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EDUCATION

- M.S., 1983, Department of Biology, Peking University
Thesis: The Purification of T4 RNA lagase with Immunoaffinity chromatography. Supervised by Prof. Tong Shen.
- B.S., 1970, Department of Biology, Peking University. Major: Biochemistry.

WORKING EXPERIENCE

- 3/86-5/89: Visiting Scholar, Washington University, School of Medicine, St. Louis, USA
- 3/89-2/91: Associate Professor, Chinese Academy of Sciences, Institute of Developmental Biology.
- 3/91-12/92: Exchange Scholar, Division of Animal Production, CSIRO, Sydney, Australia.
- 1/93-12/99: Professor, Institute of Developmental Biology,
- 1/01-12/04: Professor, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

PUBLICATIONS

- 1, Weidong Yu, Xin He, Guisheng Liu and Qingxuan Chen; Identification and analysis of stage-specific expression of lysosome-associated protein transmembrane 4 α gene during development of preimplantation rabbit nuclear transfer embryo. *Molecular Reproduction and Development*. 2004; 68: 415-421
- 2, Chong Zhang, Jingang Wang, Jinyan Cheng, Guisheng Liu and Qingxuan Chen; Seeking for senile-related gene expression in cerebral tissue of senescence-accelerated mouse. *Cellular and Molecular Neurobiology*. 2004,24(6): 741-747.
- 3, Wenyong Li, Weidong Yu, Qun Dong, Ping Wang, Qingxuan Chen; A complex prescription for vitiligo activates mitochondrial ATP synthase-6 expression in B-16 murine melanoma cells.

- Journal of Ethnopharmacology. 2004, 92: 193-196.
- 4, Chong Zhang, Jinyan Cheng, Jingang Wang, Qingxuan Chen; Seeking for ageing-associated gene expression in cerebral tissue of senescence-accelerated mouse (SAM). International Congress Series.2004, 1260: 373-378.
 - 5, Wen Yong Li, Wei Dong Yu, Bing Qi, Xin He, YU Ge Wang, Gui Sheng Liu, Miao Du, and Qing Xuan Chen; Analysis of gene expression in rabbit nuclear transfer embryos: Use of single-embryo mRNA differential display. Development Growth & Differentiation, 2003, 45(6): 543-551..
 - 6, Wenyong Li, Jianke Zhang, Weidong Yu, Guisheng Liu and Qingxuan Chen; Expression of stage-specific gene during zygotic gene activation in preimplantation mouse embryos. Zoological Sciences, 2003, 20(11): 1389-1393.
 - 7, Qingxuan Chen, Juan Zhang and Frederick Sweet; Homology of primate DNA fragments for estrous-associated oviductal glycoprotein. Hereditas, 2003, 139:75-79.
 - 8, Yu Wei-Dong, Yang Li-Xin, Li Wen-Yong, Liu Gui-Sheng, Chen Qing-Xuan; Establishment of single preimplantation embryos differential display polymerase chain reaction. Prog. Biochem. Biophys., 2003, 30(2): 308-313.
 - 9, Li Wen-Yong, Qi Bing, Wang Yu-Ge, Yu Wei-Dong, Du Miao, Chen Qing-Xuan; Differential expression analyses of development-related gene in rabbit preimplantation NT embryos. Prog. Biochem. Biophys., 2003, 30(5): 813-818.
 - 10, Sun X.S., Yue K.Z., Zhou J.B., Chen Q. X*, Tan J.H.; In vitro spontaneous parthenogenetic activation of golden hamster oocyte. Theriogenology, 2002, 57(2): 845-851.
 - 11, Qingxuan Chen, Stuart Adler and Frederick Sweet; Gene expression and intermolecular forces in Estrogen/Receptor binding. Gene Family Studies of DNA, RNA, Enzymes and Proteins, World Scientific Publishing Co., 2001, p133-139.
 - 12, Chong Zhang, Ting Yang, Jingang Wang, Guisheng Liu, Qingxuan Chen; The Chinese traditional medicine 'Busheng Yiniao Pian' increased the level of aging-related gene LRPAP-1 expression in the cerebral tissue of accelerated senescence-prone mouse 8/Ta. Journal of Ethnopharmacology, 2005, 98: 61-65.

Patents

Qingxuan Chen, Jinyan Cheng, Xin He, Weidong Li, Guisheng Liu. Patent No.: ZL00133625.8.

Date of Grant: July 14,2004. Patent Name: The application of liposome in transferring of exogenous DNA through injecting testes.

