Transgenic Animal Research Center

Proto-oncogene *MYCN* transduced pigs produced by somatic cell nuclear transfer

Neuroblastoma is a tumor of neural crest origin that comprises 8-10% of all childhood malignancies and causes 15% of cancer related death in children. Ninety percent of cases strike children before 10 years old. The transgenic mice that over-expressed proto-oncogene MYCN in neuroectodermal cells developed neuroblastoma. Although these mice are a model of neuroblastoma and are useful for study of pathogenesis and therapy, they are not optimal for research and development of surgery and chemotherapy treatments for humans. Pigs offer the advantage of having a similar size, diet, metabolism, lifespan and anatomy to humans. In the present study, we attempted to produce transgenic pigs expressing *MYCN* by nuclear transfer for use as a human disease model.

The expression vector of human *MYCN* containing the rat tyrosine hydroxylase promoter and G418 resistant gene was transfected into cultured fibroblasts derived from a male porcine fetus. After selection by G418, the isolated clones were checked by RT-PCR for *MYCN* expression. The one colony expressing *MYCN* was used for nuclear transfer as donor cells. After nuclear transfer using 2863 oocytes matured in vitro, 454 embryos at the one-cell stage and 960 embryos at 2-8 cell stage were transferred into 7 synchronized recipient pigs. Four of 7 recipients were pregnant and 3 pigs delivered 14 piglets including 2 stillbirths and 5 mummies (Fig 1). All 7 live piglets and the 2 stillbirths showed introduction of *MYCN* gene by PCR analysis. Development of neuroblastoma in the cloned pigs should be examined later.

Production of transgenic goats for 'Pharming'

We have established a system to produce transgenic (Tg) goats by using somatic cell nuclear transfer (SCNT) technique. We have produced Tg goats expressing the human selenoprotein P (hSel P) gene under control of the bovine beta-casein core promoter for expression in the mammary gland. During this year, the female Tg offspring was mated with a wild type male. After parturition, the milk was collected regularly and content of the hSel P was evaluated by specific ELISA. In the analyses, hSel P production was highest at the 2nd day of parturition, and the concentration was 15ng/ml. After that it decreased to 2ng/ml and was

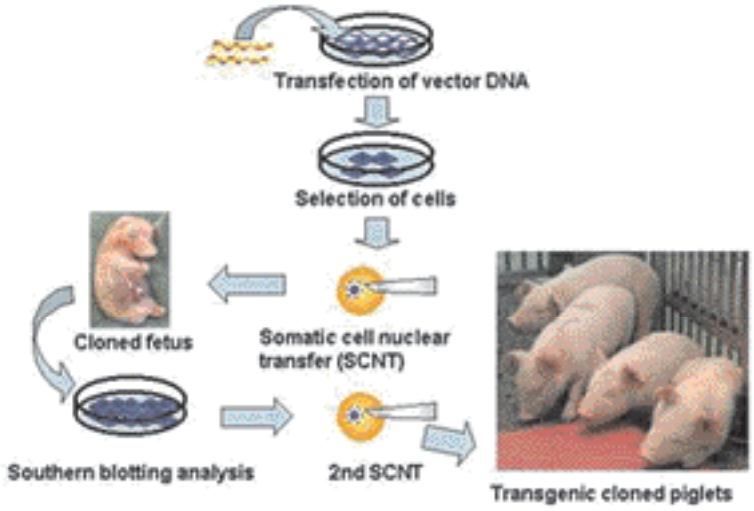

Fig 1. Production of *MYCN* transgenic pigs by somatic cell nuclear transfer.

secreted continuously for 8 weeks. Since the Tg goat carried only one copy of the hSel P gene, the concentration was lower than that found for the Tg mouse and rabbit which carried multiple copies of the same gene. The decrease in hSel P concentration may be related to the quantity of milk production during the lactation period. It is necessary to improve the expression level in the mammary gland; therefore, we have developed a new LA-SSP1 BAC vector with ~200kb DNA that includes the complete lactoalbumin gene from human BAC clone for higher control of gene expression in the mammary gland.

