Mouse Genetics Studies on Molecular Mechanisms Underlying Bone and Cartilage Disorders

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Contents

Abstract

This paper summarizes our mouse genetics studies on the molecular backgrounds of representative degenerative skeletal diosrders: osteoporosis, ossification of the posterior longitudinal ligament of the spine (OPLL), bone fracture healing, and osteoarthritis (OA).

By analyzing deficient mice, PPARγ, a key adipogenesis molecule intrinsic to bone marrow progenitors, was shown to be involved in age-related osteoporosis. Studies on deficient mice and OPLL patients revealed that insulin and insulin-like growth factor-I (IGF-I) are potent bone anabolic factors through the balance of distinct signals of the two adaptor molecules, insulin receptor substrate (IRS)-1 and IRS-2: IRS-1 for maintenance of bone turnover by up-regulating anabolic and catabolic functions of osteoblasts, while IRS-2 for retaining the predominance of the anabolic function over the catabolic function. IRS-1 was also essentil for the osteoanabolic action of parathyroid hormone (PTH) and bone fracture healing. The IRS-1 action was mediated by Akt1, which was shown to be important in both osteoblasts and osteoclasts to maintain bone mass and turnover.

In our original experimental knee OA models in mice, hypertrophic differentiation of chondrocytes was induced in the joint cartilage during OA progression. Heterozygous deficient mice of Runx2 exhibited less susceptibility to cartilage degradation. In the meantime, carminerin, a novel chondrocyte-specific protein which we identified as a chondrocyte calcification factor, was shown to be important for OA osteophyte formation by analyses of the deficient mice.

Our mouse genetics studies revealed the importance of PPARγ, insulin-IGF-I / IRS-1-IRS-2 /Akt1 signal, Runx2, and carminerin in bone and cartilage under physiological and pathological conditions. These molecules could be therapeutic targets for the skeletal disorders.

Introduction

Due to the rapidly increasing number of elderly individuals today, degenerative skeletal diosrders are now considered a major public health issue causing chronic disability in most developed countries. Among them, osteoporosis and osteoarthritis (OA) are the two representative disorders worldwide with strong social impacts.[1] Ossification of the posterior longitudinal ligament of the spine (OPLL) with a prevalence of 2-4% in Asia and somewhat lower in other countries is a disorder that causes severe tetraparesis.[2] Despite significant social demand for more information of the etiology of the disorders, the molecular mechanisms have not yet been fully elucidated. The mouse genetics approach is now considered to be a useful and efficient method due to recent progression of gene manupulating techniques in vivo. This manuscript introduces our recent mouse genetics studies attempting to elucidate the molecular mechanisms underlying the bone and cartilage disorders.

PPARγ as an intracellular molecule regulating age-related osteoporosis

Ageing is one of the major causes of osteoporosis, and the underlying mechanisms include intracellular and extracellular signals of osteoblastic cells. As an intracellular molecule regulating age-related osteoporosis, Runx2, a key transcription factor for osteoblast differentiation, is the most probable candidate, since the expression is reported to be decreased during cellular ageing of osteoblasts in vitro.[3] However, there is no in vivo evidence of its contribution to bone loss with ageing.

Osteoblasts and adipocytes are known to share a common progenitor: multipotential mesenchymal cells in bone marrow, being driven by respective key molecules Runx2 and PPARγ

(Fig. 1A).[4] In addition, ageing is associated with a reciprocal decrease of osteogenesis and an increase of adipogenesis in bone marrow.[5] Hence, we investigated the role of PPARγ in bone metabolism by analyzing heterozygous PPARγ-deficient (PPARγ+/–) mice,[6,7] since the homozygous deficient (PPARγ–/–) mice were embryonically lethal.[8] Although they showed no abnormality in major organs, PPARγ+/– mice exhibited higher bone mass than the wild-type (WT) littermates,

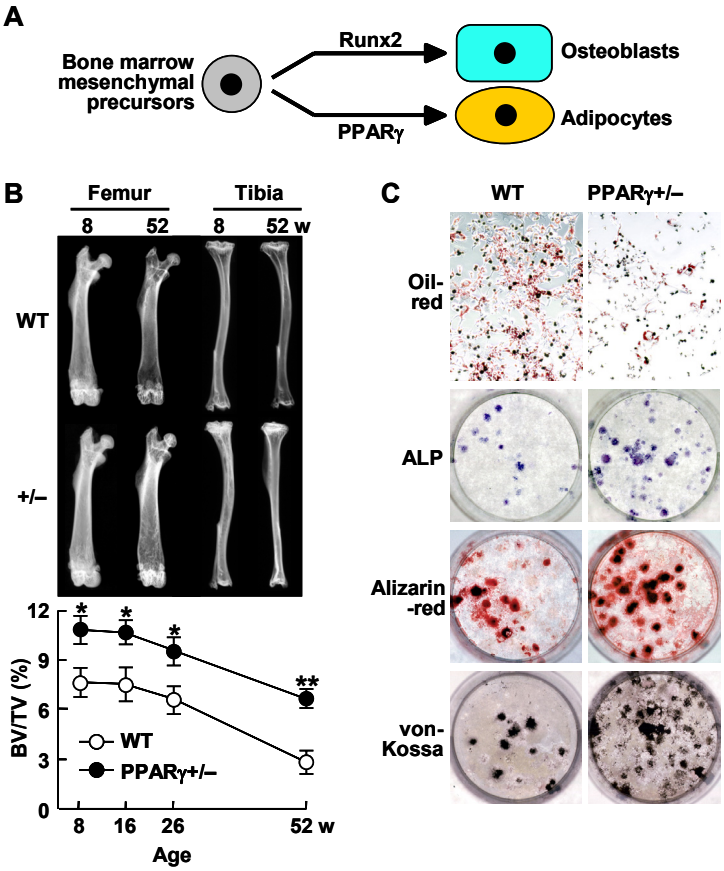

Figure 1. (**A**) A schema of differentiation of osteoblasts and adipocytes from the same progenitor cells. (**B**) Radiographs of wild-type (WT) and PPARγ+/– bones at 8 and 52 weeks of age, and time course of trabecular bone volume (BV/TV) at the distal femurs measured on CT images. *P<0.05, **P<0.01 vs. WT. (**C**) Adipogenesis by oil-red staining and osteogenesis by alkaline-phosphatase (ALP), Alizarin-red, and von-Kossa stainings in cultures of WT and PPARγ+/– bone marrow cells.

especially at older ages (Fig. 1B), indicating the involvement of the PPARγ signal in the pathophysiology of age-related osteoporosis.[6,7] Ex vivo cultures of bone marrow cells showed that PPARγ haploinsufficiency caused not only a decrease in the number of adipocytes, but also an increase of osteoblasts (Fig. 1C), indicating that PPARγ signal in marrow progenitors functions as a potent suppressor of commitment to osteoblastic lineage. Clinical involvement of PPARγ in bone loss was confirmed by an association study between the human gene polymorphism and bone density in Japanese postmenopausal women.[9]

IGF-I / IRS signal regulating age-related osteoporosis

There are several cytokines / growth factors that function as extracellular signals regulating age-related osteoporosis,[10] and among them IGF-I is the most probable candidate since the serum level is positively correlated with bone density of aged populations.[11] Patients with Laron syndrome caused by IGF-I deficiency also exhibit severe osteoporosis,[12] indicating that IGF-I is a potent bone anabolic factor clinically.

