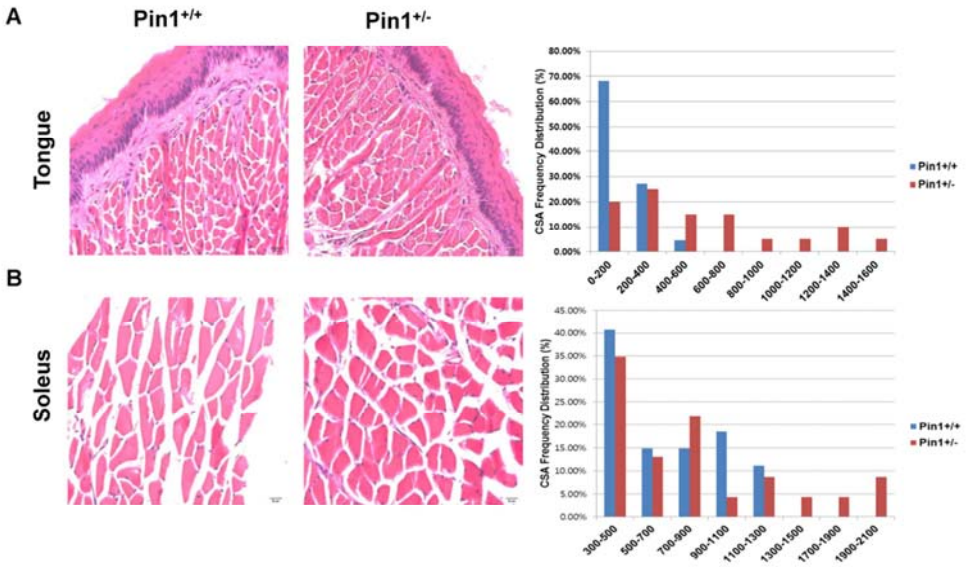


Figure III.1. Pin1$^{-/+}$ mice have more myofibers with higher surface area

(A)Tongue muscle from Pin1+/+ and Pin1+/- mice were fixed and sections were stained with H and E. CSA (cross sectional area) was measured from soleus and tongue muscle sections using the Olympus DP72 microscope and Olympus imaging software cellSens. (B) Same was done for sections of the soleus muscle. P values of the CSA measurements were separately analyzed by SPSS software and were significant (P< 0.05 in both A and B).

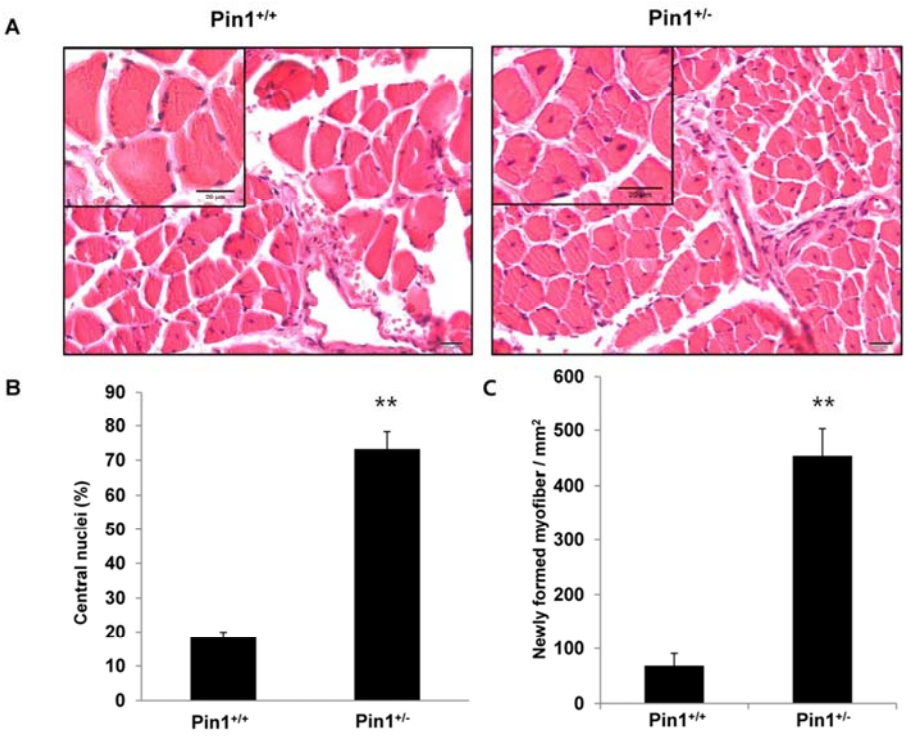


Figure III.2.Pin1$^{-/+}$ mice higher amount of secondary fusion as indicated by new myofiber formation post cardiotoxin injury

A) Representative cross sections of Pin1+/+ and Pin1+/- Tibialis anterior muscle 7 days after single cardiotoxin injury. Scale bars= 20 μM. (B) The number of myofibers with central nuclei indicating recent fusion was quantified and represented

graphically as percentage of total myofibers visible in each field of vision. (C) Newly formed myofibers in each mm^2 was calculated and shown graphically.

