The trabecular BMD in the tibias from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4–6 weeks after birth (Fig. 1*A*). In contrast, the cortical BMD in the tibias from the $Plg^{-/-}$ mice was significantly lower than that from the $Plg^{+/+}$ mice at 4–18 weeks after birth (Fig. 1*B*). Therefore, the decrease in the trabecular BMD in $Plg^{-/-}$ mice seemed to be transient; however, the decrease in the cortical BMD in the mice was consistently observed from the juvenile growth period to adulthood. In addition, TRAP staining of decalcified sections of tibias from the 5-we-old mice revealed that the intensity of TRAP staining of bone marrow tissue in the tibias from the $Plg^{-/-}$ mice was significantly stronger than that from the $Plg^{+/+}$ mice (Fig. 1, *E* and *F*). Thus, the histoenzymatic assessment indicated that the OC differentiation in bone marrow tissue of the $Plg^{-/-}$ mice might be more vigorously induced than that in the $Plg^{+/+}$ mice.

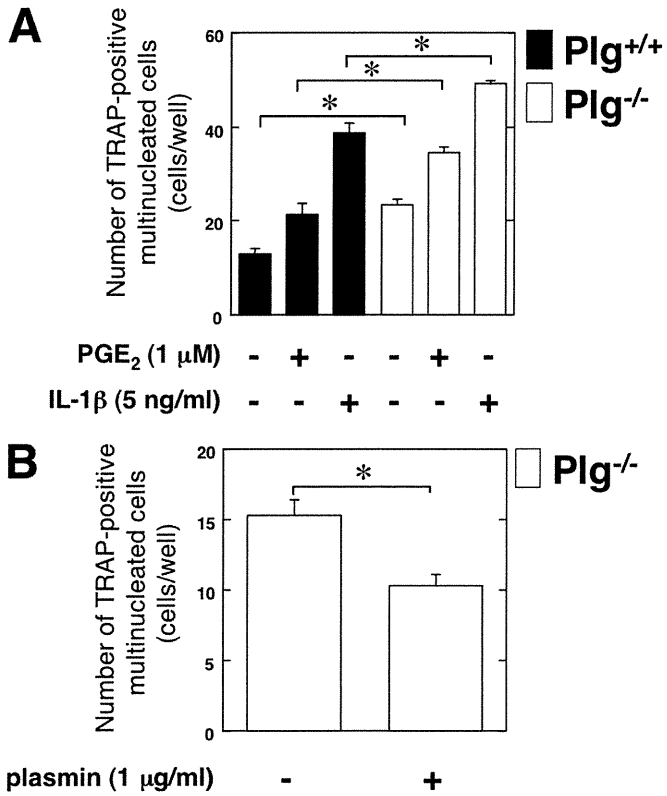

FIGURE 4. **Effects of Plg deficiency on the ability of OBs to induce osteoclastogenesis of RAW264.7 cells.** *A*, RAW264.7 cells and OBs from the $Plg^{+/+}$ and $Plg^{-/-}$ mice were co-cultured for 3 days in the absence or presence of IL-1β or PGE_2. *B*, RAW264.7 cells and OBs from the $Plg^{-/-}$ mice were co-cultured for 3 days in the absence or presence of plasmin. Mature OCs were identified as multinucleated TRAP-positive cells. The number of multinucleated TRAP-positive cells was determined from six different cultures. The data represent the mean ± S.E. *, $p < 0.01$.

