Canine osteosarcoma (OS) is an aggressive primary bone tumor, accounting for up to 85% of malignancies originating from the skeleton. Despite of advances in management modalities for canine OS, prognosis remains poor because almost 90% of dogs develop pulmonary metastasis which is the main cause of death.

Xenograft models of canine OS cells injected into immunocompromised mice are necessary for greater understanding of the biology of metastasis to improve the outcome for canine OS patients. These models can demonstrate the interaction between cancer cells and the surrounding microenvironment that is necessary for metastasis. Moreover, they also provide opportunities to evaluate novel therapeutics.

Ezrin (cytovillin/p81/80k/Villin-2), a member of the ezrin–radixin–moesin (ERM) proteins family, was identified as a key molecule during the onset and progression of the metastatic cascade in a murine OS model. Phosphorylated ezrin, an active form, was dynamically regulated in the process of murine and human OS metastasis by protein kinase C (PKC). Ezrin activation allows the tumor cells to
interact with tumor microenvironment through the link between the transmembrane receptor and the actin cytoskeleton. This process is essential for many fundamental cellular processes and integration of membrane transport with signaling pathways, such as MAPK-signaling pathway. Recently, most studies report that ezrin is associated with tumor progression and metastasis in human and murine OS. Moreover, the mechanism of ezrin activity with tumor cellular processes during metastatic cascade is still unclear.

The objective of this study was to determine the relationships between malignant behaviors and ezrin activity in canine OS.

In chapter 1, expression of ezrin and phosphorylated ERM (p-ERM) was investigated in primary tumor tissues of canine OS patients. Immunohistochemistry for ezrin and p-ERM was performed on primary tumor tissues of 15 canine OS patients surgically collected at Veterinary Medical Center of the University of Tokyo between 2006 and 2010. Correlations of the expression of ezrin and p-ERM with the clinical data and proliferation index (PI) on immunohistochemistry of Ki-67 were evaluated.

The result demonstrated that ezrin and p-ERM expression was found in all primary tissues of canine OS. High expression of ezrin and p-ERM was observed in 13 of 15 patients. The expression of ezrin was significantly correlated with p-ERM ($r=0.9932$, $p=0.0000$), but not with proliferation index ($r=0.2640$, $p=0.3417$). There were no significant correlations between high expression of ezrin and p-ERM and age, gender, breed, body weight, primary location site, histological type, serum ALP, and lung metastasis. The survival curves of patients with high and low ezrin, p-ERM, and Ki-67 expressions did not show significant differences; however, the survival curve of mice with high expression of ezrin and p-ERM tended to be worse than those with the low expression. The results suggested that expression of ezrin and p-ERM may be associated with the malignant behavior of canine OS.

In chapter 2, the effects of microenvironment at transplantation sites on the growth and metastasis in a canine OS cells-xenografted mouse model were analyzed.

In section 1, to investigate the expression of ezrin and p-ERM on 3 canine OS cell lines (HMPOS, OOS, and CHOS), Western blot analysis and immunocytochemistry were performed. Ezrin was expressed on all these cell lines, however p-ERM was not expressed on CHOS cell line by both Western blot analysis and immunocytochemistry.

Those 3 OS cell lines were transplanted via subcutaneous (SC), intratibial (IT), and intravenous (IV) injection into 5-week-old female BALB/c nude mice with 5 mice per group. The primary tumor volumes, number of lung metastatic nodules, and expression of ezrin and p-ERM were evaluated on immunohistochemistry of primary tumors and lung metastatic tissues at 1, 2, and 4 weeks after transplantation.
IT xenografts exhibited greater potential for developing primary masses and pulmonary metastasis than SC xenografts. The expression of ezrin and p-ERM in the primary tumors of IT-xenografted mice was significantly higher \( (p<0.05) \) than those in SC-xenografted mice with HMPOS and OOS cells. In IT and IV xenografts, lung micrometastases along with p-ERM overexpression were found in mice xenografted with HMPOS and OOS cells after 1 week. But decreased p-ERM expression was found at the later time points in these mice. The results suggested that the orthotopic transplantation site was associated with the primary tumor growth and the spontaneous metastasis of canine OS. In addition, ezrin phosphorylation may be involved in the early metastatic mechanism of canine OS cells.

In section 2, relationships between ezrin and p-ERM expression and PI, Ras/Raf/ERK MAPK pathway, and PKCa in tissues developed in xenografted mice were evaluated.

