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# Host-Virus Interactions of Infectious Laryngotracheitis Virus Infection in Cultured Cells

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**HOST-VIRUS INTERACTIONS OF  
INFECTIOUS LARYNGOTRACHEITIS VIRUS INFECTION IN CULTURED CELLS**

HOST-VIRUS INTERACTIONS OF  
INFECTIOUS LARYNGOTRACHEITIS VIRUS INFECTION IN CULTURED CELLS

A dissertation submitted in partial fulfillment  
of the requirements for degree of  
Doctor of Philosophy in Cell and Molecular Biology

By

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## **ABSTRACT**

Infectious laryngotracheitis virus (ILTV; Gallid herpesvirus 1) causes upper respiratory diseases in mainly chickens and exhibits 90-100% of high morbidity and up to 70% of mortality, resulting in huge economic losses in the poultry industry worldwide.

To study host-ILTV interactions, the changes in genome-wide gene expressions in response to wild-type and vaccine ILTV infections in primary chicken embryo lung cells were investigated using microarray analysis. Results provide crucial insights into host cell pathogenic and immunogenic responses against wild-type and vaccine ILTV infections. Using microarray method and Ingenuity Pathway Analysis (IPA) bioinformatics tool, 273 and 306 differentially expressed genes were identified responding to wild-type and vaccine ILTV infections, respectively. Further integrated analysis to compare differentially expressed genes revealed that eight host genes including coagulation factor II (thrombin) receptor-like 1 (F2RL1), bone morphogenetic protein 2 (BMP2), inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase subunit beta (IKBKB) interacting protein (IKBIP), thymidylate synthetase (TYMS), chromosome 8 open reading frame 79 (C8orf79), coagulation factor X (F10), prostaglandin-endoperoxide synthase 2 (PTGS2) and neuropeptide Y (NPY) were regulated differently between wild-type and vaccine ILTV infections in an opposite direction, suggesting that these host factors may play important roles in host immune responses against ILTV infection. In addition, the transcriptome changes of ILTV encoding genes were studied during infection time courses using quantitative PCR. In this study, infected-cells polypeptide (ICP) 4 showed the highest expression level and UL21 and UL42 showed unique expression patterns, unlike most of the other ILTV gene which exhibited continuous elevation of expression during lytic infection. Kinetic analysis of ILTV gene expression in host cells may provide new knowledge to understand ILTV pathogenesis.

This dissertation is approved for recommendation  
to the Graduate Council.

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**CHAPTER 1.**  
**LITERATURE REVIEW**

## 1. Herpesvirus

### 1.1. Herpesvirus virion structure and alphaherpesviruses

Herpesviruses share the same virion structure ranging from 130 to 300 nm in size. Commonly, the virion includes a linear dsDNA genome in 100 to 110 nm icosahedral capsid surrounded by tegument proteins [1]. The tegument proteins consisting of at least 20 viral proteins play various roles in viral genome replication and the modulation of cellular responses [2]. A host lipid layer with viral glycoproteins covering the tegument layer mediates attachment, penetration and immune evasion.

According to the genome sequence, replication dynamics and latency site, the *Herpesviridae* family is divided into three subfamilies; *Alpha-*, *Beta-* and *Gammaherpesvirinae* [3]. In human herpesviruses, *alphaherpesvirinae* includes herpes simplex virus (HSV) type 1 [(HSV-1; human herpesvirus 1 (HHV-1)], HSV type 2 (HSV-2; HHV-2) and varicella-zoster virus (VZV; HHV-3). *Betaherpesvirinae* includes human cytomegalovirus (hCMV; HHV-5), HHV-6 (herpes lymphotropic virus and HHV-7). *Gammaherpesvirinae* includes Epstein-Barr virus (EBV; HHV-4) and Kaposi's sarcoma-associated herpesvirus (KHSV; HHV-8).