Major Live Grants

1. The State Basic Research Development Program (973. No.G2000016107), Studies on dispelling differentiation, reprogramming and developmental totipotency of transplanted nucleus in the cytoplasm of oocyte. 2000-2005. 860,000 RMB yuan.
2. The knowledge innovation program of Chinese Academy of Sciences (CAS) (No.KSCX2-SW-316). Construction of animal and plant high effective expressive system, 2002-2006. 440,000RMB yuan.
3. National Natural Science Foundation (No. 300070423). Study of fetal sheep $3\beta,20\alpha$ -Hydroxysteroid Dehydrogenase($3\beta,20\alpha$ -HSD) gene and its function. 2002-2004. 260,000RMB yuan.
4. Cooperation project supported by Beijing Lintai Bicheng Medical Technology Limited Company. Study on the anti-aging molecular mechanism of “BuShen YiNiao Pian” 2004-2007. 150,000RMB yuan.
5. National Natural Science Foundation of China (No. 2003CB517005). Study on proteomics and family of blood stasis syndrome. 2003-2007. 600,000RMB yuan. (Cooperative project).
6. Cooperation project supported by Institute of Neuroinformatics. Study of mechanism preventing neuropathy with “YiYuan ShenEr Kang”, 2005-2008. Dalian University of Technology. 1,000,000RMB yuan.

Laboratory Members

Investigator/Professor: 1 .

Research Assistants: 2.

Graduate students: 4.

Cloning and Functional Analysis of Development-Related Genes in Mammalian Preimplantation Transfer Nuclear Embryos

A Progress Report

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Background

In recent years, animal cloning technology develops rapidly and new cloned animals have been acquired continually, including sheep(Wilmot et al.,1997), mouse(Wakayama et al., 1998), cow(Galli et al., 1999), goat(Baguisi et al., 1999), pig(Polejaeva et al., 2000), rabbit(Chesne et al.,2002), cat(shin et al., 2002), mule(Woods et al., 2003), horse(Galli et al.,2003) and rat(Zhou et al., 2003). Although somatic nuclear transfer has successfully achieved in various species, its efficiency has been very low until recently. The inefficiency lies in many areas, such as donor cell type, cell cycle stage(Dinnyes et al., 2002; Renard et al., 2002), et al. Studies also show that some cytoplasmic factors, like nucleoplasmin, play important roles in erasure of repressive chromatin accompanied by demethylation, the mechanism of which is not yet clear.

Cell Cycle Coordination and NT Efficiency

Cell cycle synchronization has traditionally been thought the best way to improve the efficiencies of nuclear transfer. The benefit of using early-stage donor nuclei was confirmed by the enhanced rate of development of manipulated embryos to blastocysts with donor blastomeres in the early cell cycle stage (G1). Bypassing the S phase was also considered important for effective nuclear transfer (Collas et al., 1992). After the delivery of the first cloned adult mammal, Dolly (which was produced by inducing donor nuclei into the quiescent state), many living offspring were produced using quiescent, cultured donor cells. It is generally believed that a diploid, G0/G1 stage of the cell cycle is required to initiate reprogramming following transfer of the donor nucleus into an inactivated, oocyte cytoplasm. This stage is also thought to ensure that the diploid of the cloned embryo is normal. Other groups have used cycling cells in presumptive G1 stage and have also obtained offspring. As the majority of cumulus cells are presumed to be in the G0/G1 stage, they have also been used for donor cells.

Studies show that the cell cycle stage of the donor cells could significantly interfere with in vitro development of stem cell generated NT embryos. However, the implantation rate at day 7 is quite similar between the three types of nuclei (G2, 23%; G1, 16%; and M-phase, 25%). The pup delivery rates are also similar between metaphase and interphase groups at day 19 (M-phase, 2.0% vs. I-phase G1, 1.6% and G2, 1.9%). The survival rate of the cloned pups after one week is also similar between these two groups (M-phase, 38% vs. I-phase G1, 33% and G2, 40%). However, our results show that up to 85.1% of the cloned embryos develop to blastocysts when metaphase nuclei are injected, whereas this rate drops to about 20% when interphase nuclei are used (G1 and G2). What does cell cycle synchronization alter? Evidence suggests that cell cycle synchronization can only change the rate of blastocyst formation. When examining the chromatin remodeling of the injected nucleus during activation, it was found that metaphase donor nuclei reformed a metaphase plate rapidly after transferring. Although 20% of the spindles were abnormal, with disordered chromosomal arrangement, 93.3% could form one pseudo pronucleus (PN) and one polar body (PB) 6 hrs after activation. Interphase nuclei underwent premature chromatin condensation (PCC), after which only 50% of the G1 formed 2 PN and 63% of the G2 formed one PN and one PB. In 20% of the cloned embryos derived from interphase donor nuclei, fragment chromatin and condensed chromatin block were found. MII cytoplasm is known to induce donor chromatin remodeling, a process that greatly depends upon the donor cell cycle stage. Data show that cell cycle synchronization changes the pattern of chromatin remodeling. By avoiding PCC, which may induce chromosomal abnormalities, metaphase donor cells are able to achieve a higher in vitro development rate. However, although pre-implantation development improved significantly in our research, post-implantation and full-term development were similar in every cell cycle stage analyzed. These data indicate that restoration of the nuclear totipotency depends more on the nature of the donor nucleus than its initial cell cycle stage.