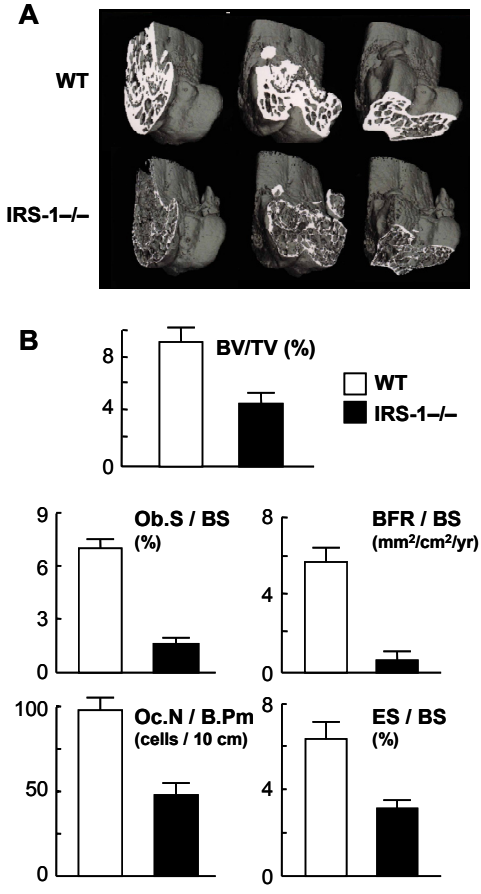

Figure 2. (**A**) 3D-CT of distal femurs of WT and IRS-1–/– littermates at 8 weeks of age. (**B**) Trabecular bone volume (BV/TV) and bone histomorphometric analysis of the proximal tibiae. Both bone formation parameters (Ob.S/BS and BFR/BS) and bone resorption parameters (Oc.N/B.Pm and ES/BS) were decreased in the IRS-1–/–.

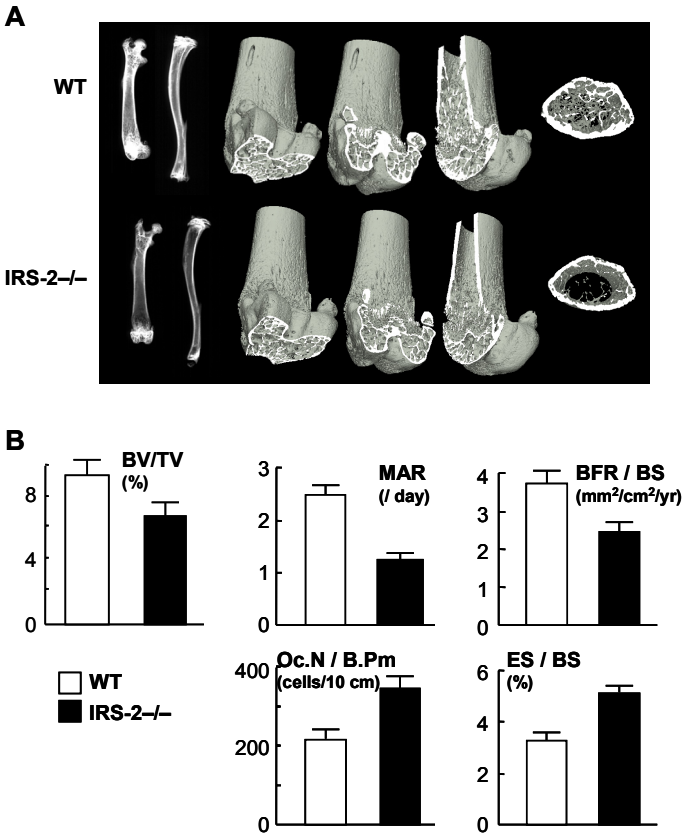

Figure 3. (**A**) Radiographs of femurs and tibias, and 3D-CT of distal femurs of WT and IRS-2–/– littermates at 8 weeks of age. (**B**) Trabecular bone volume (BV/TV) and bone histomorphometric analysis of the proximal tibiae. Bone formation parameters (MAR and BFR/BS) were decreased while bone resorption parameters (Oc.N/B.Pm and ES/BS) were increased in the IRS-2–/–.

IGF-I as well as insulin initiate cellular responses by binding to their respective cell-surface receptors, and then activate essential adaptor molecule insulin receptor substrate (IRS) followed by downstream signaling pathways like phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs).[13] Among the IRS family, we found that IRS-1 and IRS-2 are expressed in bone.[14,15] Our further studies on mice lacking IRS-1 (IRS–1–/–) or IRS-2 (IRS–2–/–) revealed that these mice exhibited severe osteopenia with distinct mechanisms: IRS-1–/– mice showed a low bone turnover in which both bone formation and resorption were decreased (Fig. 2),[14] whereas IRS-2–/– mice showed an uncoupling status with decreased bone formation and increased bone resorption (Fig. 3).[15] It therefore seems that IRS-1 is important for maintaining bone turnover, while IRS-2 for remodeling balance retaining the predominance of anabolic function over catabolic function of osteoblasts. IGF-I and insulin may up-regulate bone formation without affecting bone resorption through the balance of the two IRS signals in osteoblasts (Fig. 4).[16]

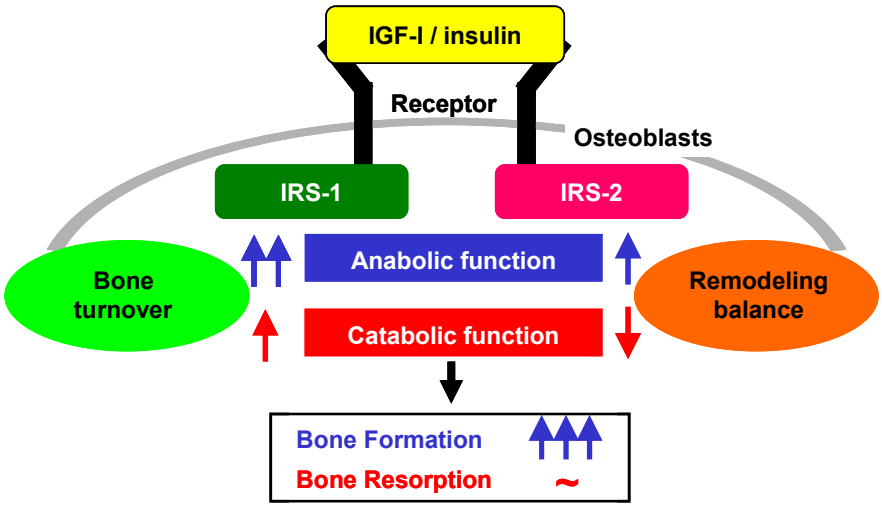

Figure 4. A schema of mechanism underlying the osteoanabolic function of IGF-I and insulin via distinct functions of IRS-2 and IRS-2 in osteoblasts.

Insulin signal in OPLL

Patients with type 1 diabetes with decreased insulin level are often associated with

osteoporosis,[17] probably via the suppression of IRS-1 and IRS-2 signals above. Besides osteoporosis, the insulin/IGF-I signal may possibly be involved in other skeletal disorders. Although the relationship between osteoporosis and type 2 diabetes is controversial, most OPLL patients have been reported to accompany type 2 diabetes.[18] Our clinical study examining the relationship between the extent of ossification and glucose intolerance in OPLL patients revealed that the insulin secretory response, but not the severity of glucose intolerance or diabetes, was associated with the ossification severity (Fig. 5),[19,20] confirming the clinical osteoanabolic action of insulin under the pathological condition.

