INTRODUCTION: Hedgehog proteins are essential morphogens and hedgehog signaling pathway plays an important role in embryonic development. Hedgehog signaling pathway is activated by the binding of the hedgehog protein to the transmembrane receptor PTCH. In the absence of hedgehog protein, PTCH suppresses the second transmembrane protein SMO. Binding of hedgehog to PTCH inhibits suppression, and then SMO is activated. Activated SMO stabilizes the transcription factor GLI and GLI promotes the expression of target genes. Recent studies have shown that constitutive activation of hedgehog signaling promotes various types of malignancies, including breast cancer, lung cancer, prostate cancer, B cell lymphoma, and medulloblastoma. However, it remains unclear whether hedgehog pathway is activated in human osteosarcoma. In an attempt to better understand osteosarcoma pathogenesis, we investigated the expression and activation of hedgehog related proteins in osteosarcoma and examined the effect of inhibition to osteosarcoma growth by using the specific inhibitor cyclopamine, Smo shRNA and Gli2 shRNA. And also, we examined the molecular mechanism of osteosarcoma growth inhibition.

Materials and Methods: Human osteosarcoma cell lines and human osteoblast cells (NHOst) were used. RT-PCR: we investigated the expression of hedgehog related genes in both osteosarcoma cell lines and human specimens. Immunohistochemistry: we investigated the expression of hedgehog related proteins in both osteosarcoma cell lines and human specimens. MTT assay: to confirm the suppression of osteosarcoma growth by the hedgehog pathway inhibition, we performed MTT assay using cyclopamine and Smo or Gli2 shRNA. Cell cycle analysis and Ki67 staining: to examine the relationship between hedgehog pathway and cell cycle in vitro and in vivo, we performed cell cycle analysis by flow cytometry and Ki67 staining. Quantitative RT-PCR and western blot: to investigate changes in expression of hedgehog related genes and proteins by cyclopamine treatment, we performed quantitative RT-PCR and western blot. Animal experiments: 143B cells (1 × 10⁶) were mixed with a collagen gel at a 1:1 volume, and were inoculated subcutaneously in the 5-week-old nude mice. Cyclopamine(10 25mg/kg) were administrated by intraperitoneal injection. The tumor size was measured with calipers weekly, and tumor volume was calculated by a formula of LW²/2 (L and W represent the length and width of tumors). And also, Smo or Gli2 shRNA transfected osteosarcoma cell were inoculated subcutaneously.

RESULTS : RT-PCR revealed high expression of hedgehog related genes in both osteosarcoma cell lines and human specimens. Immunohistochemistry showed high expression of hedgehog related proteins in both osteosarcoma cell lines and human specimens. These results suggested that the components of hedgehog pathway were overexpressed in human osteosarcoma. Moreover, MTT assay showed that cyclopamine and Smo or Gli2 shRNA suppressed the growth of the osteosarcoma cell lines in vitro. Next we performed cell cycle analysis by flowcytometry. As a result, cyclopamine promoted G1 arrest in vitro. Next we examined the transcription of the cell cycle related genes which transcription was regulated in cell cycle. Real time RT-PCR and western blot revealed that cyclopamine suppressed transcription of cyclin D1, cyclin E1, Skp2, phosphorylated Rb. And also, P21^{WAF} protein was upregulated by cyclopamine treatment. These data suggest that cyclopamine suppressed osteosarcoma growth and promoted G1 arrest by regulation of cell cycle regulators expression. In xenograft model, we showed 25mg/kg of cyclopamine treatment reduced the Ki67 positive cells. These data suggest that inhibition of hedgehog signaling prevents osteosarcoma growth by cell cycle regulation. But, all mice died by undetermined reason after 1 month cyclopamine treatment. Next we performed 10mg/kg cyclopamine treatment, there was no difference of osteosarcoma growth between cyclopamine treatment and control group. Unfortunately, we could not achieve a therapeutic dose in 143B xenograft model. On the other hand, xenograft model showed that transfection of Gli2 shRNA prevented osteosarcoma growth compared to control.