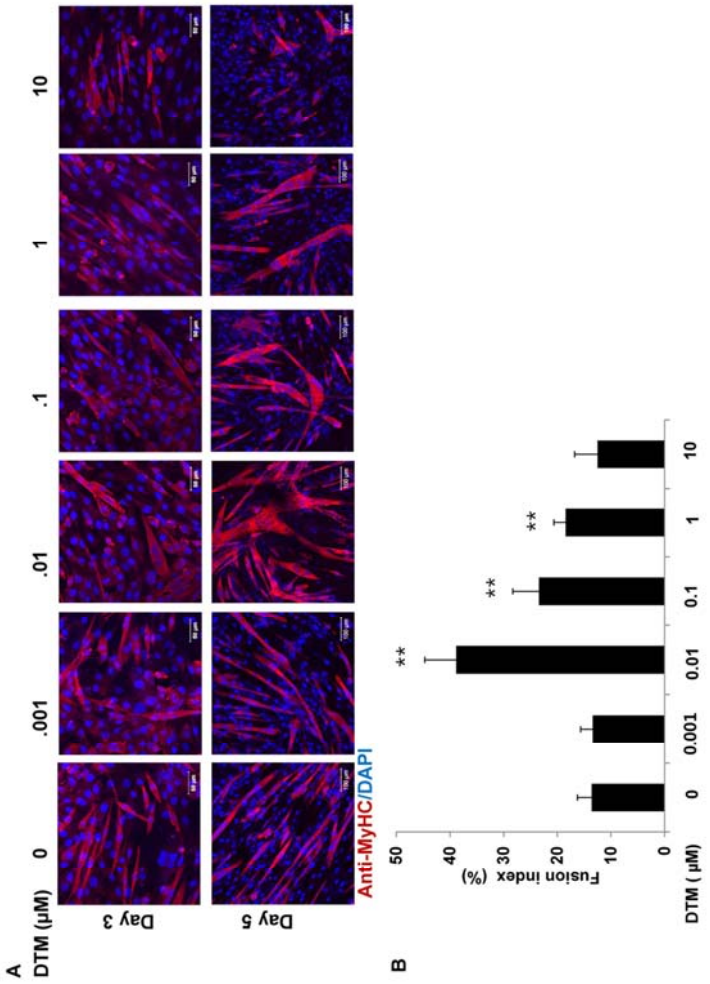


Figure III.3. Inhibition of Pin1 enhances fusion of C2C12 myoblasts

A) C2C12 cells treated with Pin1 inhibitor DTM at different dosage were stained with MyHC antibody after 3 days of differentiation and (B) Fusion index was calculated from the confocal images by calculating the ratio between total nuclei number in each myotubes and total nuclei number in each field. (** indicates $P<0.001$ compared to control).