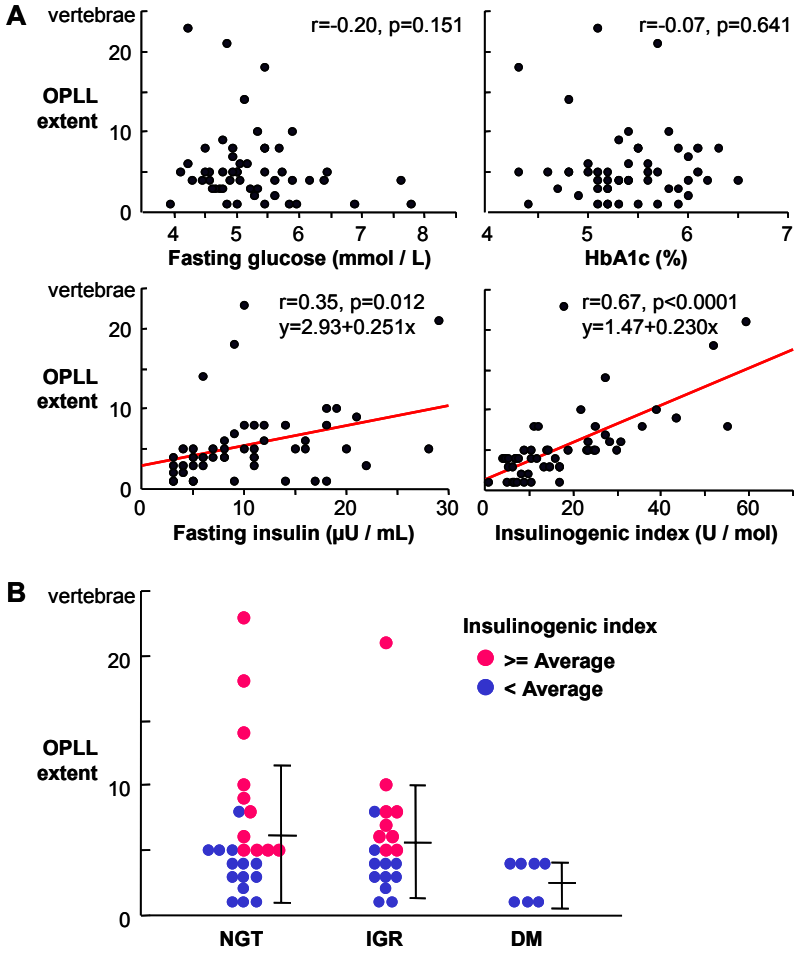

Figure 5. (**A**) Correlation between OPLL extent and serum levels of diabetes-related factors in OPLL patients (n=52). (**B**) OPLL extent of patients with three grades of diabetes severity: non-glucose tolerance (NGT), intermediate glucose resistance (IGR), and diabetes mellitus (DM). Patients with insulinogenic index equal to or higher than the average are shown as red circles and those less than that are shown as blue circles.

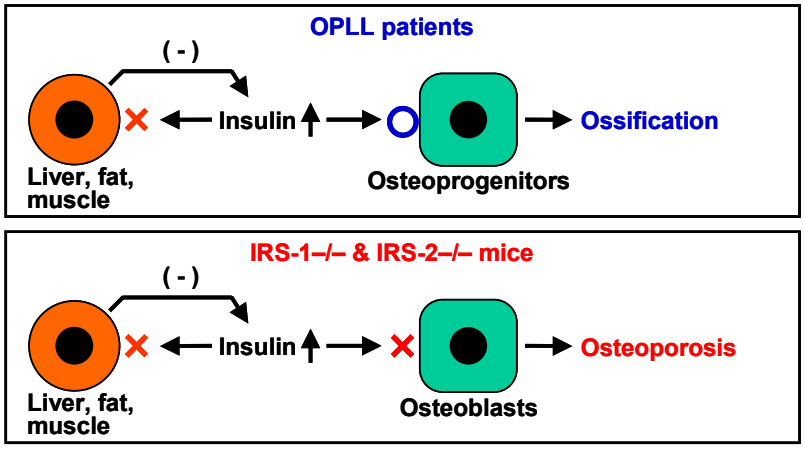

Figure 6. A schema of mechanisms underlying the ossification in OPLL patients, and osteoporosis in IRS-1–/– and IRS-2–/– mice.

Taking the clinical and mouse genetics studies together, in OPLL patients with type 2 diabetes the up-regulation of insulin production as a feedback mechanism of the impairment of insulin sensitivity in the target cells may stimulate osteoprogenitor cells in the ligament to induce ossification. Contrarily, in IRS-1–/– and IRS-2–/– mice even the increased insulin may not affect osteoblasts, resulting in osteoporosis (Fig. 6).

IRS-1 signal in PTH osteoanabolic action and bone fracture healing

Bone anabolic action of PTH has also been suggested to be mediated by induction of IGF-I in osteoblasts, which acts locally as an autocrine/ paracrine factor.[21] To learn the involvement of IRS-1 and IRS-2 in the bone anabolic action of PTH in vivo, IRS-1–/– and IRS-2–/–mice and their respective wild-type littermates were given daily injections of PTH or vehicle for 4 weeks (Fig. 7).[22] In the wild-type mice, the PTH injection increased bone mineral densities of the femur, tibia, and vertebrae. These stimulations were

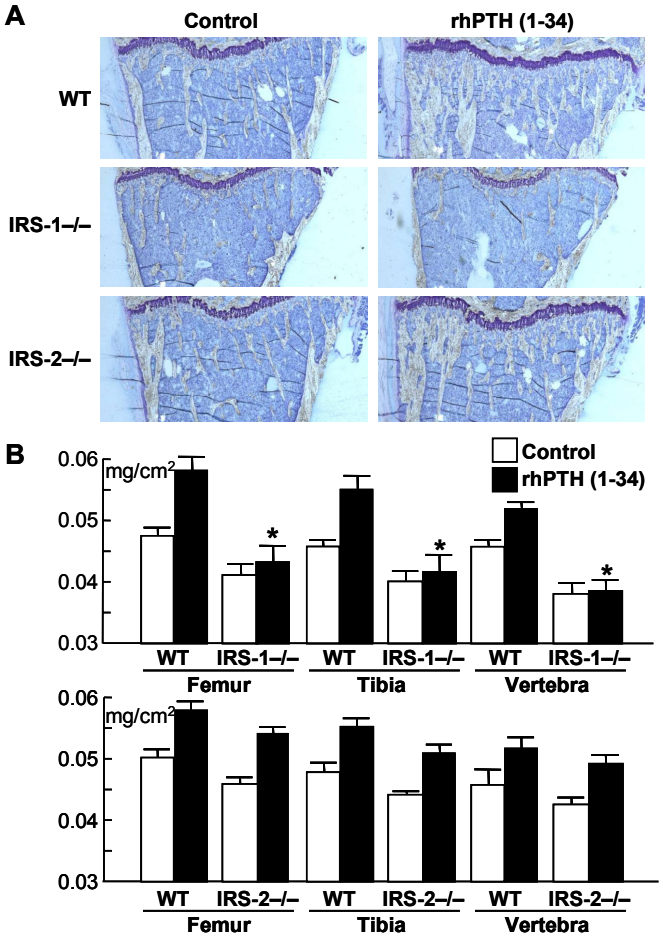

Figure 7. Effects of in vivo injection of recombinant human (rh)PTH (1-34) in IRS-1–/– and IRS-2–/– mice. The knockout mice and the respective wild-type (WT) littermates underwent daily injections of rhPTH (80 g/kg) or the vehicle for 4 weeks. (A) Representative toluidine-blue stainings of proximal tibias. (B) Bone mineral densities of the entire femurs, tibias, and vertebral bodies determined by dual-energy X-ray absorptiometry. *P<0.01 vs. WT with rhPTH treatment.

similarly seen in IRS-2–/–mice; however, they were markedly suppressed in IRS-1–/–mice. These results indicate that the PTH bone anabolic action is mediated by the activation of IRS-1, but not IRS-2, as a downstream signal of IGF-I.[22]