The expressions of molecules in Ras/Raf/ERK MAPK pathway (Ras, B-Raf, C-Raf, p-ERK1/2, and ERK1/2) and PKCa were investigated in OS cell lines on immunocytochemistry and Western blot analysis. In addition, Ki-67, Ras, B-Raf, C-Raf, p-ERK1/2 and PKCa in xenograft mouse tissues were investigated on immunohistochemistry.

High metastatic HMPOS and OOS cells expressed high B-Raf and C-Raf but low p-ERK1/2 when compared to non-metastatic CHOS cells. The PI, Ras, C-Raf, and p-ERK1/2 in primary tissues of IT-xenografted mice increased in a time-dependent manner and were significantly higher \( (p<0.05) \) than those of SC-xenografted mice in the later progression time after transplantation, relating to tumor growth. These results suggested that ezrin in phosphorylated form may be involved in a short time at the beginning step for signal transduction of MAPK pathway and then dephosphorylated form may be involved in the later time for proliferation of OS.

The expression of PKCa in primary tissues of IT-xenografted mice was significantly higher \( (p<0.05) \) than that of SC-xenografted mice at the earlier time of progression and was significantly correlated with expression of ezrin and p-ERM. Moreover, expression of p-ERM and PKCa was low in the central portions of the primary and metastatic lesions in progression time at 4 weeks after transplantation. This result suggested that activated ezrin may be associated with PKCa.

In chapter 3, the effect of suppression of p-ERM in canine OS cells by a PKC inhibitor was investigated in both \textit{in vitro} and \textit{in vivo} orthotopic xenografted mouse model.

Chelerythrine (CHE) was used as a PKC inhibitor. Cell viability MTT assay was performed for HMPOS, OOS, and CHOS cell lines treated with CHE at 0.5, 1, 1.25, 2.5, 5, 10 and 20 \( \mu \)M for 24 hours. Cell motility using wound-healing, transwell, and \textit{xCELLigence} real time migration assays as well as cell
invasion using matrigel invasion and xCELLigence real time invasion assays were also performed for those cells treated with the dosage of CHE without affecting cell viability. The expression of PKCα, ezrin, p-ERM, Ras, B-Raf, C-Raf, p-ERK1/2 and ERK1/2 was investigated in HMPOS cells by Western blot analysis.

The IC50 values of CHE were 7.42, 7.10, and 7.56 μM for HMPOS, OOS, and CHOS cell lines, respectively. CHE had the anti-migratory and anti-invasive effects in all cell lines exposed to CHE at 1 and 5 μM, respectively. HMPOS cells exposed to CHE showed suppression of PKCα and p-ERM with increased p-ERK1/2 in a time-dependent manner.

In in vivo experiment, IT-xenografted mouse model with HMPOS cell line was used. Mice were divided into 3 groups (n = 5 mice/group). The control group received no treatments. The treatment 1 group received 5 mg/kg CHE i.p. at day 0, 2, and 4 after transplantation. The treatment 2 group received 5 mg/kg CHE i.p. at day 8, 10, and 12 after transplantation. At 1, 2, and 4 weeks after transplantation, the primary tumor volume and lung metastatic nodules were measured, and immunohistochemistry was performed for ezrin, p-ERM, p-ERK1/2, and PKCα in primary tumors and lung metastatic tissues.

Lung metastasis was not detected in the treatment 1 group at 1 week after transplantation. At 4 weeks after transplantation, number of metastatic nodules of mice in the control group was significantly higher (p<0.05) than those of treatment 1 and 2 groups. However, the tumor volumes were not significantly different between the control and treatment groups. The expressions of p-ERM and PKCα in primary and lung metastatic lesions at 2 weeks after transplantation in the treatment 1 and 2 groups were significantly lower (p<0.05) than that in the control group. These results suggested that decreased lung metastatic potential was associated with the regulation effect of p-ERM by PKC through suppression of migration and invasion of canine OS cells.

In conclusion, ezrin and p-ERM may play an important role in the proliferation and the earlier phase of lung metastasis in canine OS. These functions may be due to increase in motility and invasion of OS cells. The high expressions of ezrin and p-ERM may suggest shorter survival time in OS patients. In addition, a PKC inhibitor may suppress their expressions and decrease the incidence of lung metastasis in canine OS.