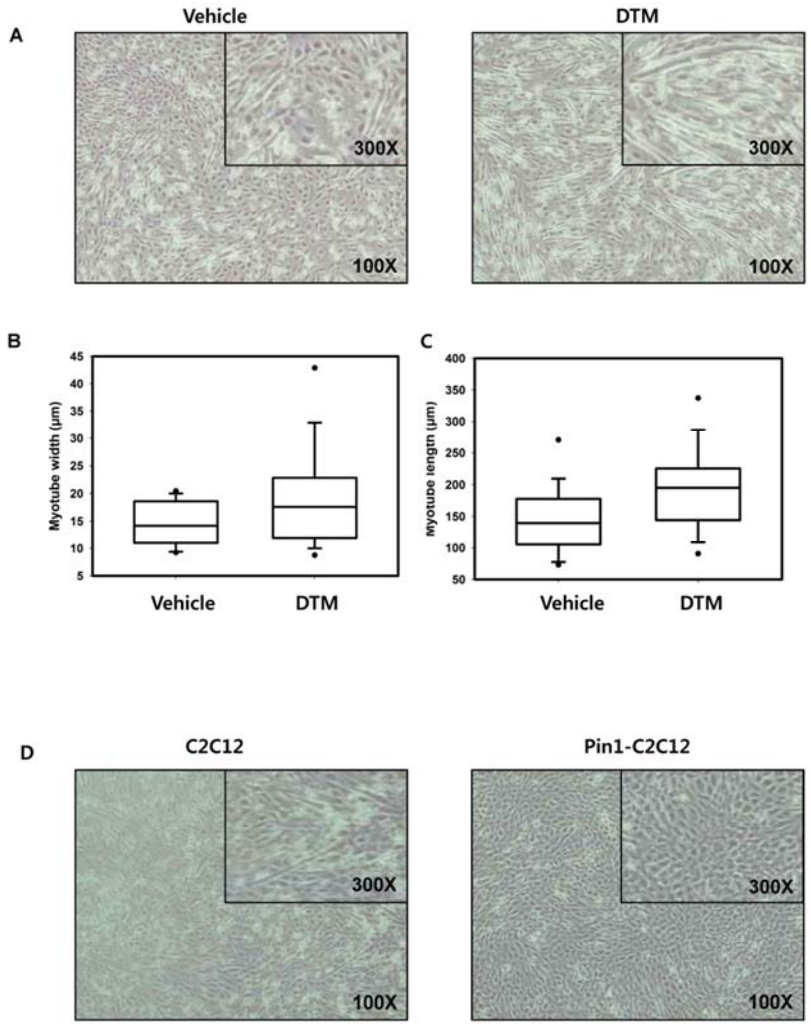


Figure III.4. Pin1 regulates muscle fiber size

A) C2C12 cells treated with DTM (.01 μM) were differentiated for 3 days in differentiating media and analyzed under a phase contrast microscope. (B) Myotube width and (C) Myotube length of DTM treated cells were also measured and

distribution or the measurements were observed in a graphical manner with box and whisker plots. (# indicates $P<0.05$). (D) Stably Pin1 overexpressing C2C12 (Pin1-C2C12) cells were cultured in myogenic differentiation medium (DMEM with 5% FBS) for 3 and 5 days and observed under inverted bright field microscope at 200x magnification.

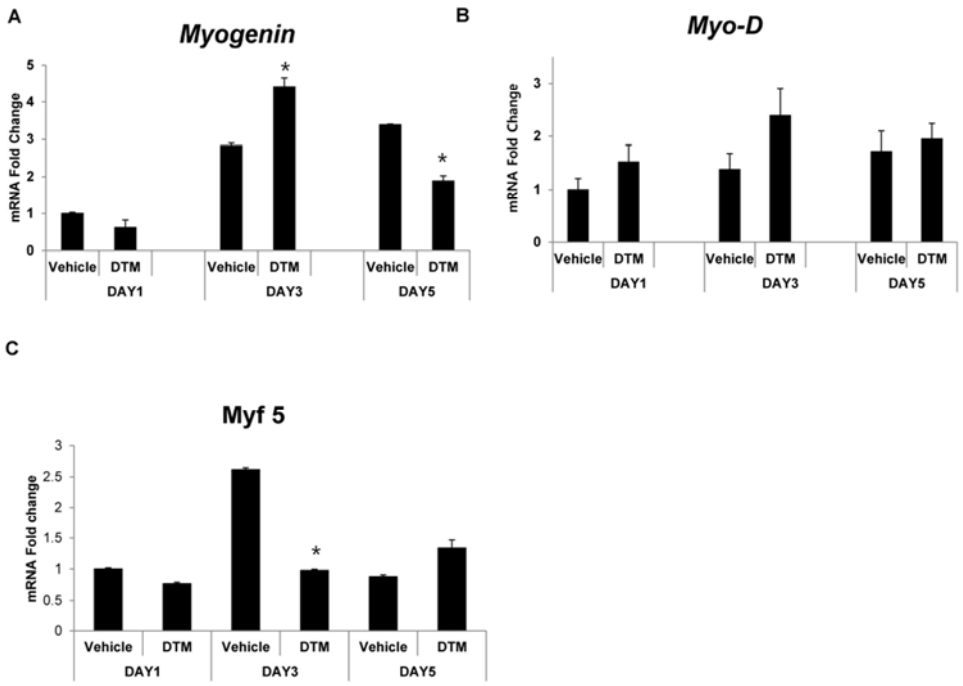


Figure III.5. Inhibition of Pin1 does not affect the mRNA level of myogenic markers highly.