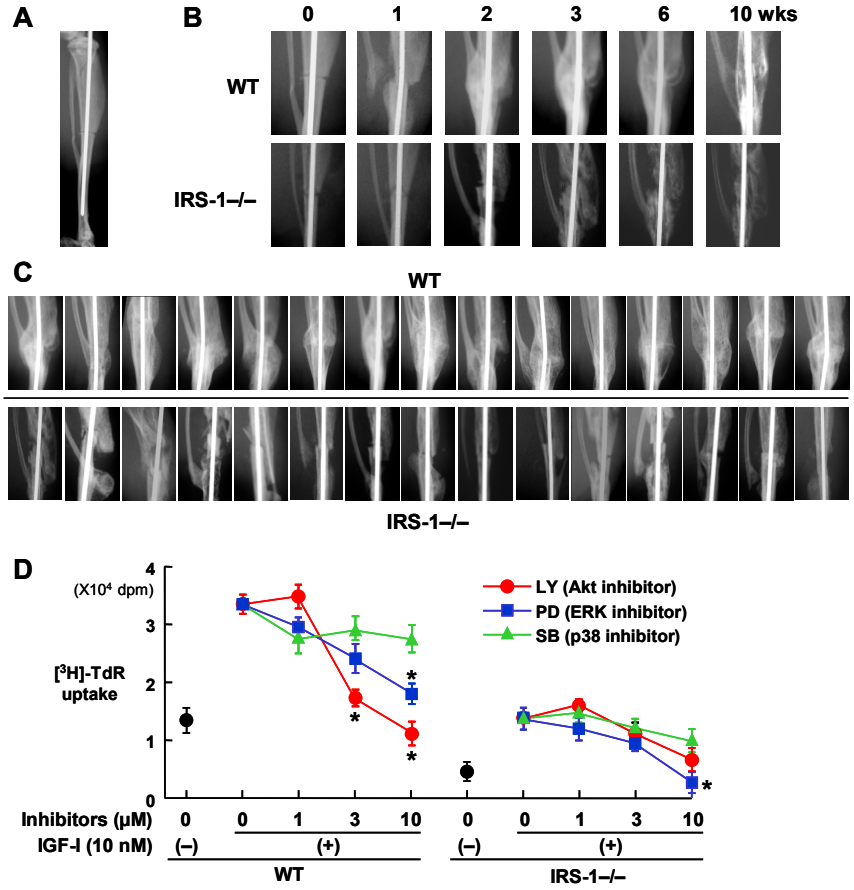

Figure 8. (**A**) The mouse fracture model. (**B**) Time course of fracture healing in representative WT and IRS-1–/– mice. (**C**) Radiographs of fracture sites of all WT mice (n=15) and IRS-1–/– mice (n=15) 3 weeks after the fracture. (**D**) Proliferation of primary osteoblasts determined by [^{3}H]-TdR uptake by IGF-I and specific inhibitors. *p<0.01 vs. IGF-I alone.

To further know the involvement of IRS-1 in bone formation, we compared the healing of bone fracture which was created at the midshaft of mouse tibia and fixed with an intramedullary nail in IRS-1–/– mice (Fig. 8A).[23] After 3 weeks, all 15 WT mice showed bone union, while in IRS-1–/– mice, only four showed bone union but with small fracture callus, and the other 11 mice remained non-union (Fig. 8B & C). Contrarily, the fracture healing was not suppressed in IRS-2 mice (data not shown). In the ex vivo cultures of WT osteoblasts, cell proliferation stimulated by IGF-I was suppressed strongly by an inhibitor of Akt signal, moderately by an ERK inhibitor, but not by an p38 MAPK inhibitor (Fig. 8D). Meanwhile, IRS-1–/– osteoblasts were less respondent to IGF-I and were

inhibited only by the ERK inhibitor. These indicate that the osteoanabolic action of IGF-I/IRS-1 is mainly mediated by the Akt pathway.

Akt1 signal in bone formation and resorption

Akt, a phosphoinositide-dependent serine-threonine protein kinase, is one of the key players in the signal of potent bone anabolic factors. Our analyses of the knockout mice with Akt1, a major Akt in osteoblasts and osteoclasts, revealed that the mice exhibited osteopenia with decreased both bone formation and resorption (Fig. 9A-C).[24] Figure 9D summarizes the mechanisms underlying the Akt1 function to maintain bone mass and turnover which were obtained from further ex vivo cell culture analyses.[24] Briefly, Akt1 suppresses the susceptibility to mitochondria-dependent apoptosis of osteoblasts by inhibiting the transcription factor FoxO3a nuclear entry and the proapoptotic Bim transactivation, and stimulates the differentiation and function by enhancing the Runx2 transcriptional activity, resulting

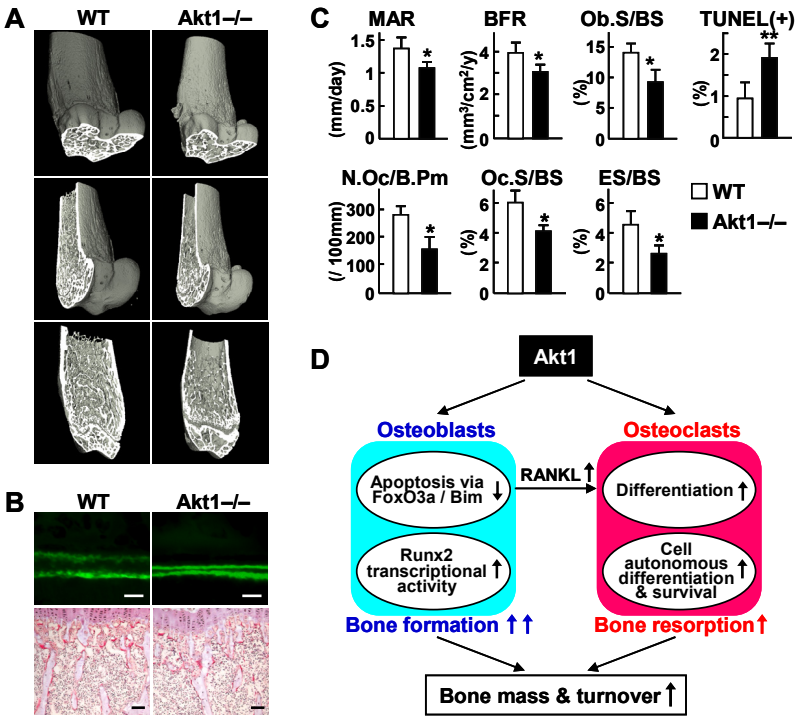

Figure 9. (**A**) 3D-CT of distal femurs of WT and Akt1–/– littermates at 8 weeks of age. (**B**) Calcein double labelings (bars, 20 mm) and TRAP staining (bars, 100 mm) of proximal tibias of the two genotypes. (**C**) Bone histomorphometric analysis of the proximal tibiae. The upper row shows bone formation parameters and the lower row shows bone resorption parameters. *p<0.05, **p<0.01 vs. WT. (**D**) Schematic diagram of the mechanisms underlying the Akt1 function to maintain bone mass and turnover (Details are shown in ref. 24).

in increased bone formation. Akt1 also induces receptor activator of nuclear factor-*k*B ligand (RANKL), a major determinant of osteoclastogenesis, in osteoblasts to support osteoclast differentiation, and shows cell-autonomous effects in osteoclasts to stimulate the differentiation and survival, resulting in increased bone resorption.

Taken together, the insulin-IGF-I / IRS-1-IRS-2 / Akt1 signal may play crucial roles for bone formation under various physiological and pathological conditions. It maintains bone volume and turnover for prevention of osteoporosis, enhances OPLL progression, mediates the PTH bone anabolic action, and facilitates bone fracture healing. Further understanding of the molecular network related to this signal will provide a basis for rational therapeutic targets for these disorders.

Runx2 regulating cartilage degradation in OA

Osteoarthritis (OA), a chronic degenerative joint disorder characterized by articular cartilage degradation and osteophyte formation, is a major cause of disability in aged people. Despite significant social demand for more information, the approved risk factors have to date been limited to age, obesity, trauma history, occupation, and gender.[25] Since these factors are closely related to the accumulation of mechanical loading to joints, mechanical instability of the joints may play a major role in the OA pathogenesis. In efforts to clarify the mechanisms whereby the joint instability leads to OA development, we have established mechanical instability-induced OA experimental models in mice that are reproducible and resemble the human OA, using a microsurgical technique.[26] Our examination of the time course of histology of the mouse joint cartilage using the OA model revealed that type X collagen (COL10), a specific marker of

hypertrophic chondrocytes, appeared in the superficial and middle zones above the tidemark at 4 weeks, and MMP13 expression appeared in the hypertrophic chondrocytes at 8 weeks (Fig. 10).[27] These findings suggest that articular chondrocytes undergo hypertrophic differentiation in response to joint instability, and the hypertrophic chondrocytes express MMP13 that may degrade the cartilage matrix.

