Introduction: Autocrine motility factor (AMF) stimulates cell motility in various types of tumor cells in an autocrine manner and enhances tumor metastasis angiogenesis. AMF/PGI activity is elevated in the serum or urine in patients with malignant tumors. We purified AMF from conditioned medium of murine metastatic fibrosarcoma, and demonstrated that AMF is identical to phosphoglucose isomerase (PGI), a ubiquitous enzyme catalyzing the interconversion of glucose-6-phosphate to fructose-6-phosphate, neuroleukin (NLK). AMF stimulation shows result in stress-fiber formation, which is associated with activation of two family members of the Rho like GTPases, RhoA and Rac1, but not Cdc42 activation. It is, however, still unknown which molecules involve in downstream of AMF-AMFR receptor (AMFR) signals. In this study, we constructed an amf/pgi transfection system using adenovirus, and then investigated its secretion, effects on cell motility, metastasis, molecules related to downstream reaction of AMF/PGI stimulation was analyzed with microarray.

Materials and Methods: To investigate the function of AMF/PHI as an autocrine cytokine, we used two cell lines able to grow in a protein-free condition. One was a murine fibrosarcoma cell line, Gunma clone-4 protein-free (Gc-4 PF), in which the motility and metastatic capacity show high responses to AMF by increased expression of the receptor. Another was Dunne osteosarcoma protein-free (DPF), a mouse osteosarcoma cell line. cDNA of AMF/PHI was cloned from a mouse liver, inserted into TA cloning vector, cut with restriction enzymes and again inserted into another vector containing CMV promoter lesion. The whole sequence of the insert was analyzed and contained no mutation. An expression unit was excised and inserted into a cosmid cassette pAdex1cw. After plating transduced Escherichia coli, we could obtain a clone containing the desired insert. Recombinant adenovirus was constructed by homologous recombination in human embryonic kidney cell line 293 cells. Two kinds of vectors were constructed, AdlacZ and Adamf/pgi containing lacZ and amf/pgi gene, respectively. The cells were infected by viral solutions to cell monolayers and analyzed. To assess the efficiency of the adenovirus-mediated gene transfer, beta-galactosidase activity of Gc-4 PF and DPF cells infected by adenovirus at 10 multiplicity of infection (MOI) and 100 MOI was examined 2 and 7 days after infection. Immunoblot analysis was performed to detect AMF/PGI and the receptor, gp78 in Gc-4 PF and DPF cells after infection by adenovirus at 10 and 100 MOI. For random motility assay, uniform carpets of gold particles were prepared on glass coverslips. The coverslips were placed tissue culture dishes, and Gc-4 PF and DPF cells were plated onto them. After 24h incubation with or without PGI proteins, the phagokinetic tracks produced by the migratory cells were visualized under a microscope. To examine the ability of experimental metastasis formation, 20 000 cells infected by adenovirus at 10 and 100 MOI were injected intravenously at the tail vein into C3H/He mice. Mice were killed 3 weeks later, and the lungs were examined for lungocloning nodules. Gc-4 PF cells were separated into three conditions; infected by Adamf/pgi or adenovirus alone at 100 MOI where infection rate was almost 100%, or not infected. The isolated total RNA of each cell 2 days after transfection was analyzed with microarray containing probe sets for 1081 mouse genes. The genes whose expression was up- and down regulated more than two-fold in response to adenovirus-mediated overexpression of AMF/PGI were selected and analyzed.

Results: The proportions of beta-galactosidase activity-positive Gc-4 PF1 and DPF cells infected AdlacZ at 100 MOI were both almost 100% at 2 and days after infection. On the other hand, the proportions of positive cells at 10 MOI were 17.0 and 54.5% at 2 days after infection respectively, which impelled us to use adenovirus at 100 MOI in microarray assay. The expression of intra- and extracellular AMF/PGI was detected in Gc-4 PF cells under all conditions. Both intra- and extracellular expressions were augmented by Adamf/pgi at 100 MOI. The extracellular molecules in DPF cells were detected only in cells infected by Adamf/pgi at 100 MOI. Both intra- and extracellular expressions were similar to that in Gc-4 PF cells. The expression of AMFR was found in Gc-4 PF cells as described previously. In contrast, no expression of AMFR was observed in DPF cells even in the cells transfected by amf/pgi gene at 100 MOI, suggesting absence of the receptor for AMF in DPF cells. Transfection of the amf/pgi gene stimulates motility of Gc-4 PF cells in a dose-dependent manner. The motility of Gc-4 PF cells infected by Adamf/pgi was significantly higher than those of controls. In contrast, there was no change in the phagokinetic activity of DPF cells after the cells were transfected by amf/pgi gene even at 100 MOI. Transfection of amf/pgi gene resulted in an increase in the lung metastatic nodules of Gc-4 PF cells in a dose-dependent manner. In contrast, no metastatic pulmonary nodules were found after intravenous injection of DPF even after transfection of Adamf/pgi at 100 MOI. With use of microarray analyses, we detected two augmented genes, rho GDP dissociation inhibitor beta (GDI beta) and kinesin motor 3A (KIF3A), as well as AMF itself. RNA message and protein expression of these two molecules were confirmed to be upregulated.

Discussion: In the present study, immunoblot analysis showed that AMF/PGI secretion was augmented by the amf/pgi gene transfection in a dose-dependent manner in Gc-4 PF cells. Interestingly, AMF/PGI molecules appeared in conditioned medium following high-amount gene transfection even in DPF cells that did not secrete AMF/PGI before transfection. These results indicated that high production of amf/pgi gene RNA message could allow the cells to secrete AMF/PGI even if the cells do not originally secrete AMF/PGI. Such quantitative factors may play an important role in the extracellular secretion of AMF/PGI molecule regardless of the cell type, while qualitative factors such as biochemical modification have still not been fully elucidated. Gc-4 PF cells exhibited an increased metastatic ability in response to the transfection of amf/pgi gene in a transfection rate-dependent manner. On the other hand, in non-metastatic DPF cells, the metastatic ability was not obtained by gene transfection. Probably, metastatic ability regulated by AMF/PGI may require the receptor, gp78 expression. GDI-beta in invasion and metastasis of cancer cells is still controversial at present. GDI-beta is reported to involves in progression of ovarian carcinoma corresponding well to our present results. In contrast, however, another group has reported the GDI-beta as an invasion and metastasis suppressor gene Uregulation of GDI-beta in the present study might be induced as a negative feedback mechanisms against excess signals from AMF/PGI. In the present study, amf/pgi-transfected cells exhibited not only augmented expression of KIF3A but also a tendency of distribution shift to the cell periphery. KIF3A may play an important role in the focal delivery of a component(s) that modulates the dynamics of adhesive structures at the focal contact, and then stimulate cell motility.