Aberrant Reprogramming and Physically Normal Cloned Adults

Considering the frequency of abnormal gene expression, it could be considered surprising that physically, functionally, and histologically normal cloned adults have been produced in several species. When a fertile cloned animal is delivered, the donor cells should be reprogrammed into a state compatible with embryonic development. However, most cloned embryos have been observed to fail to develop to term, and some of the surviving cloned animals have shown abnormalities. The major cause may reside in faulty or incomplete epigenetic

reprogramming of the donor nucleus, which affects the gene expression needed for every developmental stage of cloned embryos and offspring. Most cloned embryos lose their developmental abilities during pre-implantation and gastrulation. Moreover, the surviving adults often show abnormalities.

To better understand the issues controlling incomplete epigenetic reprogramming, scientists have compared the long-term viability of mice derived from ES nuclei and somatic nuclei. Reconstructed embryos were transferred into foster mothers, and caesarean sections were performed at day 19 of gestation. The combined weight of placentas for all cloned pups obtained was approximately double the weight of the control. From the standpoint of live birth cloned pups, more than half of the pups suffered from respiratory failure and general weakness and died only a few hours after delivery. The weight curves of NT mice that did survive were followed for 12 to 19 months and were similar to those of the controls. This study also show no marked differences could be detected between normal and cloned animals. Although some apparently normal cloned mice were produced, an aberrant expression pattern in NT embryos was observed even in these mice. This pattern concerned genes thought to be involved in stress adaptation, trophoblastic function, and DNA methylation during pre-implantation development. It has been shown that the culture of pre-implantation embryos affects the regulation of various imprinted and nonimprinted genes, leading to aberrant fetal growth and development (Natale et al.,2001).No significant correlation between the anomalous fetal growth of cloned mice and abnormal expression of any single gene was seen. The accumulated actions of abnormal gene expression at multiple loci ultimately resulted in embryonic or postnatal abnormalities. The high incidence of embryonic loss after implantation and postnatal death in clones suggests a need for characterizing molecular parameters that can be used to assess the developmental potential of preimplantation embryos. Researchers are currently experimenting with different methods of identifying gene expression in nuclear transfer.

A cohort of such identified genes will provide a useful tool when analyzing the developmental potential of preimplantation embryos. Another valued marker when assessing embryonic developmental potential is genomic methylation. The evidences showed that aberrant methylation patterns at the two-cell stage zygote are an indicator of early developmental failure. These authors have used an antibody to 5-methylcytosine to examine the immunostaining patterns of methylated genomic sites in two-cell zygotes developed from superovulated females, nonsuperovulated matings, and in vitro fertilization. A major conclusion of their work is that a methylcytosine staining pattern has been shown to be a valuable indicator of early developmental methylation reprogramming of the two parental genomes in normal or in vitro fertilized zygotes. The authors indicate that this immunostaining approach promises to be potentially useful for determining the safety and efficiency of technologies that assist with reproduction. To best explain aberrant reprogramming and the acquisition of apparently normal adult animals, there is an increasing need to explore how NT is affected by varying genetic backgrounds, the nuclear

transfer procedure, and the synchronization of donors and recipients.

In the past years, we have identified several development related genes by mRNA differential display through comparing normal and cloned embryonic development in order to explore the mechanism of animal cloning. Therefore, I will summarize our work and state future research plan here.

Progress

Establishment of Modified Single-Embryo mRNA DDRT-PCR Method

Nowadays, there are many ways to clone novel genes, such as subtractive hybridization (Zimmermann *et al.*, 1994) and mRNA differential display reverse transcription polymerase chain reaction (DDRT-PCR, Liang and Pardee, 1992;), gene chip (Sasaki *et al.*, 2003) and so on. Comparatively, DDRT-PCR is one of the most effective methods to validate novel expressive genes during early embryonic development so far (Li and Han, 1997), but it is hampered by the paucity of starting biological materials for early mammalian embryos, especially, for nuclear transfer (NT) embryos. The limited availability of mammalian NT embryos requires modifications of previously established techniques to achieve sufficient sensitivity and efficiency. mRNA differential display method has been used to detect differences in gene expression between distinct cells or tissues. However, this method is difficult to apply to early stage mammal embryo due to (1) the limitation on the number of preimplantation embryo, (2) the small amount of mRNA that is present in these embryos (i. e., picogram amounts), and (3) the technical difficulties in isolating picogram amount of poly(A)⁺ RNA. Several modifications of the method of DDRT-PCR were necessitated due to the limitations of the number of preimplantation embryos and the small amount of mRNA that is present in these embryos. Total RNAs were prepared from rabbit preimplantation NT embryos (from M II-egg to 4-cell stage embryos) using RNeasy Mini Kit coupled with Micro RNA Isolated Kit. Reverse transcription and PCR were performed under optimized conditions using anchored primers. Comparison of banding patterns revealed several discernable fragments, which are differentially expressed in different stages.