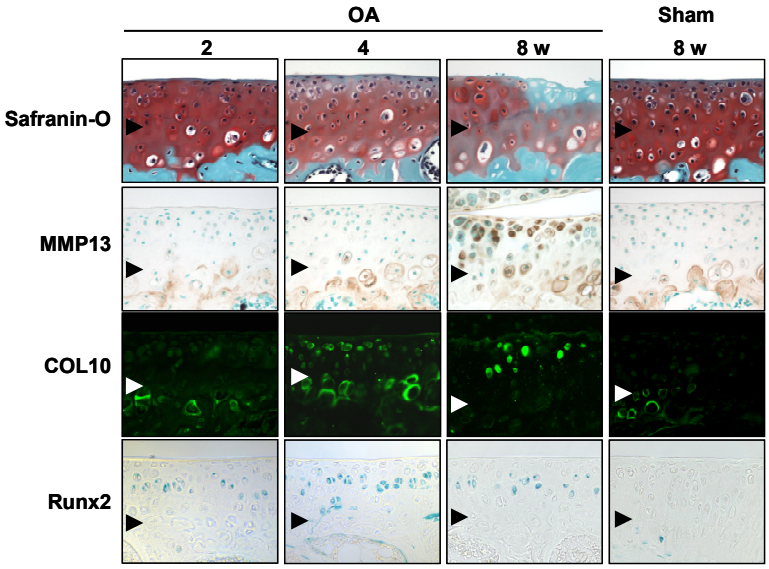

Figure 10. Time course of expressions of MMP13, COL10, and Runx2 in the medial tibial cartilage of OA-induced and sham-operated knee joints in the experimental mouse model. Localization of MMP13 and COL10 was detected by immunohistochemistry, and Runx2 localization was detected by X-gal staining of heterozygous Runx2 deficient mice with LacZ knock-in at the site of Runx2 deletion (Runx2+/lacz). Arrowheads indicate the level of tidemark.

Since a transcriptional activator Runx2 is known to induce both chondrocyte hypertrophy and MMP13 expression,[28,29] we then examined the involvement of Runx2 during OA development.[27] Runx2 expression was induced above the tidemark in the cartilage as early as 2 weeks, enhanced at 4 weeks, and decreased thereafter by the OA induction, which was not observed in the sham-operated cartilage (Fig. 10).

For the functional analyses of Runx2, we used heterozygous Runx2-deficient mice (Runx2+/–), since homozygous Runx2-deficient (Runx2–/–) mice died just after birth. The Runx2+/– mice showed normal skeletal development and articular cartilage under physiological conditions (data not shown). When the OA progression was compared between WT and

Runx2+/– joints, the cartilage degradation in Runx2+/– was much milder than that of the WT cartilage at 8 weeks and thereafter (Fig. 11A).[27] The Runx2+/– joint also showed decreased osteophyte formation (Fig. 11B). These findings demonstrate that Runx2 contributes to cartilage degradation and the subsequent osteophyte formation under the joint instability.

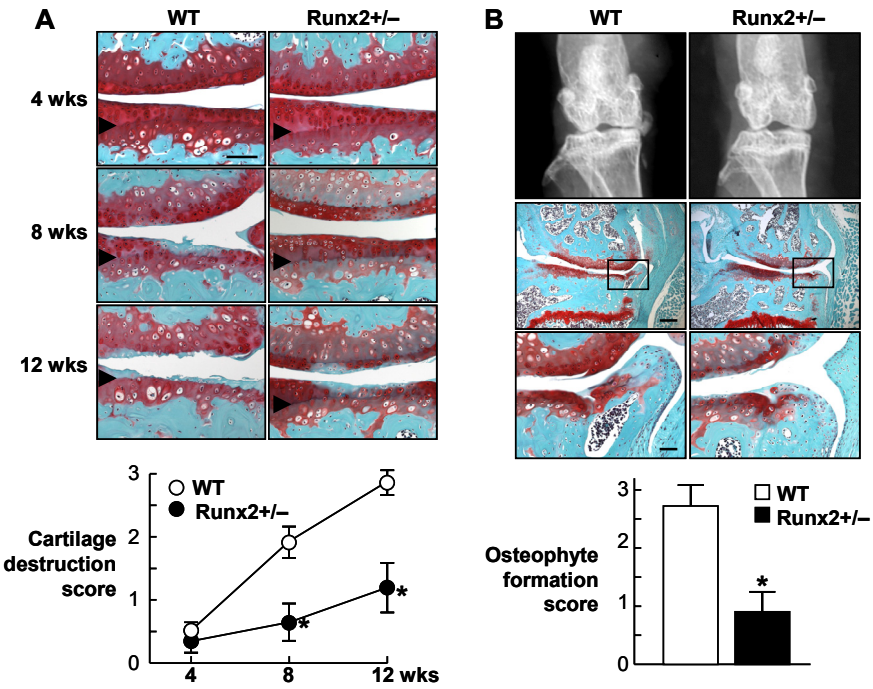

Figure 11. (**A**) Safranin-O staining and the cartilage destruction score of the medial tibial cartilage. Arrowheads indicate the level of tidemark. (**B**) Anteroposterior X-ray features, Safranin-O staining, and the osteophyte formation score 12 weeks after surgery. Means (symbols or bars) ± SEM (error bars). *P<0.01 vs. WT.

In addition to hypertrophic differentiation of chondrocytes, chondrocyte apoptosis is known to be involved in OA development.[30,31] When we created the experimental OA medial model in the hetero-knockout mice of osteoprotegerin, joint destruction was enhanced as compared to the wild-type littermates.[32] On the contrary, an intraarticular injection of recombinant osteoprotegerin suppressed joint destruction with a decrease of apoptotic chondrocytes. Since tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a ligand of osteoprotegerin, induces chondrocyte apoptosis, osteoprotegerin might inhibit the apoptosis induced by TRAIL.

Pro-inflammatry factors like prostaglandins (PGs), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1), and IL-6 have been suggested to be involved in the OA development.[33]

However, our previous report showed that levels of TNF-α, IL-1, and IL-6 in the synovial fluid from knee joints of OA patients were much lower than those of rheumatoid arthritis patients.[34] In addition, although we created the experimental OA model in the knockout mice of microsomal PGES-1 (mPGES-1), a terminal enzyme for the PGE_2 synthesis, the cartilage degradation and osteophyte formation were comparable to the wild-type littemates (Fig. 12).[35] We therefore believe that inflammation may be associated with the OA process as a consequence, but might not have a central role in the cause of OA initiation or progression.

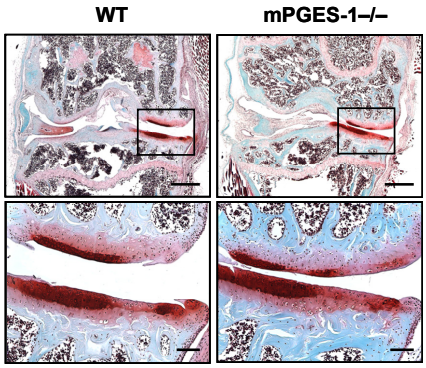

Figure 12. Cartilage degradation in the medial portion of tibial cartilage of 8-week-old wild-type (WT) and mPGES–/– mice in the experimental OA model (Safranin O staining). The inset boxes indicate the regions of the lower panels. Bars, 500 μm (upper) & 50 μm (lower).