GFP gene expression in gonads of chicken embryos

The present study was carried out to develop techniques for introducing exogenous DNA into primordial germ cells (PGCs) and expressing the introduced DNA efficiently in the gonads of developing chicken embryos. PGCs circulating in the bloodstream were transfected with GFP gene in vitro or in vivo by lipofection or nucleofection. The manipulated PGCs successfully migrated to the germinal ridges and expressed GFP gene efficiently in the gonads of developing embryos (Fig 2). Intense GFP gene expression was observed in the gonads during the first 8 days following the transfection, during which period the sexual differentiation of gonads and germ cells (GCs) takes place. The GFP gene expression then gradually declined during subsequent embryonic development until hatching. When PGCs were transfected in vivo by lipofection with linearised plasmid DNA, GFP gene was detected in the gonads of 4.3% (19/442) of embryos examined at 20.5 days of culture. In comparison, the frequency of detection was less than 1% in the gonads of embryos in which PGCs were transfected in vitro or with circular form plasmid DNA. In two of the embryos in which PGCs were transfected in vivo by lipofection with linearised plasmid DNA, GFP gene was expressed clearly in limited areas of the gonads of 20.5-day cultured embryos. The results obtained in this study suggest that the present in vitro and in vivo techniques for PGC manipulation provide a useful experimental system for studying gene functions in the sexual differentiation of gonads and GCs in early chicken embryos.

Development of immortalized cell lines and novel tissue culture models

Immortalized bovine brain cell lines provide ideal in vitro cellular infection models for bovine spongiform enthephalopathies (BSE) prion without enduring a species barrier. Here, we established an immortalized brain

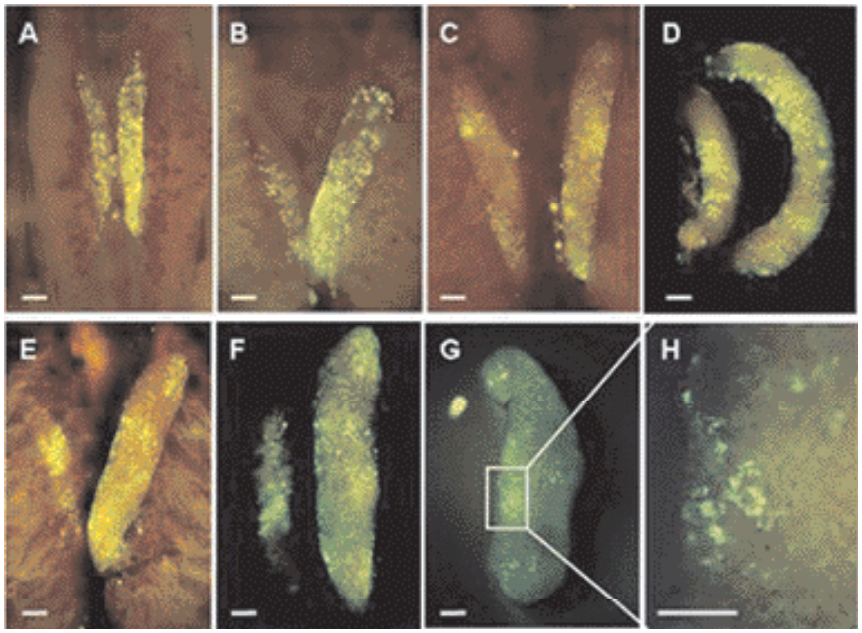

Fig 2. Expression of GFP gene in the gonads of developing chicken embryos at (A) 6.5 days, (B) 8.5 days, (C, D) 10.5 days, (E, F) 12.5 days, and (G, H) 20.5 days of culture.

cell line (FBBC-1) from primary cultures of cryopreserved fetal bovine brain tissues after transfection with SV40 large T antigen (Fig 3). FBBC-1 cells have spindle-like morphology and are stably passaged at a doubling time of about 24 hr up to at least 100 population doublings after single cell cloning. After the treatment with dibutyryl-cyclic AMP, these cells ceased proliferation and extended neurite-like processes that were immunostained with the antibody against tubulin ßIII, a marker of immature neurons. Up-regulation of tubulin ßIII expression was also confirmed by immunoblotting. FBBC-1cells expressed cellular prion protein, so these bovine cells may provide the opportunity to develop an in vitro propagation model of cattle BSE prion.