A) C2C12 cells treated with and without (concentration) DTM in myogenic medium were harvested at 1, 3 and 5 days post addition of differentiation medium and RNA was extracted from them followed by cDNA making and Real-time PCR for *myogenin*, (B) *Myo-D* and (C) *Myf-5.* (* indicates P<.05).

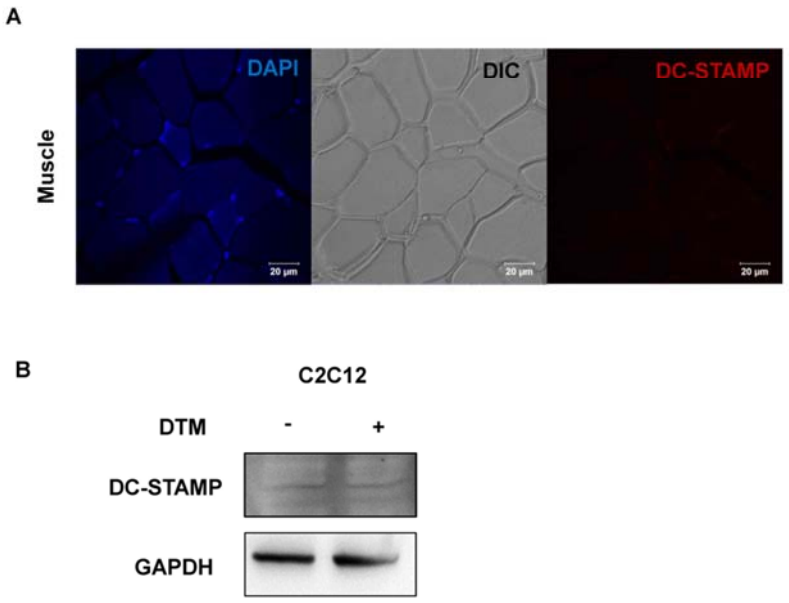
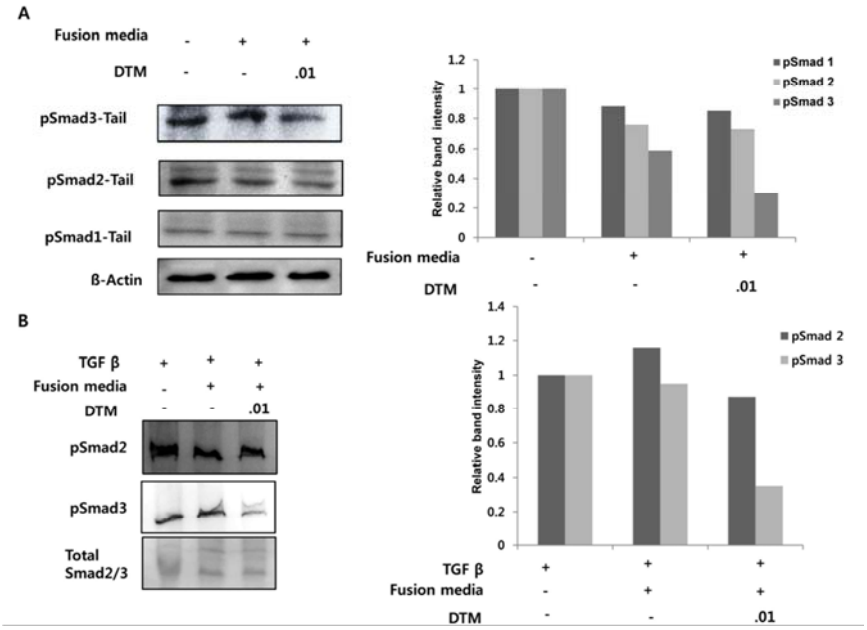


Figure III.6. Pin1 inhibitor does not increase mitosis.

A) C2C12 cells in growth media (GM) and differentiation media that is fusogenic media (DM) were treated with and without DTM (1 µM) for 1 day post starvation. Cells were lysed in RIPA buffer and the level of pH3 (phospho Histone 3) compared to total H3 was observed. (B) C2C12 cells cultured on a glass coverslip were serum starved after reaching confluence followed by culture in differentiation media with and without DTM. Cells were then stained for mitotic marker pH3 and nuclear stain DAPI.