Taken together, chondrocyte hypertrophy and apoptosis, which constitute endochondral ossification, play some roles in cartilage degradation during OA development under mechanical stress. Our recent search by the COL10 promoter analyses has so far identified several factors including cyclic GMP-dependent protein kinase II and S100 proteins as potent inducers of endochondral ossification, which may possibly be crucial for the OA development.[36-40]

Carminerin regulating osteophyte formation in OA

Considering that osteophyte of OA is formed by chondrocyte calcification during endochondral ossification, we have been attempting to isolate molecules that are involved in this process. Our differential display analysis identified a novel molecule that was up-regulated by a high phosphate diet in association with calcification of auricular cartilage in the naturally occurring

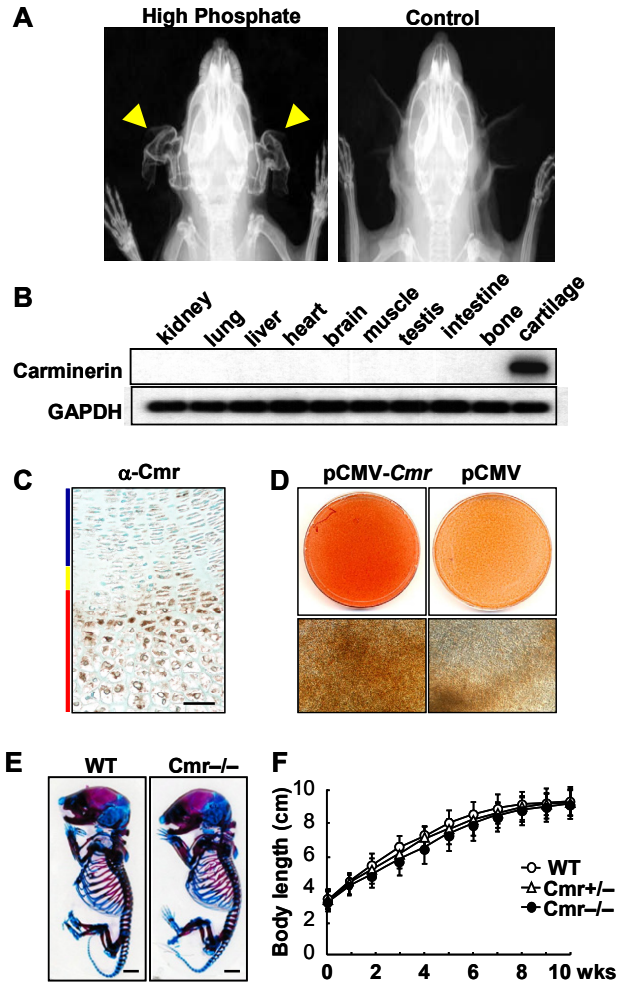

Figure 13. (**A**) Radiographs of ttw mice showing enhanced calcification in the auricular cartilage (arrowheads) by a high phosphate diet. (**B**) Tissue expression of carminerin by RT-PCR. (**C**) Immunohistochemistry of carminerin in the growth plate of an embryonic mouse (E18.5). Blue, yellow, and red bars indicate proliferative, prehypertrophic, and hypertrophic layers, respectively. (**D**) Alizarin red staining of carminerin-overexpressing pCMV-Cmr and the control pCMV/ATDC5 cells. (**E**) The skeleton of WT and Cmr–/– littermate embryos (E17.5) stained with Alizarin red and Alcian blue. (**F**) Growth curves of WT, Cmr+/– and Cmr–/– mice.

mouse mutant ttw (tiptoe walking) with generally enhanced calcification (Fig. 13A).[41] This molecule, termed carminerin, was also called cystatin 10 due to the amino acid sequence containing a cystatin consensus, with about 40% homology to cystatin C. Expression of carminerin was specific to cartilage (Fig. 13B), mainly localized in the cytoplasm of hypertrophic chondrocytes (Fig. 13C). Mouse chondrogenic ATDC5 cells overexpressing carminerin showed an increased calcification, suggesting that carminerin promotes chondrocyte calcification (Fig. 13D). To further investigate the in vivo role of carminerin, we generated mice lacking carminerin (Cmr–/–), and found that the mice developed and grew similarly to WT and Cmr+/– littermates without abnormality of major organs (Fig. 13F & F).[42]

Radiological analyses, however, revealed that Cmr–/– mice experienced decreases in trabecular bones mainly at the metaphysis, as compared to the WT littermates at 8 weeks of age (Fig. 14A). When the OA model was created in the WT and Cmr–/– knee joints, the joint

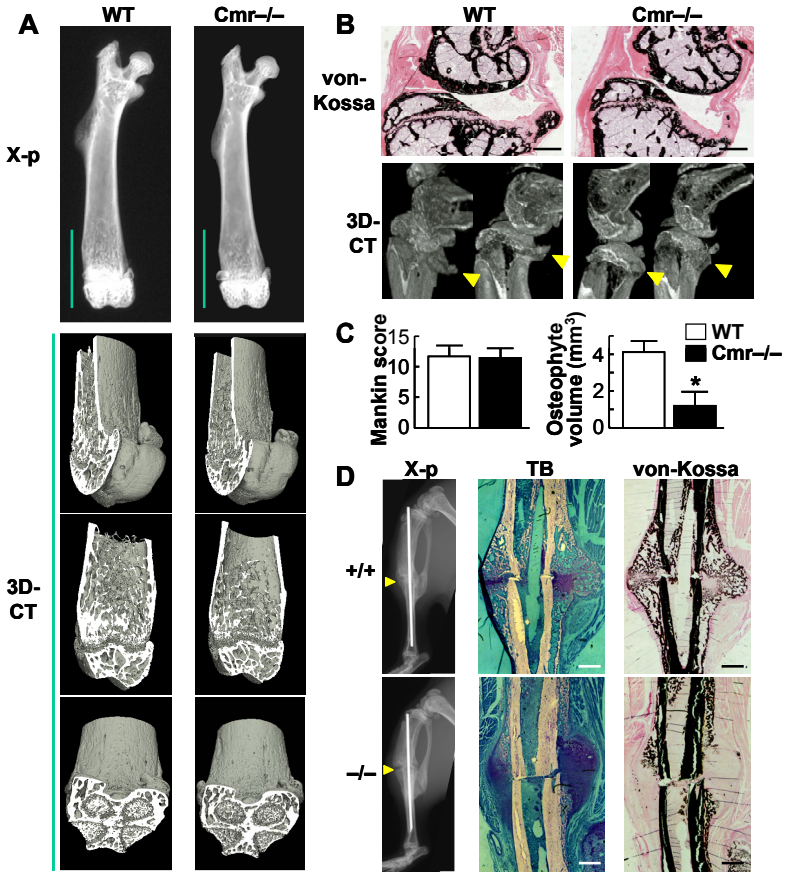

Figure 14. (**A**) Radiographs and 3D-CT of femurs of WT and Cmr–/– littermates under physiological conditions. (**B**) von Kossa staining and 3D-CT of knee joints of the two genotypes in the experimental OA model. (**C**) Quantification of the cartilage destruction and the osteophyte formation in the OA model by Mankin score and the osteophyte volume measured on 3D-CT images, respectively. *P<0.01 vs. WT. (**D**) Radiographs, toluidine blue (TB) and von Kossa stainings 3 weeks after fracture at the tibias.

cartilage degradation was similarly visible at the posterior part of the tibias (Fig. 14B, top), and Mankin's grading score showed comparable values (Fig. 14C, left) in both genotypes. However, osteophyte formation at the posterior tibias shown by von-Kossa staining (Fig. 14B, top) and 3D-CT analysis (Fig. 14B, bottom; Fig. 14C, right) was significantly decreased. These findings indicate that carminerin contributes to osteophyte formation without affecting cartilage degradation.[42] We further examined the involvement of carminerin in bone fracture healing at the midshaft of tibias. Cmr–/– mice showed a bone gap on the radiograph 3 weeks after the fracture, with substantial soft callus formation but impaired calcification (Fig. 14D). This model confirms that the carminerin deficiency impaired endochondral ossification by suppressing chondrocyte calcification.

Figure 15 summarizes our hypothesis of the molecular backgrounds of OA progression under mechanical stress in joints. As the mechanism involved in this stress causing production of proteinases like MMP13, we hereby propose the importance of chondrocyte hypertrophy and apoptosis, essential steps for endochondral ossification that occurs physiologically in the growth plate cartilage.[43] The proteinases produced by hypertrophic chondrocytes cause cartilage degradation at the center of the joint and osteophyte formation at the periphery. The difference of the two sites may depend on the vascularity. At the periphery, vascularity is accessible from synovium or tendon, which causes endochondroal ossification to occur and make osteophytes, just like at the growth plate cartilage. Carminerin may play a role in the chondrocyte calcification at this stage. However, in the center, the vascularity is not accessible from the edge, so that it ends up with cartilage degradation without being replaced by bone.