We previously succeeded in preparing a thin (thickness ca. 20μm), strong, transparent and protein-permeable membrane made of collagen fibrils in high density like corneal parenchyma in vivo. We named it collagen vitrigel membrane because its fabrication process utilizes a concept for the vitrification of heat-denatured proteins. As a first step to develop a corneal epithelial model useful for replacing the eye irritation test (Draize test), normal rabbit corneal epithelial (NRCE) cells were cultured on the collagen vitrigel membrane and their growth activity and morphology were analyzed. As a result, NRCE cells cultured on the collagen vitrigel membrane proliferated well and formed a monolayer of polygon-shaped cells the same as those on the control plastic dish did (Fig 4). These data suggest that the collagen vitrigel membrane can provide an appropriate scaffold for corneal epithelial cells and can contribute to an in vitro corneal epithelial reconstruction model.

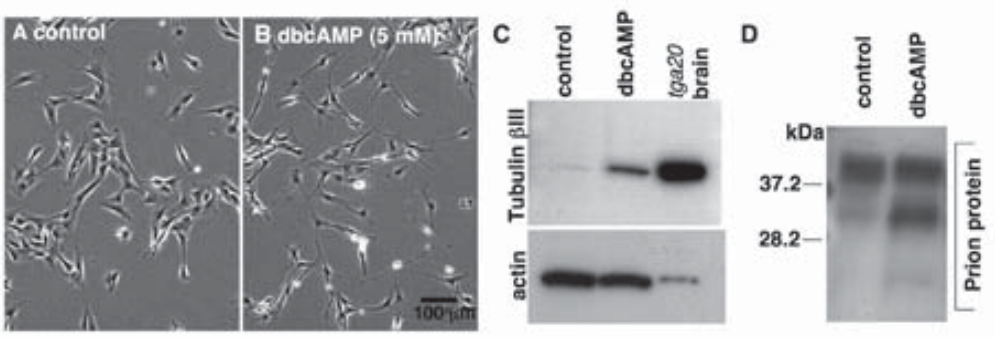

Fig 3. Establishment of an immortalized bovine brain cell line, FBBC-1
(A) and its neuronal differentiation by the treatment with dibutyryl-cyclic AMP (B and C). FBBC-1 cells express cellular prion protein (D).

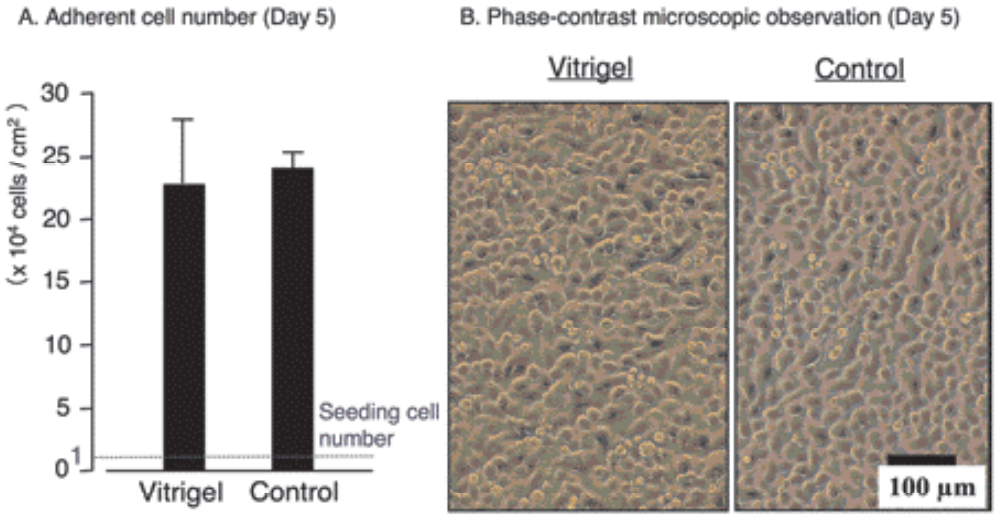

Fig 4. Growth activity (A) and morphology (B) of NRCE cells cultured on collagen vitrigel or control plastic dish.