A primary obstacle that has delayed molecular analysis of this developmental program is the difficulty of collecting and analyzing large numbers of eggs and embryos. Usually, one unfertilized egg contains about 0.43 ng RNA; one fertilized egg contains about 0.35 ng RNA; one two-cell embryo contains about 0.24 ng RNA; while one four-cell embryo only contains about

0.60 ng RNA. Total RNAs are extracted and purified using RNeasy Mini Kit, removing DNA contaminant, and then using MicroRNA Isolation Kit to purify it. Negative control was designed in reverse transcription (without reverse transcriptase). No band was found in negative control lane, indicating no DNA contaminant in total RNAs. There is only picogram amounts of mRNAs in these embryos, so, it is difficult to isolate the poly (A)⁺mRNA from total RNA. Therefore, total RNAs were used for DDRT-PCR directly. Based on experimental comparison, many parameters for performing reverse transcription, PCR and electrophoresis were improved. In order to obtain cDNAs from lower amount of mRNAs, Superscript II and M-MLV were used coordinately in reverse transcription system. The cDNAs obtained from DDRT-PCR are less qualified and producing high rate of pseudo clones. To identify positive or negative clones is important for availability of DDRT-PCR. For the present studies, a new validation approach was designed based on reverse northern hybridization. In this study, the single preimplantation embryo mRNA differential display reverse transcription and polymerase chain reaction (SPEDDRT-PCR) was developed from the method of Liang & Pardee (1992) and the method of Shimono A & Behringer RR(2000), which makes it possible to study gene expression pattern during mammalian preimplantation embryo development. This modified technique is very sensitive and reproducible. A broad spectrum of expressed genes from MII-oocyte to 8-16 cell NT embryos were defined using SPEDDRT-PCR method. Several different bands were shown prominently in a portion of autoradiogram. When using SPEDDRT-PCR method to compare the mRNA content in rabbit NT embryos at different stages during the preimplantation period, it was found that it is very important for obtaining good results to remove the zona plooocida of MII -eggs and NT embryos. All MII-eggs and NT embryos were placed in Acid Tyrode's Solution (pH 2.2) and at the same time observed under a dissection microscope and immediately transferred to PBS once they are just dissolved (about 30 sec). The embryos will disintegrate and adhere to vessels if they are over-dissolved. On the contrary, deficient dissolution interferes in the release of total RNA from cytoplasm in lysis buffer, which may lead to incorrect data.

Identification and Analysis of Differential Display Fragments

Little has been known on what happens to the nucleus of the somatic cell after it was transferred into the cytoplasm of an oocyte. Expression analyses of individual genes showed differences between NT embryos and normal embryos at the different developmental stages. So differential gene expression is a remarkable characteristic for the development of a single-celled zygote into a patterned multicellular organism with differentiated cell types and tissues. Therefore, the identification of differentially expressed genes provides an important molecular resource for functional studies to define genetic pathways in mammal development. In order to study the

molecular mechanism of the NT embryo development, here sixty interested fragments were obtained, and fifty four of them were reamplified. And thirty four (Table 1) of the reamplified fragments were successfully subcloned and sequenced. To identify eight fragments of the thirty four amplicons, the Reverse Northern Blot which was improved from normal Northern Blot was used to suit the lack of starting material. cDNA pools were constructed successfully from different stages of NT embryos. Housekeeping gene, β -actin was used as positive control to detect the quantity of cDNA pools. There are four fragments (A023, A028, C043, C048) which are consonant with the pattern of DDRT-PCR and are validated to be positive clones (4/8,50%). Thirty-four successful cloned fragments were compared with deposited sequences in NCBI using BLAST tool. Eleven cDNAs (11/34, 32.4%) have no homology sequences in EST and nr, such as A023 and A026 that may be the new genes. Northern blot indicated that A023 is expressed highly in 8-16-cell stage. So the full cDNA length of A023 can be cloned from a single 8-16-cell cDNA library of NT embryos. Twelve cDNAs (12/34, 35.2%) displayed sequence homology to established sequence tags, but their functions were unknown (such as A023, A026, C052). Eleven cDNAs (11/34, 32.4%) showed sequence homology to protein of sequences in the EMBL and GenBank databases. Three of these genes (A020, A025, and A028) were probably related to preimplantation embryo development and might play an important role in development of rabbit NT embryo. A020 is homologous to retinoblastoma binding protein 4 (RBBP4, identity=95%), which may be involved in mitogen stimulation (Yang *et al.* 2002). A025 is homologous to Homo sapiens coronin, actin binding protein 1C (CORO1C, identity=91%), which is expressed in a tissue-specific manner. Immunocytochemical staining demonstrated that CORO1C was co-localized with β -actin. The gene product is likely to be important in cytokinesis, motility, and signal transduction (Iizaka *et al.* 2000). Transcript A028 displayed high homology (identity=93%) to CstF3 gene for cleavage stimulation factor, subunit3, 77KD, which was involved in pre-mRNA 3'-end processing and was required for progression through mitosis. In rabbit, the translation of many maternal mRNAs is correlated with a post-transcriptional modification, namely, polyadenylation. During mRNA 3'-end formation, cleavage stimulation factor (CstF) binds to a GU-rich sequence downstream from the polyadenylation site and helps to stabilize the binding of cleavage polyadenylation specificity factor (CPSF) to the upstream poly-adenylation sequence (AAUAAA). A decrease in CstF-77 activity preferentially affects 3'-end formation of particular

mRNAs, some of which are involved in mitosis. Some maternal mRNAs undergo cytoplasmic polyadenylation. This polyadenylation was functionally correlated with the translational activation of these mRNAs. The elongation of the poly (A) tail is associated with increased translation, while poly(A) tail shortening correlated with decreased translation. Cytoplasmic polyadenylation has been shown to be an absolute requirement for the translational activation of some maternal mRNAs. Furthermore, it may affect developmental events (Hatton *et al.* 2000). Through analysis of all the data obtained, we suggest that the transcript A028 possibly plays an important role during the early development of rabbit nuclear transfer embryos.