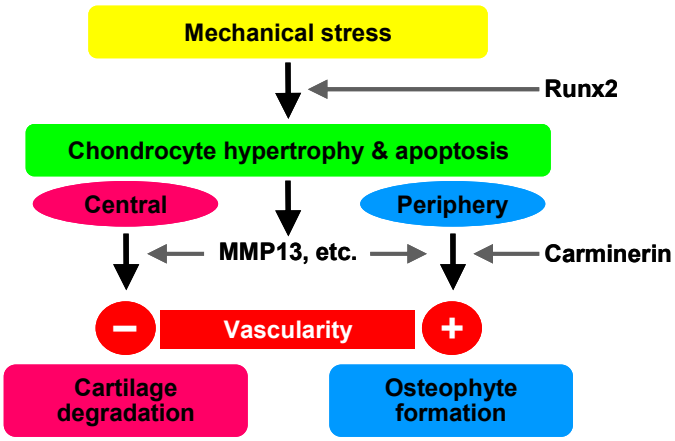

Figure 15. A schema of possible molecular backgrounds of OA under mechanical stress.

Conclusion

This paper has introduced our mouse genetics studies on the molecular backgrounds of osteoporosis, OPLL, fracture healing, and OA. In the mechanism of age-related osteoporosis, we have proposed involvement of PPARγ and the IRS-1-IRS-2/Akt1 signal. These signals may work with putative molecules like vitamin D, estrogen, Runx2 and other osteoanabolic cytokines to

maintain bone mass. One of the next tasks ahead of us will be to elucidate the network system of these many factors. Bone formation seen in OPLL, PTH osteoanabolic action, and fracture healing may also be mediated by the insulin-IGF-I / IRS-1 / Akt signal. For OA, Runx2 and carminerin were shown to be involved in distinct OA features: cartilage degradation and osteophyte formation, respectively.

The ultimate aim of the present study is to identify the molecular targets for clinical treatments of the degenerative skeletal disorders. Although we mainly used the mouse genetics approaches, we have attempted to confirm the reproducibility of the mouse findings in humans using human gene polymorphism or clinical biochemical studies.[44-49] Among the molecules we identified in this study, there are some whose suppression ameliorated skeletal disorders under pathological conditons but did not affect physiological conditions, indicating that targeting on these molecules may lead to an ideal treatment without side effects on physiological functions. In fact, trials based on the present findings are being practically planned for clinical application.

References

1. Yelin E, Callahan LF. 1995. The economic cost and social and psychological impact of musculoskeletal conditions. National Arthritis Data Work Groups. Arthritis Rheum 38: 1351-1362.
2. Matsunaga S, Sakou T. Overview of epidemiology and genetics. In: Yonenobu K, Nakamura K, Toyama Y, editors. OPLL: Ossification of the Posterior Longitudinal Ligament. 2nd ed. Springer; 2006. p. 7-9.

3. Christiansen M, Kveiborg M, Kassem M, Clark BF, Rattan SI. 2000. CBFA1 and topoisomerase I mRNA levels decline during cellular aging of human trabecular osteoblasts. J Gerontol A Biol Sci Med Sci 55: B194-200.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284: 143-147.
5. Meunier P, Aaron J, Edouard C, Vignon G. 1971. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. Clin Orthop 80: 147-154.
6. Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung U, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, and Kawaguchi H. 2004. PPARγ insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 113: 846-855.
7. Kawaguchi H, Akune T, Yamaguchi M, Ohba S, Ogata N, Chung UI, Kubota N, Terauchi Y, Kadowaki T, Nakamura K. 2005. Distinct effects of PPARγ insufficiency on bone marrow cells, osteoblasts, and osteoclastic cells. J Bone Miner Metab 23: 275-279.
8. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Kadowaki T. 1999. PPARγ gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol Cell 4: 597-609.
9. Ogawa S, Urano T, Hosoi T, Miyao M, Hoshino S, Fujita M, Shiraki M, Orimo H, Ouchi Y, Inoue S. 1999. Association of bone mineral density with a polymorphism of the peroxisome

proliferator-activated receptor gamma gene: PPARγgamma expression in osteoblasts. Biochem Biophys Res Commun 260: 122-126.

10. Harada S, Rodan GA. 2003. Control of osteoblast function and regulation of bone mass. Nature 423: 349-355.
11. Rosen CJ. 1994. Growth hormone, insulin-like growth factors, and the senescent skeleton: Ponce de Leon's Fountain revisited? J Cell Biochem 56: 348-356.
12. Laron Z, Klinger B, Silbergeld A. 1999. Patients with Laron syndrome have osteopenia/osteoporosis. J Bone Miner Res 14: 156-157.
13. Kadowaki T, Tobe K, Honda-Yamamoto R, Tamemoto H, Kaburagi Y, Momomura K, Ueki K, Takahashi Y, Yamauchi T, Akanuma Y, Yazaki Y. 1996. Signal transduction mechanism of insulin and insulin-like growth factor-1. Endocr J 43: S33-S41.
14. Ogata N, Chikazu D, Kubota N, Terauchi Y, Tobe T, Azuma Y, Ohta T, Kadowaki T, Nakamura K, and Kawaguchi H. 2000. Insulin receptor substrate-1 in osteoblast is indispensable for maintaining bone turnover. J Clin Invest 105: 935-943.
15. Akune T, Hoshi K, Kubota Y, Terauchi K, Tobe Y, Azuma T, Ohta T, Nakamura K, and Kawaguchi H. 2002. Insulin receptor substrate-2 maintains predominance of anabolic function over catabolic function of osteoblasts. J Cell Biol 159: 147-156.
16. Kawaguchi H. 2006. Molecular backgrounds of age-related osteoporosis from mouse genetics approaches. Rev Endocr Metab Disord 7: 17-22.
17. Thrailkill KM, Lumpkin CK Jr, Bunn RC, Kemp SF, Fowlkes JL. 2005. Is insulin an anabolic agent in bone? Dissecting the diabetic bone for clues. Am J Physiol Endocrinol Metab 289: E735-745.

18. Shingyouchi Y, Nagahama A, Niida M. 1996. Ligamentous ossification of the cervical spine in the late middle-aged Japanese men. Its relation to body mass index and glucose metabolism. Spine 21: 2474-2178.

19. Akune T, Ogata N, Seichi A, Ohnishi I, Nakamura K, and Kawaguchi H. 2001. Insulin secretory response is positively associated with the extent of ossification of the posterior longitudinal ligament of the spine. J Bone Joint Surg (Am) 83: 1537-1544.

20. Kawaguchi H, Akune T, Ogata N, Seichi A, Takeshita K, and Nakamura K: Contribution of metabolic conditions to ossification of the posterior longitudinal ligament of the spine. In: Yonenobu K, Nakamura K, Toyama Y, editors. OPLL: Ossification of the Posterior Longitudinal Ligament. 2nd ed. Springer; 2006. p. 37-40.

21. Canalis E. 1993. Insulin like growth factors and the local regulation of bone formation. Bone 14:273–276.

22. Yamaguchi M, Ogata N, Shinoda Y, Akune T, Kamekura S, Terauchi Y, Kadowaki T, Hoshi K, Chung UI, Nakamura K, and Kawaguchi H. 2005. Insulin receptor substrate-1 is required for bone anabolic function of parathyroid hormone in mice. Endocrinology 146: 2620-2628.

23. Shimoaka T, Kamekura S, Chikuda H, Hoshi K, Chung U, Akune T, Maruyama Z, Komori T, Matsumoto M, Ogawa W, Terauchi Y, Kadowaki T, Nakamura K, and Kawaguchi H. 2004. Impairment of bone healing by insulin receptor substrate-1 deficiency. J Biol Chem 279: 15314-15322.