The binding of RANKL to its receptor RANK triggers intricate and distinct signaling cascades that control lineage commitment and osteoclast activation (13). OPG inhibits osteoclast formation and bone resorption by blocking RANKL/RANK interactions (14). This study showed that plasmin increased the OPG expression in WT OBs (Fig. 3, *A–D*). Moreover, the expression level of OPG was decreased in $Plg^{-/-}$ OBs compared with $Plg^{+/+}$ OBs (Fig. 3, *G* and *H*), suggesting that absence of plasmin may result in an acceleration of OB-mediated osteoclastogenesis of pre-OCs in accordance with the depletion of OPG expression in OBs. In fact, the number of TRAP-positive multinucleated RAW264.7 cells co-cultured with $Plg^{-/-}$ OBs was significantly higher than that of the cells co-cultured with $Plg^{+/+}$ OBs (Fig. 4*A*). Intriguingly, plasmin significantly inhibited the M-CSF- and RANKL-induced OC differentiation of bone marrow cells derived from the $Plg^{+/+}$ and $Plg^{-/-}$ (Fig. 2*D*), suggesting that plasmin might attenuate osteoclastogenesis by its direct effects on pre-OCs. In addition, there was a larger population of pre-OCs in bone marrow-derived cells from the $Plg^{-/-}$ mice in comparison with the $Plg^{+/+}$ mice (Fig. 2, *A–C*). The level of ALP activity in $Plg^{-/-}$ OBs was similar to that in $Plg^{+/+}$ OBs (Fig. 5), thus suggesting that the bone-mineralizing activity of OBs in the $Plg^{-/-}$ mice might be comparable with that in the $Plg^{+/+}$ mice. Consequently, the $Plg^{-/-}$ mice display decreased bone mineral density in accordance with the enhanced ability of OBs to induce osteoclastogenesis of pre-OCs, the loss of the direct and suppressive effect of plasmin on pre-OCs differentiating into mature OCs, and the increased pre-OC population in bone marrow cells. In fact, the injection of plasmin into the $Plg^{-/-}$ mice clearly rescued the diminished trabecular BMD during the juvenile growth period (Fig. 6).

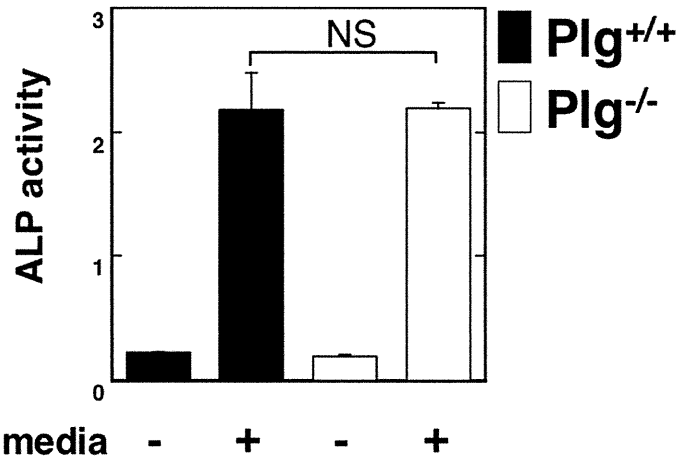

FIGURE 5. **Effect of Plg deficiency on the ALP activity in OBs.** ALP activity in OBs from the $Plg^{+/+}$ and $Plg^{-/-}$ mice was evaluated ($n = 4$). The data represent the mean ± S.E. *NS*, not significant.