Table 1, Properties of analyzed amplicons.

Clone#	Size (bp)	GenBankdatabase		From Stage(s)	Description (E-value, Putative Function, Homology identity)
		nr	est		
A002	501	--	+	BM693428	8-16-cell Transcript of unknown function, 6e-50, 89%
A003	114	--	--		8-16-cell unknown
A004	232	+	+	AF151885	8-16-cell Homo sapiens CGI-127 protein mRNA, 1e-24, 86%
A005	535	+	+	AC006038.3	8-16-cell COX7A2L cytochrome c oxidase subunit VII a polypeptide 2 like, 7e-31, 82%
A007	414	--	+	BM263656	M II Transcript of unknown function, 4e-26, 90%
A008	407	--	--		M II, 2-cell unknown
A010	318	+	+	NM003769.1	8-16-cell Homo sapiens splicing factor, arginine/serine-rich 9 (SFRS9), 4e-41, 97%
A011	355	+	+	BC028424.1	8-16-cell Similar to fring, 1e-47, 86%
A013	249	+	--	XM065712.3	8-16-cell Homo sapiens similar to transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kD), 3e-41, 85%
A017	284	--	--		2-cell unknown
A018	277	+	+	AB011165	8-16-cell mRNA for KIAA0593 protein, partial cds, 3e-44, 84%
A020	276	+	+	BC003092	8-16-cell Similar to retinoblastoma binding protein 4, 1e-52, 95%
A021	263	--	+	BE681627	M II Transcript of unknown function, 7e-70, 89%
A022	253	--	--		M II unknown
A023	260	--	+	BE286250	8-16-cell Transcript of unknown function, 1e-71, 90%
A025	240	+	+	NM014325.1	2-cell Homo sapiens coronin, actin binding protein, 1C (CORO1C), 1e-31, 91%
A026	243	--	+	BM735551	4-cell Equus caballus cDNA, Transcript of unknown function, 5e-58, 88%
A028	215	+	+	HSDJ85M6	8-16-cell, M II, 2-cell Human DNA sequence from clone RP1-85M6 on chromosome 11, p12-13 Contains the CSTF3 gene for cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kD, PIGCP1 pseudogene (phosphatidylinositol glycan, class C), the gene for a novel protein similar to testis> 2e-72, 93%
C032	484	--	--		M II unknown
C033	106	--	--		4-cell unknown
C034	280	+	+	MRBPR17B	2-cell M.musculus mRNA for ribosomal protein L7, 9e-85, 91%
C036	436	--	--		8-16-cell unknown
C038	237	+	+	AC072028.14	M II Homo sapiens 3 BAC RP11-190C21 (Roswell Park Cancer Institute Human BAC Library) complete sequence Length = 186739, e-131, 100%
C039	426	+	+	ACO15987	M II, 2-cell Homo sapiens chromosome 7 clone RP11-207O19, e-164, 95%
C041	95	+	+	BF710377	M II, 2-cell MI-P-AY1-nqr-c-03-0-ULs1 MI-P-AY1 Sus scrofa cDNA clone 1e-34, 98%
C042	383	--	--		8-16-cell unknown
C043	378	+	+	OCNAT23	8-16-cell, 4-cell Rabbit NAT2 gene for arylamine N-acetyltransferase (EC2.3.1.5), 8e-46, 94%
C044	385	--	--		unknown
C047	356	+	+	AC019205.4	M II, 2-cell Homo sapiens BAC clone RP11-398K22 from 6, 4e-38, 95%
C048	296	+	+	OCAJ1588	8-16-cell, 4-cell <i>Oryctolagus cuniculus</i> complete mitochondrial genome, e-166, 99%
C049	323	--	--		8-16-cell unknown
C050	312	--	--		8-16-cell unknown
C051	294	+	+	AY011158.1	M II, 2-cell Rabbit osteoclast, similar to 16S ribosomal RNA gene, 89%
C052	286	--	+	BI400964	8-16-cell Transcript of unknown function, 1e-31, 81%

So far, several genes that are differentially expressed in different stages of NT rabbit embryos were identified using SPEDDRT-PCR. Although the functions in detail of these genes and their

products are still unclear, this study may provide a basis for future research of the possible causes of NT embryos developmental failure. With the better understanding of gene expression profiling in preimplantation embryos derived from NT nucleus, particularly, those important genes are necessary for the attainment of developmental totipotency and lack or abnormal expressions of developmentally important genes is believed to hamper early development of NT embryo.