A
Fusion media - + +
DTM - - .01
pSmad3-Tail
pSmad2-Tail
pSmad1-Tail
β-Actin
Relative band intensity
pSmad 1
pSmad 2
pSmad 3
Fusion media - + +
DTM - - .01
B
TGF β + + +
Fusion media - + +
DTM - - .01
pSmad2
pSmad3
Total Smad2/3
Relative band intensity
pSmad 2
pSmad 3
TGF β + + +
Fusion media - + +
DTM - - .01

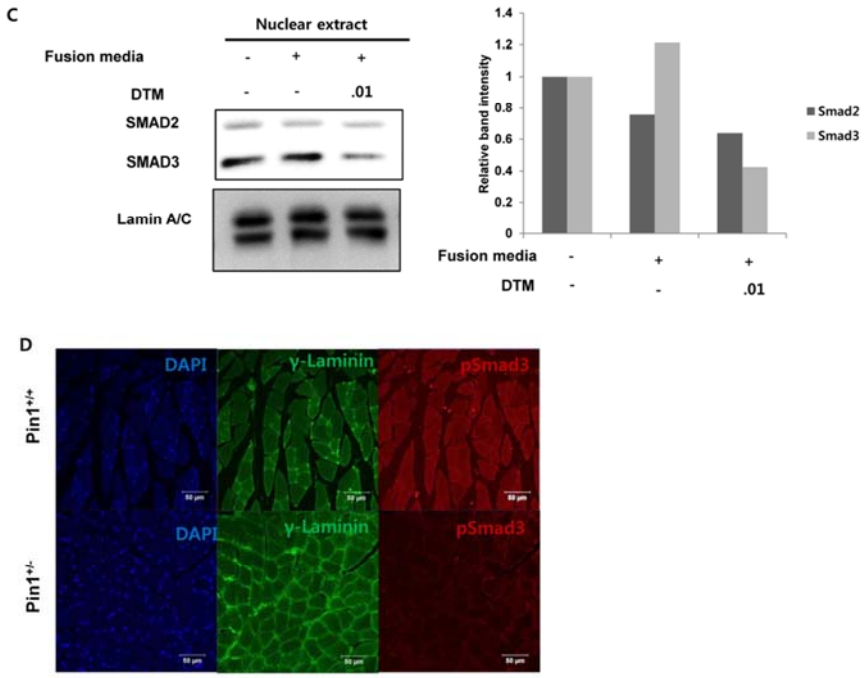


Figure III.7. Pin1 inhibition in lower dosage suppresses the SMAD2/3 signaling

(A) C2C12 cells treated for 24 hours in growth media and fusion media with low dosage of DTM (.01μM) and without any treatment were lysed and levels of tail phosphorylated Smad 2, Smad 3 and Smad1 were observed with densitometry utilizing image J software after western blot. (B) In the presence of TGF-β and low concentrations of DTM for 1 hr in fusogenic/differentiation media after 12 hrs of starvation the level of expression of pSmad2 and pSmad3 were analyzed. (C)

In the same condition as nuclear localization of Smad 2/3 was observed with subcellular fractionation and western blot followed by immune labelling. The amount of nuclear Smad 2/3 was quantified with densitometry. (D) Histological cross-sections of the tibialis anterior muscle from $Pin1^{+/+}$ and $Pin1^{+/-}$ mice were stained for pSmad3 (Cy3-red) and γ-Laminin (Alexa 488-green).