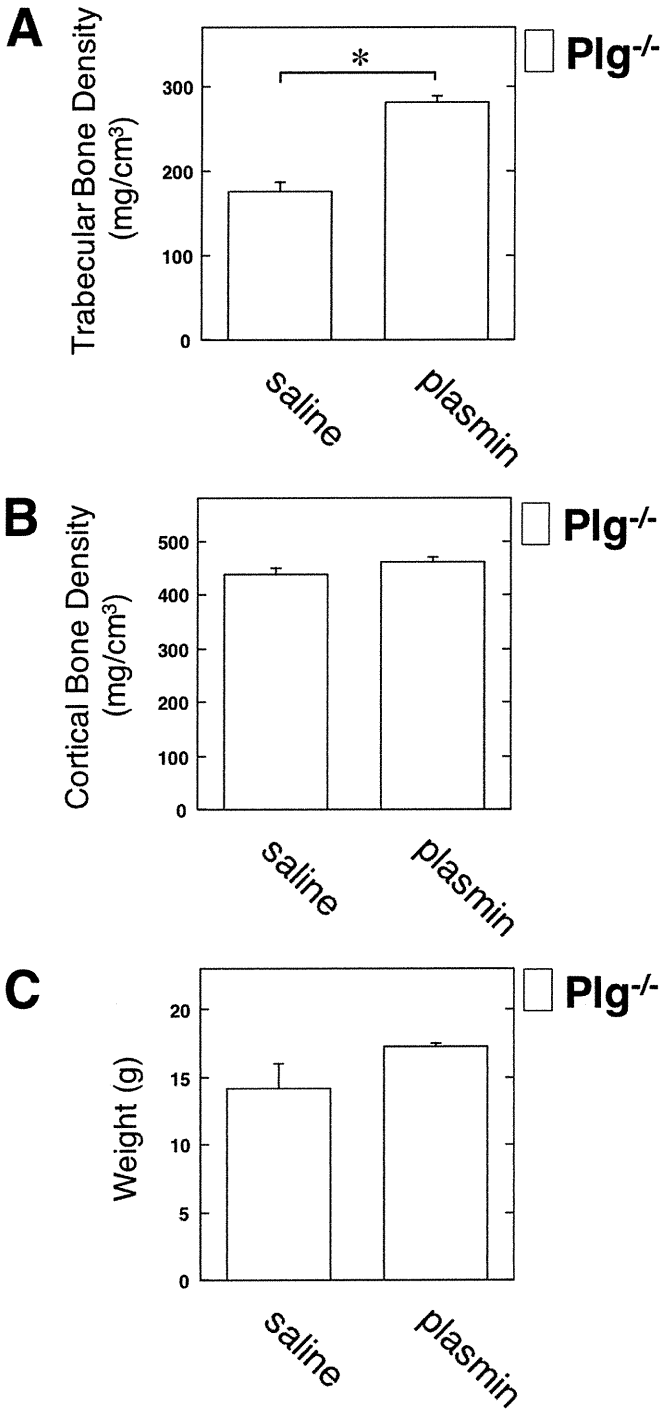

FIGURE 6. **Rescue of the down-regulated BMD in Plg-deficient mice by the injection of plasmin.** Intraperitoneal injection with saline or plasmin (1 mg/kg) in the 5-week-old male $Plg^{-/-}$ mice was carried out weekly for up to 3 weeks. Then the trabecular BMD (*A*), the cortical BMD (*B*), and the weight (*C*) in the male $Plg^{-/-}$ mice were measured by pQCT ($n = 3$). The data represent the mean ± S.E. *, $p < 0.01$.

Plasmin activates a latent transforming growth factor β (TGF-β) (24, 25) trapped in extracellular matrix to induce an OPG expression in extracellular matrix-harbored OBs. The accelerated expression of OPG on OBs might result in the suppression of the OB-mediated osteoclastogenesis. It is under investigation by us whether deficiency of activated TGF-β causes decreased bone mineral density and decreased body weight in $Plg^{-/-}$ mice. However, plasmin directly activates various intracellular signaling through annexin A2 in macrophage (26). Plasmin activates macrophages via the annexin A2 heterotetramer composed of annexin A2 and S100A10 with subsequent stimulation of Janus kinase JAK1/TYK2 signaling. JAK1/TYK2 leads to STAT3 activation, Akt-dependent nuclear factor-κB (NF-κB) activation, and phosphorylation of ERK1/2 and p38 MAPK. Furthermore, inhibitors of JAK, p38 MAPK, and NF-κB revealed that these signaling pathways are indispensable for the plasmin-mediated tumor necrosis factor-α and IL-6 induction in the cells. In addition, angiostatin, a fragment of plasmin(ogen), is a ligand and an antagonist for integrin α9β1 (27). Angiostatin, representing the kringle domains of plasmin, alone did not induce the migration of Chinese hamster ovary (CHO) cells, but simultaneous activation of the G protein-coupled protease-activated receptor-1 with an agonist peptide induced the migration on angiostatin. These facts suggest that plasmin directly stimulates various cell lineages without an indirect cell stimulation through an activation of some growth factors such as TGF-β. We showed that plasmin activated ERK1/2 and p38 MAPK, and the inhibition of ERK1/2 and p38 MAPK attenuated plasmin-induced OPG expression (Fig. 3, *E* and *F*). In addition, plasmin activated JNK, but the inhibition of JNK did not attenuate plasmin-induced OPG expression (data not shown). These data suggest that plasmin induces OPG expression through the ERK1/2 and p38 MAPK pathways. However, the time lag between the activation of p38 MAPK and ERK1/2 after plasmin stimulation in OBs might depend on the hierarchy of ERK1/2 and p38 MAPK in the plasmin-induced signal transduction. The ERK1/2 might be the downstream target of p38 MAPK directly activated by plasmin in OBs. Further investigations would be required to clarify the details.

These results strongly suggest that the plasmin activity regulates both OB and OC functions and then plays an important role in bone metabolism. These findings may provide new insights into the development of clinical therapies for the prevention of bone loss-related disorders.

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