24. Kawamura N, Kugimiya F, Oshima Y, Ohba S, Ikeda T, Saito T, Shinoda Y, Kawasaki Y, Ogata N, Hoshi K, Akiyama T, Chen WS, Hay N, Tobe T, Kadowaki T, Azuma Y, Tanaka

S, Nakamura K, Chung UI, and Kawaguchi H. 2007. Akt1 in osteoblasts and osteoclasts controls bone remodeling. PLoS ONE 2: e1058.

25. Sharma L, Kapoor D. Epidemiology of osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg VM, editors. Osteoarthritis: Diagnosis and Medical/Surgical Management. 4th ed. Lippincott Williams & Wilkins; 2007. p. 3-26.
26. Kamekura S, Hoshi K, Shimoaka T, Chung UI, Chikuda H, Yamada T, Uchida M, Ogata N, Seichi A, Nakamura K, and Kawaguchi H. 2005. Osteoarthritis development in novel experimental mouse models induced by knee joint instability. Osteoarthritis Cartilage 13: 632–641.
27. Kamekura S, Kawasaki Y, Hoshi K, Shimoaka T, Chikuda H, Maruyama Z, Komori T, Sato S, Takeda S, Karsenty G, Nakamura K, Chung UI, and Kawaguchi H. 2006. Runx2 contributes to pathogenesis of osteoarthritis in mice after induction of knee joint instability. Arthritis Rheum 54: 2462–2470.
28. Takeda S, Bonnamy JP, Owen MJ, Ducy P, Karsenty G. 2001. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. Genes Dev 15: 467–481.
29. Jimenez MJ, Balbin M, Lopez JM, Alvarez J, Komori T, Lopez-Otin C. 1999. Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol 19: 4431–4442.
30. Kühn K, D'Lima DD, Hashimoto S, and Lotz M. 2004. Cell death in cartilage. Osteoarthritis Cartilage 12: 1-16.

31. D'Lima D, Hermida J, Hashimoto S, Colwell C, and Lotz M. 2006. Caspase inhibitors reduce severity of cartilage lesions in experimental osteoarthritis. Arthritis Rheum 54: 1814-21.

32. Shimizu S, Asou Y, Itoh S, Chung, UI, Kawaguchi H, Shinomiya K, and Muneta T. 2007. Prevention of cartilage degradation with intraarticular osteoclastogenesis inhibitory factor/osteoprotegerin in a murine model of osteoarthritis. Arthritis Rheum 56, 3358-3365.

33. Vincenti MP and Brinckerhoff CE. 2002. Transcriptional regulation of collagenase (MMP-1. MMP-13) genes in arthritis, integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res 4: 157–164.

34. Manabe N, Oda H, Nakamura K, Kuga Y, Uchida S, and Kawaguchi H. 1999. Involvement of fibroblast growth factor-2 in joint destruction of rheumatoid arthritis patients. Rheumatology (Oxford). 38: 714–720.

35. Yamakawa K, Kamekura S, Kawamura N, Saegusa M, Kamei D, Murakami M, Kudo I, Uematsu S, Akira S, Chung UI, Nakamura K, and Kawaguchi H. 2008. Association of microsomal prostaglandin E synthase 1 deficiency with impaired fracture healing, but not with bone loss or osteoarthritis, in mouse models of skeletal disorders. Arthritis Rheum 58: 172-183.

36. Chikuda H, Kugimiya F, Hoshi K, Ikeda T, Ogasawara T, Shimoaka T, Kawano H, Kamekura S, Tsuchida A, Yokoi N, Nakamura K, Komeda K, Chung UI, and Kawaguchi H. 2004. Cyclic GMP-dependent protein kinase II is a molecular switch from proliferation to hypertrophic differentiation of chondrocytes. Genes Dev 18: 2418-2429

37. Chikuda H, Kugimiya F, Hoshi K, Ikeda T, Ogasawara T, Kamekura S, Ogata N, Nakamura K, Chung UI, and Kawaguchi H. 2005. Mutation in cGMP-dependent protein kinase II causes dwarfism in a rat mutant KMI through uncoupling of proliferation and differentiation of chondrocytes. J Bone Miner Metab 23: 200-204.

38. Kugimiya F, Chikuda H, Kamekura S, Ikeda T, Hoshi K, Ogasawara T, Nakamura K, Chung UI, and Kawaguchi H. 2005. Involvement of cyclic guanosine monophosphate-dependent protein kinase II in chondrocyte hypertrophy during endochondral ossification. Mod Rheumatol 15: 391-396.

39. Saito T, Ikeda T, Nakamura K, Chung UI, and Kawaguchi H. 2007. S100A1 and B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. EMBO Rep 8: 504-509.

40. Kawasaki Y, Kugimiya F, Chikuda H, Kamekura S, Ikeda T, Kawamura N, Saito T, Shinoda Y, Higashikawa A, Yano F, Ogasawara T, Ogata N, Hoshi K, Hofmann F, Woodgett JR, Nakamura K, Chung UI, and Kawaguchi H. Phosphorylation of GSK-3β by cGMP-dependent protein kinase II promotes hypertrophic differentiation of murine chondrocytes. J Clin Invest (in press).

41. Koshizuka Y, Yamada T, Hoshi K, Ogasawara T, Chung U, Kawano H, Nakamura Y, Nakamura K, Ikegawa S, and Kawaguchi H. 2003. Cystatin 10, a novel chondrocyte-specific protein, may promote the last steps of the chondrocyte differentiation pathway. J Biol Chem 278: 48259-48266.

42. Yamada T, Kawano H, Koshizuka Y, Fukuda T, Yoshimura K, Kamekura S, Saito T, Ikeda T, Kawasaki Y, Azuma Y, Ikegawa S, Hoshi K, Chung UI, Nakamura K, Kato S, and

Kawaguchi H. 2006. Carminerin contributes to chondrocyte calcification during endochondral ossification. Nature Med 12: 665-670.

43. Kawaguchi H. 2008. Endochondral ossification signals in cartilage degradation during osteoarthritis progression in experimental mouse models. Mol Cells 25: 1-6.
44. Ogata N, Shiraki M, Hosoi T, Koshizuka Y, Nakamura K, and Kawaguchi H. 2001. A polymorphic variant at the Werner helicase (wrn) gene is associated with bone density, but not spondylosis, in postmenopausal women. J Bone Miner Metab 19: 296-301.
45. Koshizuka Y, Kawaguchi H, Ogata N, Ikeda T, Mabuchi A, Seichi A, Nakamura Y, Nakamura K, and Ikegawa S. 2002. Nucleotide pyrophosphatase gene polymorphism associated with ossification of the posterior longitudinal ligament of the spine. J Bone Miner Res 17: 138-144.
46. Ogata N, Matsumura Y, Shiraki M, Kawano K, Koshizuka Y, Hosoi T, Nakamura K, Kuro-o M, and Kawaguchi H. 2002. Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. Bone 31: 37-42.
47. Ogata N, Koshizuka Y, Miura T, Iwasaki M, Hosoi T, Shiraki M, Seichi A, Nakamura K, and Kawaguchi H. 2002. Association of bone metabolism regulatory factor gene polymorphisms with susceptibility to ossification of the posterior longitudinal ligament of the spine and its severity. Spine 27: 1765-1771.
48. Kawano K, Ogata N, Chiano M, Molloy H, Kleyn P, Spector T D, Uchida M, Hosoi T, Suzuki T, Orimo H, Inoue S, Nabeshima Y, Nakamura K, Kuro-o M, and Kawaguchi H. 2002. Klotho gene polymorphisms associated with bone density of aged postmenopausal women. J Bone Miner Res 17: 1744-1751.

49. Koshizuka Y, Ogata N, Shiraki M, Hosoi T, Seichi A, Takeshita K, Nakamura K, and Kawaguchi H. 2006. Distinct association of gene polymorphisms of estrogen receptor and vitamin D receptor with lumbar spondylosis in postmenopausal women. Eur Spine J 15: 1521-1528.