Cloning and Identification of Stage-Specific Expression of LAPT_M4 α Gene

Lysosome-associated protein transmembrane 4 α (LAPT_M4 α) is a member of the LAMP (Lysosome –Associated Transmembrane Protein) family, which was found in mammals, insects and nematodes. It owns four predicted transmembrane spanning domains and requires two arranged tyrosinase-based sorting signals for efficient and strict localization in endosomes and lysosome (Hogue et al.,2002). In mammals, there are three homologues from human, mouse and rabbit which was firstly cloned by our laboratory recently. As a nucleotide transporter protein, the possible function of LAPT_M4 α is to regulate the intercellular compartmentalization of amphipathic solutes and, the sensitivity of cells toward anthracyclines, antibiotics, ionophores, nucleosides and organic cations (Che et al.,1995). In addition, LAPT_M4 α was able to alter intercellular steroid distribution in a drug-sensitive strain of *Saccharomyces cerevisiae* as well as in cultured mammalian cells (Hogue et al.,1996).

In the study on gene differential expression during preimplantation development of rabbit nuclear transfer embryos, we found LAPT_M4 α stage-specific expression at 8-cell stage as well as MII oocyte using methods of the single preimplantation embryonic differential display and reverse Northern Blotting (Yu et al.,2003a,2003b). LAPT_M4 α homology 85% with LAPT_M5 α , and the later may have a special functional role during embryogenesis. The facts of its function to alter intercellular steroid distribution in cultured mammalian cells and specific expression in MII oocyte indicated LAPT_M4 α may play a potential role in the follicle-genesis (or oogenesis) and embryogenesis (Hogue et al.,2002; Yu et al.,2003a,2003b).

In the present study, we have cloned the full length LAPT_M4 α cDNA, analyzed the gene expression during the preimplantation NT embryo development and identified LAPT_M4 α gene expression distribution in intra-ovary during follicle-genesis by *in situ* hybridization(ISH) .The stage-specific expression of Lysosome-associated protein transmembrane 4 α (LAPT_M4 α) in

preimplantation rabbit nuclear transfer (NT) embryo was identified with the DDRT-PCR and reverse Northern Blot. The full length(1364bp) cDNA of LAPTM4 α was screened out from cDNA library constructed with rabbit ovary and In Situ Hybridization was used to trace the distribution of the LAPTM4 α mRNA in intra-ovary, especially the follicle which proved that the LAPTM4 α gene expression is involved in the follicles development, maturation, ovulation, luteinization , and preimplantation development in the rabbit (*Oryctolagus cuniculus domestica*). To our knowledge, this is the first characterization of LAPTM4 α gene expression and mRNA distribution in the rabbit ovary and first evidence for this gene involved in follicle development and rabbit preimplantation development.

Rabbit IFRG Gene Expression During Preimplantation Normal and NT Embryo Development

Previous studies showed that there are about 147 different expression fragments, among which a fragment is abnormal expressed in NT blastocyst stage embryo. After screening the cDNA library and performing 5'-RACE, the full length cDNA (2794bp) of this gene is first cloned, which consists of an ORF encoded 131 amino acids. It shared 98% homologous to interferon responsive gene, 15KD, so we named it IFRG (Accession No. AJ584672).

In our studies, IFRG is expressed in all stage of normal preimplantation embryos including oocytes. But some of the NT blastocysts have no expression. During the perimplantation period, expression of several cytokines and their cognate receptors by the recipient uterus and the developing embryo allows for a complex interplay between the two partners, which normally results in a mutual interaction and leads to a successful implantation. Interferon-gamma(IFN γ) is highly expressed by mammalian trophoblast cells during implantation. Truchet found that mouse oocytes and preimplantation embryos bear the two sub-units of IFN γ receptor, suggesting that this cytokine could play a role during early development (Truchet S, 2001). IL-6 is an important developmental gene. Cross talk exists between the IFN- α/β and IL-6 signaling pathways, in that weak signaling by spontaneously produced IFN- α/β contributes to an efficient IL-6 signaling. And the weak IFN- α/β signaling is also critical for efficient IFN- γ signaling (Takaoka A, 2000).

In ruminants, interferon- τ (IFN- τ) is an pregnancy factor and is secreted from the

trophoblast during the time of implantation. The major function of IFN- τ are to involve maternal and fetal recognition. IFN- τ binds to the IFN receptor(IFNR) located and the uterine endometrium and IFNR is thought to be expressed only in the denometrium (Thatcher WW, 1992). However, a recent study showed that the expression of IFNR at earlier stages in ovine conceptuses. In a recent study, Takahashi suggested a novel function for IFNs in promoting embryonic development and the effect may be related to type-I IFN receptor expressed in the early stages of preimplantation embryos (Imakawa K, 2002). Although IFN- α like IFN- τ binds to the same IFN receptor to promote embryo development, they could result in a different amplification of signal transduction. In mammalian preimplantation embryos other than bovine embryos, the expression of IFN α gene is observed from oocytes through to preimplantation embryo in mouse (Riego E,1995).

More and more experiments showed that interferon may play a very important role in early embryo development, but the physiological role of these interferons in early embryonic development is not clear. In our present study, for the first time we find that IFRG exists in oocytes and preimplantation embryos, which provided some clue for understanding the role of interferons. IFRG is an abnormal expression gene in some cloned embryos, which may provide a marker for the diagnosis of cloned embryos viability prior to implantation. The abnormal expression of IFRG may be one of the reasons which lead to low efficiency of animal cloning.