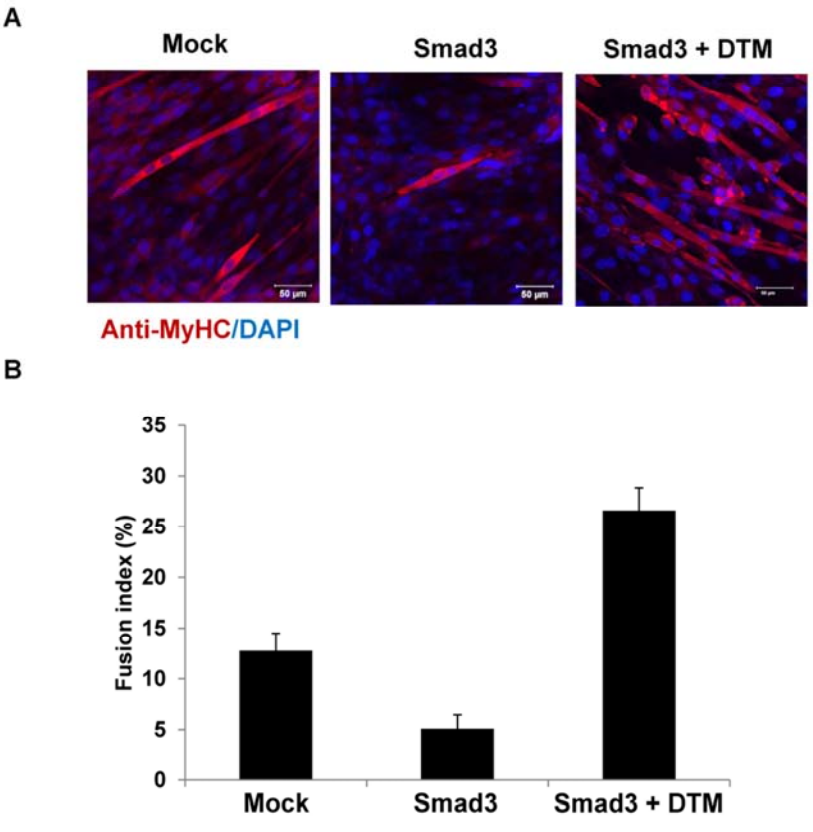


Figure III.8. Pin1 inhibition can rescue the repressed fusion observed with overexpression of Smad3 protein

A) C2C12 cells were transfected with PCDNA3.1(Mock) and PCMV-Smad3 with or without DTM treatment and allowed to differentiate for 5 days post transfection. (B) Fusion index was calculated as percentage of nuclei number in multinucleated cells among total nuclei.

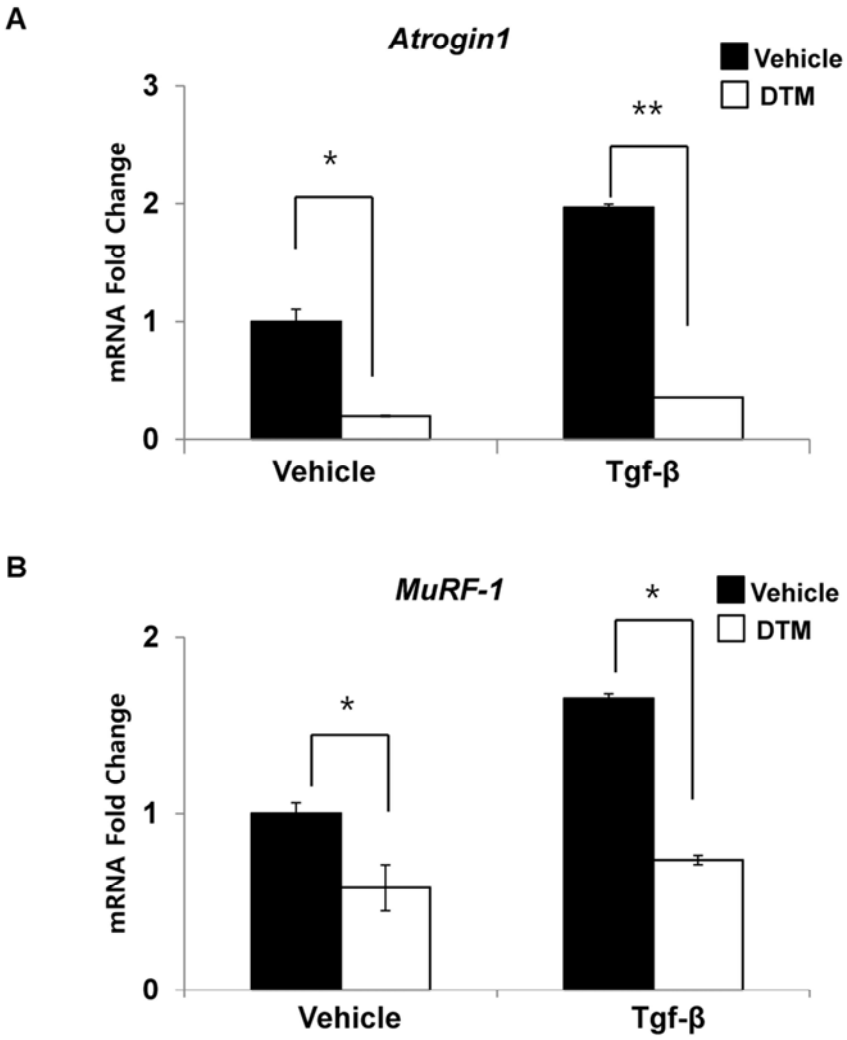


Figure III.9. Pin1 inhibition shows decreased expression of down stream genes of Smad3 that is *atrogin-1* and *MuRF-1*

C2C12 cells treated with DTM and Tgf-β in myogenic media for 12 hours were harvested and RNA was extracted from them. Realtime

PCR was done for (A) Atrogin-1 and (B) MuRF-1 (* and ** indicates P value is <.05 and <.001 respectively).

