type 5a showed larger number of granulosa cells, higher estradiol-17β production and higher proportion of follicles with TGF-β1 positive granulosa cells.

In conclusion, this study showed that type 5a follicles could develop normally *in vitro* and produce oocytes with developmental competence to the blastocyst stage. Results in the analysis of TGF-β1 immunolocalization in ovarian and *in-vitro* grown follicles indicated that normally developing follicles express TGF-β1 in the granulosa cells around the time of antrum formation. Furthermore, the usefulness of the present follicle culture system in the investigations of follicular physiology was demonstrated.

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A fundamental study on the gene therapy of human bladder cancer using the dominant negative H-*ras* mutant N 116 Y

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To clarify the significance of the Ras guanine-nucleotide exchange reaction in human bladder cancer, I investigated the expression of epidermal growth factor (EGF) - receptor, growth-factor receptor binding protein 2 (Grb 2), and son of sevenless (Sos) proteins upstream of Ras in several human bladder cancer cell lines. Grb 2 and Sos proteins were overexpressed in all cell lines, suggesting that the Ras guanine-nucleotide exchange reaction was significant for the proliferation of human bladder cancer cells. These results led us to postulate that the dominant negative H-*ras* mutant N 116 Y (N 116 Y *ras* mutant), which has been suggested to be an inhibitor of Ras guanine-nucleotide exchange reaction, could be a potential tool of cancer gene therapy for human bladder cancer. To demonstrate the therapeutic effects of N 116 Y *ras* mutant on human bladder cancer, I performed the experiments as follows.

First, I transfected an efficient N 116 Y *ras* mutant expression vector, pZIP-N 116 Y, into 3 human bladder cancer cell lines (T 24, UMUC-2, KU-7) by lipofection procedure. Thus the high expression of the N 116 Y *ras* mutant, under the control of the murine leukemia virus (MLV) retroviral promoter, significantly suppressed the growth of all human bladder cancer cell lines examined.

Next, to investigate the effects of N 116 Y *ras* mutant at a low expression level, I established stable transfectants of UMUC-2 cells in which the N 116 Y *ras* mutant was constitutively expressed under the control of the hMT IIa promoter. I examined the cellular morphology and the ability to grow in soft agar of the N 116 Y *ras* mutant transfectants.

Although UMUC-2 cells were the most resistant to the expression of the N 116 Y *ras* mutant under the control of the murine leukemia virus (MLV) retroviral promoter, constitutive expression of the N 116 Y *ras* mutant caused a dramatic change of cellular morphology and a corresponding significant impairment in the anchorage-independent growth. These results suggested that the N 116 Y *ras* mutant suppressed tumorigenic transformation of human bladder cancer cells. Furthermore, I examined the phosphorylation of Jun kinase/stress-activated protein kinases (JNKs/SAPKs) and extracellular signal-regulated protein kinases (ERKs) by Western-blotting using antibodies specific for the phosphorylated form of ERKs and JNKs. The N 116 Y *ras* mutant could suppress the phosphorylation of JNKs, but not of ERKs, in UMUC-2 cells when it was weakly expressed under the control of the hMT IIa promoter. Considering that JNK activation is crucial for cellular transformation by oncogenic Ras, suppression of transformed phenotypes in UMUC-2 cells appears to be significantly associated with the inhibition of JNK phosphorylation by the N 116 Y *ras* mutant.

Finally, I investigated therapeutic effects of the adenovirus vector expressing the N 116 Y *ras* mutant (AdCMV-N 116 Y), on human bladder cancer cells. I infected the AdCMV-N 116 Y to human bladder cancer cell lines, KU-7 and UMUC-2, *in vitro*. AdCMV-N 116 Y significantly suppressed cellular proliferation and induced apoptosis *in vitro*. Furthermore, I investigated the effect of AdCMV-N 116 Y on the *in vivo* growth of orthotopically-implanted human bladder cancer cells. AdCMV-N 116 Y significantly suppressed the growth of human bladder cancer cells that were orthotopically implanted in the urinary bladder in nude mice. The adenoviral transgene expression in the mouse bladder was confirmed by the expression of β-galactosidase gene following infection by AdCMV-LacZ. The signal of β-galactosidase gene expression was observed in the epithelium, lamina propria, and the parts of the tumor mass. These results suggested that the inoculation of AdCMV-N 116 Y into the bladder was effective for the remission of human bladder cancer. These effects also might be seen in animals because the sequence homology of *ras* gene is very high among the mammals. Thus, this therapy also would be effective as a novel treatment for bladder cancer. Few studies of the cancer gene therapy in veterinary medicine have been reported. Results of present studies would be useful for the cancer gene therapy to the veterinary medicine.

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