Recent publications of the laboratory

- 1, Weidong Yu, Xin He, Guisheng Liu and Qingxuan Chen; Identification and analysis of stage-specific expression of lysosome-associated protein transmembrane 4 α gene during development of preimplantation rabbit nuclear transfer embryo. *Molecular Reproduction and Development*. 2004; 68: 415-421
- 2, Wen Yong Li, Wei Dong Yu, Bing Qi, Xin He, YU Ge Wang, Gui Sheng Liu, Miao Du, and Qing Xuan Chen; Analysis of gene expression in rabbit nuclear transfer embryos: Use of single-embryo mRNA differential display. *Development Growth & Differentiation*, 2003, 45(6): 543-551..
- 3, Wenyong Li, Jianke Zhang, Weidong Yu, Guisheng Liu and Qingxuan Chen; Expression of

- stage-specific gene during zygotic gene activation in preimplantation mouse embryos. *Zoological Sciences*, 2003, 20(11): 1389-1393.
- 4, Chong Zhang, Jingang Wang, Jinyan Cheng, Guisheng Liu and Qingxuan Chen; Seeking for senile-related gene expression in cerebral tissue of senescence-accelerated mouse. *Cellular and Molecular Neurobiology*. 2004,24(6): 741-747.
 - 5, Wenyong Li, Weidong Yu, Qun Dong, Ping Wang, Qingxuan Chen; A complex prescription for vitiligo activates mitochondrial ATP synthase-6 expression in B-16 murine melanoma cells. *Journal of Ethnopharmacology*. 2004, 92: 193-196.
 - 6, Qingxuan Chen, Juan Zhang and Frederick Sweet; Homology of primate DNA fragments for estrous-associated oviductal glycoprotein. *Hereditas*, 2003, 139: 75-79.
 - 7, Chong Zhang, Ting Yang, Jingang Wang, Guisheng Liu, Qingxuan Chen; The Chinese traditional medicine 'Busheng Yiniao Pian' increased the level of aging-related gene LRPAP-1 expression in the cerebral tissue of accelerated senescence-prone mouse 8/Ta. *Journal of Ethnopharmacology*, 2005, 98: 61-65.
 - 8, Bing Qi, Wenyong Li, Zhe Liu, Guisheng Liu, Xin He, Qingxuan Chen, Cloning and analysis of IFRG (interferon responsive gene) in rabbit oocytes and preimplantation embryos. *Zoological Science*, 2005 (in press).

Future work

Based on the previous work, the plan of continuous studies as follows:

- (1), To isolate and identify more development related genes from normal and NT mammalian embryos;
- (2), To construct expressive vector of genes cloned before, to express their corresponding proteins, to prepare antibodies; and to locate the gene expressive products by using tissue in situ hybridization;
- (3), To do the functional studies with the techniques of RNA interference, gene Knock out, transgenic animals and so on;
- (4), To improve the technology of animal cloning.

•Literatures Cited

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempe MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, et al. (1999), Production of goats by somatic cell nuclear transfer. *Nat Biotechnol*, **17**: 456–461.

Che M, Ortiz DF, Arias IM, (1995), Primary structure and functional expression of a cDNA encoding the bile canalicular, purine-specific Na⁺-nucleoside cotransporter. *J Biol Chem*, **270**: 13596-13599.

Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP, (2002), Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat Biotechnol*, **20**: 366–369.

Collas P, Pinto-Correia C, Ponce de Leon FA, Robl JM. (1992), Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. *Biol Reprod*, **46**: 501–11.

Dinnyes A, De Sousa P, King T, Wilnut I, (2002), Somatic cell nuclear transfer: recent progress and challenges. *Cloning Stem Cells*, **4**: 81–90.

Galli C, Duchi R, Moor RM, Lazzari G. (1999), Mammalian leukocytes contain all the genetic information necessary for the development of a new individual. *Cloning*, **1**: 161–170.

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G, (2003), A cloned horse born to its dam twin. *Nature*, **424**: 635.

Hatton LS, Eloranta JJ, Figueiredo LM, Tatagaki Y, Manley JL, Hare KO', (2000), The drosophila homologue of the 64 kDa subunit of cleavage stimulation factor interacts with the 77kDa subunit encoded by the suppressor of forked gene. *Nucleic Acids res.* **28**: 520-526.

Hogue DL, Nash C, Ling V, Hobman TC, (2002), Lysosome-associated protein transmembrane 4 α (LAPTM4 α) requires two tandemly arranged tyrosine-based signals sorting to lysosomes. *Biochem J.* **365**: 721-730.

Hogue DL, Elliso MJ, Young JD, Cass CE, (1996), Identification of novel membrane transporter associated with intracellular membranes by phenotypic complementation in the yeast *Saccharomyces cerevisiae*, *J Biol Chem*, **271**: 9801-9808.