V.4. Discussion

Although virtually pathognomonic of viral infections and neoplasia, spontaneous cell-cell fusion, or syncytialization, is quite restricted in healthy tissues. Normally seen in muscle (Abmayr and Pavlath, 2012) and in bone (Soe et al., 2011) and crucial for their proper functioning.

Pin1 is a ubiquitous protein which is important to maintain the balance of many biological processes (Liou et al., 2002). As only a small modification of Pin1 level can enhance muscle bulk in a generous amount it can be a very useful target to utilize for developing muscle bulk enhancing drugs that have less toxic side effects.

Pin1 inhibition increases fusion which can be utilized for diseases with muscle atrophy and dystrophy. An approach to increase muscle bulk can benefit other forms of therapy also as cell fusion is a key factor that can help enhance the efficacy of many drugs being researched now for different purposes. Muscle injury and atrophy in normal healthy adults for example people who simply suffer an injury like a compound fracture of the knee or have their legs in cast for a

long time suffer from muscle atrophy. And in a lot of cases as hard as we try, the muscle mass never comes back. This problem can be solved with a muscle bulk enhancing drug. Cancer related muscle atrophy-Cachexia, which is characterized by the progressive loss of skeletal muscle mass, affects up to 80% of patients with advanced cancer (Tisdale, 2002). It seriously reduces quality of life and is estimated to be responsible for ~25% of deaths in these patients (Tse, 2010). Therapeutic strategies to prevent muscle wastage are limited and the underlying mechanisms are unclear. Finding the mechanism of Pin1 inhibition based muscle fusion can lead to the discovery of other drug targets for the same purpose. Pin1 inhibition might have adverse effects like neurodegeneration or osteoporosis. But, from my data it is evident that only a low dose of Pin1 inhibition can enhance muscle fusion.

Most fusion events lead to cytoskeletal rearrangements. Finding the relation of Pin1 inhibition with the cytoskeleton (Shilagardi et al., 2013) which is the main player in cell to cell fusion can resolve many unanswered questions about cell to cell fusion in general. And this knowledge can be utilized for study of cancer therapeutics and other diseases.

From this experimental data it can be inferred that Pin1 is biologically important to maintain the balance in SMAD signaling to regulate the muscle bulk and also for muscle fusion. A small amount of inhibition in Pin1 activity can cause a very significant amount of increase in the muscle size and fusion by specifically targeting the tgf-β pathway.

V.5. References

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VI. Conclusion

I found that Pin1 binds to runx1 and modulates the transcription of PU-1. Pin1 inhibits excessive fusion in osteoclasts through the regulation of DC-STAMP expression and localization. Myoblast fusion is also modulated by Pin1. Effect of Pin1 on Smad 1/5 and Smad 3 is crucial for the muscle bulk maintainance. In low dosage of Pin1 inhibition pSmad 3 is only reduced and muscle hypertrophy occurs.