Imakawa K, Tamura K, Lee RS, Ji Y, Kogo H, Sakai S, Christenson PK, (2002), Temporal expression of type 1 interferon receptor in the periimplantation ovine extra-embryonic membranes: demonstration that human IFN alpha can bind to this receptor. *Endocr J*, **49**: 195-205.

Liang P, Pardee AB, (1992), Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. *Science*, **257**: 967-971.

Li SH, Han Wa HF, (1997), mRNA differential display and its application to the identification of development related genes. *Dev Rep Biol.* **6**: 67-75.

Lizaka M, Han HJ, et al, (2000), Isolation and chromosomal assignment of a novel human gene, CORO1C, homologous to coronin-like actin-binding proteins. *Cytogenet cell Genet*, **88**: 221-224.

Natale D, DeSousa PA, Westhusin ME, Watson A, (2001), Sensitivity of bovine blastocyst gene expression patterns to culture environments assessed by differential display RT-PCR. *Reproduction*, **122**: 687–693.

- Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, et al.** (2000), Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, 407: 86–90.
- Renard JP, Zhou Q, LeBourhis D, Chavatte-Palmer P, Hue I, Heyman Y, Vignon X.** (2002), Nuclear transfer technologies: between successes and doubts. *Theriogenology*, 57: 203–222.
- Riego E, Perez A, Martinez R, Castro FO, Leonart R, Fuente J,** (1995), Differential constitutive expression of interferon genes in early mouse embryos. *Mol Reprod Dev*, 41: 157-166.
- Sasaki T, Arai H, Beppu T, Ogasawara K,** (2003), Detection of gene amplification and deletion in high-grade gliomas using a genome DNA microarray (GenoSensor Array 300). *Brain Tumor Pathol*, 20(2): 59-63.
- Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M.** (2002), A cat cloned by nuclear transplantation. *Nature*, 415(6874): 859.
- Shimono A, Behringer RR,** (2000), Differential screens with subtracted PCR-generated cDNA libraries from subregions of single mouse embryos. *Meth mol Biol*, 136: 333-347.
- Takaoka A, Mitani Y, Suemori H, Sato M, Yokochi T, Noguchi S, Tanaka N, Taniguchi T,** (2000), Cross talk between interferon- γ and $-\alpha / \beta$ signaling components in caveolar membrane domains. *Science*, 288: 2357-2360.
- Thatcher WW, Danet, Desnuyers G, Wetzels C,** (1992). Regulation of bovine endometrial prostaglandin secretion and the role of bovine trophoblast protein-1 complex. *Reprod Fertil Dev*, 67: 2797-2811.
- Truchet S, Wietzerbin J, Debey P,** (2001), Mouse oocytes and preimplantation embryos bear the two subunits of interferon-gamma receptor. *Mol Reprod Dev*, 60: 319-330.
- Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R,** (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature*, 394: 369–374.
- Wilmot I, Schnieke AE, McWhir J, Kind AJ, Campbell KH,** (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385: 810–813.
- Woods, GL.; White, KL.; Vanderwall, DK.; Li, GP.; Aston, KI.; Bunch, TD.; Meerdo, LN.; Pate, BJ.** (2003), A Mule Cloned from Fetal Cells by Nuclear Transfer. *Science*, 301(5636): 1063.
- Yu WD, Li WY, Wang YG, Yang LX, Liu GS, Du M, Chen QX,** (2003a). Isolation and identification of reprogramming genes related to the development of the rabbit reconstructed embryos. *Chin J Biotech*, 19: 30-34.
- Yu WD, Yang LX, Li WY, Liu GS, Chen QX,** (2003b). Establishment of single preimplantation embryos differential polymerase chain reaction. *Pro Biochem Biophys*, 30: 309-311.
- Zimmermann JM, Schultz RM,** (1994). Analysis of gene expression in the preimplantation mouse embryo: use of mRNA differential display. *Dev. Biol*, 91: 5456-5460.
- Zhou, Q.; Renard, JP.; Le Fric, G.; Brochard, V.; Beaujean, N.; Cherifi, Y.; Fraichard, A.; Cozzi, J.** (2003). Generation of Fertile Cloned Rats by Regulating Oocyte Activation. *Science*, 302(5648): 1179.

Key publications (see attachment)

- 1, Weidong Yu, Xin He, Guisheng Liu and Qingxuan Chen; Identification and analysis of stage-specific expression of lysosome-associated protein transmembrane 4 α gene during development of preimplantation rabbit nuclear transfer embryo. *Molecular Reproduction and Development*. 2004; 68: 415-421
- 2, Wen Yong Li, Wei Dong Yu, Bing Qi, Xin He, YU Ge Wang, Gui Sheng Liu, Miao Du, and Qing Xuan Chen; Analysis of gene expression in rabbit nuclear transfer embryos: Use of single-embryo mRNA differential display. *Development Growth & Differentiation*, 2003, 45(6): 543-551..
- 3, Wenyong Li, Jianke Zhang, Weidong Yu, Guisheng Liu and Qingxuan Chen; Expression of stage-specific gene during zygotic gene activation in preimplantation mouse embryos. *Zoological Sciences*, 2003, 20(11): 1389-1393.