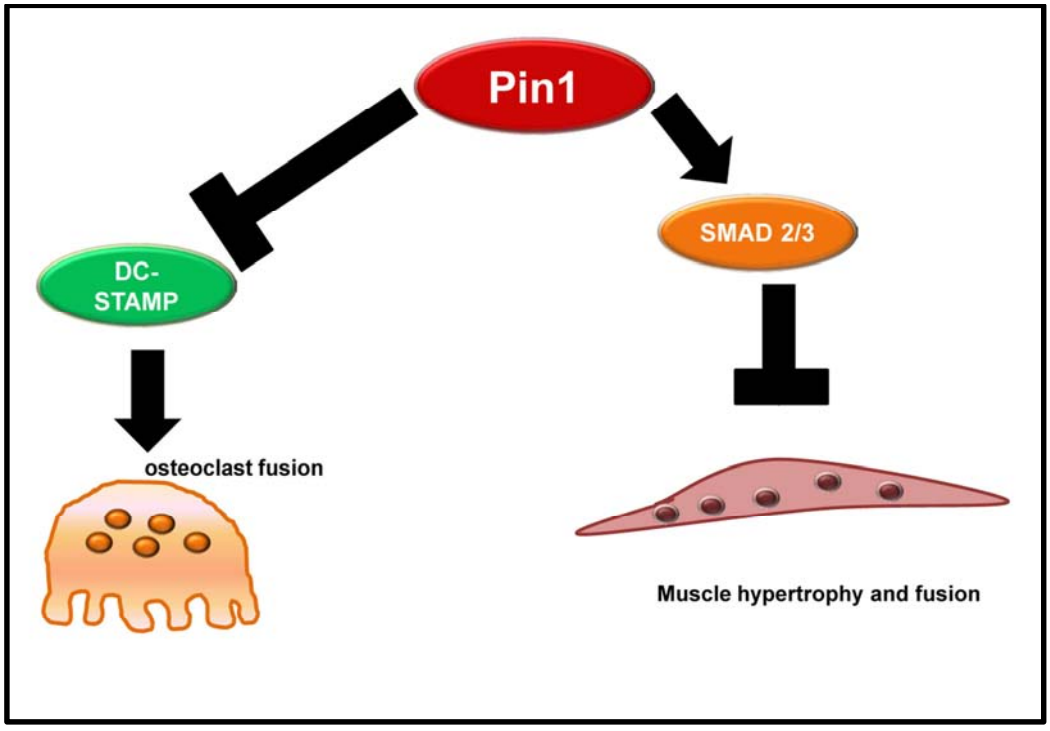

Figure - Summary of the findings for the mechanism how Pin1 regulates fusion in osteoclasts and myoblasts.

These findings can be utilized for a cell fusion based approach to strengthen muscle and inhibit osteoporosis. Resolving the mechanism of muscle bulk regulation by Pin1 can open new avenues for therapeutic advance in myopathies, neurodegenerative diseases, cancer and inflammations.

VII. Abstract

생물학적으로 막 융합은 정상적 (생식세포 융합과 근육모세포 융합) 그리고 병리학적 (바이러스와 세포간의 융합) 사건을 조절하는 근본적인 과정이다. 세포간의 융합은 수정, 발달 그리고 다양한 기관의 생리학적 기전에 매우 중요하다. 세포 내 융합생성 단백질과 세포골격을 이루는 액틴은 세포간 융합과정에 관련되어 있음에도 불구하고, 아직 어떻게 그것들이 협력하여 세포막 융합을 촉진하는지는 명확히 밝혀지지 않고 있다. 장골의 조직 단면에서 Pin1을 발현하지 않는 마우스의 경우 Pin1을 정상적으로 발현하는 마우스에 비해 낮은 골 량과 증가된 TRAP 염색 결과를 관찰할 수 있다. 또한, 골수에서 유래한 단핵백혈구와 대식세포에서 생체 외 파골 세포 형성 정도를 분석한 결과 Pin1이 없는 마우스의 파골 세포는 정상 마우스의 파골 세포보다 더 크고 더 많은 핵 수를 가지는 것을 확인하였고, 이는 융합이 더 증가되었음을 나타낸다. Pin1이 없는 마우스는 생체 내에서와 생체 외에서 모두 이물거대세포의 형성 또한 매우 증가하였다. 알려진 융합 단백질 중, 오직 DC-STAMP만이 Pin1이 없는 파골 세포에서 매우 증가되었는데, 이는 종아리 뼈 단면의 면역조직화학 결과에서도 관찰되었다. 우리는 Pin1이 DC-STAMP와 결합하여 DC-STAMP를 이성질화 시키고, 이는 DC-STAMP의 단백질 수준과 세포막에서의 위치 결정에 영향을 끼친다는 것을 발견하였다. 이와 같은 연구결과들을 통틀어 살펴본 결과, Pin1이 파골 세포 융합 단백질 DC-STAMP의 작용을 조절하여 골 량을 조절하는 결정인자라는 결론을 내릴 수 있다. Pin1이 세포융합기전에 관련되어 있다는 사실의 규명은 학문적으로는 골다공증을 포함한 파골 세포 관련 질환의 이해를 도울 뿐만 아니라 상업적으로는 골 질환 치료제 개발 시장에서 새로운 타겟 인자를 밝혔다는 점에서 큰 의미를 갖는다.

근육모세포에서 Pin1이 미치는 영향을 살펴본 결과, Pin1이 근육모세포의 융합을 억제하고 있음을 확인할 수 있었고 이는 Pin1을 억제하자 근육모세포 융합이 더 활성화되는 것으로 입증되었다. 만약 Pin1이 파골 세포와 근육세포의 융합 모두에 영향을 준다면 이것은 근육 감소증과 같은 다양한 근 골격계 질환을 조절할 수 있는 핵심인자로 작용할 것이며 그 밖에도 암이나 노화와 같은 일반적인 질환과도 연관 지어 적용할 수 있을 것이다.

키워드 : 세포 융합, 근육 세포, 파골 세포, 프롤린 이성질화 효소(Pin1)