In animal *herpesviridae*, *alphaherpesvirinae* is categorized into genera of *simplexvirus*, *varicellovirus*, *mardivirus* and *iltovirus*. Simplexvirus includes bovine herpesvirus 2 (BHV-2), cercopithecine herpesvirus 1 (herpes B virus) and ateline herpesvirus 1. *Varicellovirus* genus includes bovine herpesviruses 1 (BHV-1) and 5 (BHV-5), caprine herpesvirus 1, porcine herpesvirus 1 (pseudorabies herpesvirus; SuHV-1), equine herpesvirus 1 (EHV-1), 3 (EHV-3) and 4 (EHV-4), canine herpesvirus 1 (CHV-1), feline herpesvirus 1 (FVR-1) and duck herpesvirus 1 (DEV-1). *Mardivirus* genus includes gallid herpesvirus (GaHV) 2 (Marek's disease virus; MDV) and 3 (GaHV-3 or MDV-2), and herpesvirus of turkeys (HVT). *Iltovirus*

genus includes GaHV-1 (infectious laryngotracheitis virus; ILTV) and pittacid herpesvirus 1 (PsHV-1). *Betaherpesvirinae* has only one rahdinovirus genus including porcine herpesvirus 2. *Gammaherpesvirinae* includes alcelaphine herpesvirus 1 (AIHV-1) and 2 (AIHV-2), bovine herpesvirus 4 (BHV-4), equine herpesvirus 2 (EHV-2) and 5 (EHV-5) and murid herpesvirus 4 [murine gammaherpesvirus-68 (MHV-68)] [4].

Alphaherpesviruses initially infect epithelial cells such as skin cells, replicate quickly and cause sores on the membranes of skin or mucous. Since the virus is neurotropic, the viruses travel to the central nervous system (CNS) to establish latent infection in the ganglia of sensory neurons without active virus replication. The latently infected viruses are reactivated by the environmental changes of host such as ultraviolet (UV) light stimulation, the immunosuppression, trauma of latently infected CNS and even social stress [5]. The alphaherpesviruses have been used as a model to study other herpesviruses and host-virus interactions due to the rapid replication [6]. Generally, betaherpesviruses produce progenies slower than other subfamilies and latently infect leukocytes, while gammaherpesviruses propagate at variable rates and latently infect only lymphocytes [7-9].

## **1.2. Herpesvirus life cycle**

As an ancestor type of herpesviruses, the HSV-1 entry event follows three steps; the sequential-, intermediate hemifusion- and a stable entry pore forming stages [10]. During the sequential stage, herpesvirus entry is initiated by the interaction between the viral envelope glycoproteins and host cellular surface receptors. Viral glycoprotein (g) C (gC) binds to heparan sulfate on a cell surface. In turn, gD binds to one of three host cell entry receptors named herpesvirus entry mediator (HVEM), nectin-1 and 3-O heparan sulfate to establish a strong

attachment. In the hemifusion stage, gD changes its structural conformation and interacts with the complex of gH and gL. At the last entry step, gB also interacts with the gH and gL complex to form a pore for the viral capsid to enter into the host cell cytoplasm [10, 11].

The viral capsid in the cytoplasm moves to a nuclear entry pore on the nuclear membrane, and the viral DNA is released into the nucleus through the capsid portal consisting twelve of proteins encoded by UL6 genes [12, 13].

The DNA released into nucleus transcribes the genes in three stages: immediate-early (IE), early (E) and late (L). In the IE stage, infected-cells polypeptide (ICP) 4 is mainly expressed and functions as a transcriptional transactivator for other virus genes by binding to viral promoters [14]. A cellular alpha trans-inducing factor ( $\alpha$ -TIF) protein helps ICP4 to regulate the viral transcription activity in the IE stage [15]. The viral proteins expressed in the E stage play regulatory roles in virus genome replication. The most important E protein is the virion host shutoff (VHS) protein encoded by UL41 genes since VHS blocks host protein synthesis by cleaving host mRNA to support both viral genome replication and viral gene expression [16, 17]. The proteins expressed during the L stage mainly function in capsid formation and in packaging virus particles containing the virus genome, core and capsid proteins. Herpesvirus maturation and tegumentation occur in the perinuclear space and in the cytoplasm, respectively. Following two envelopment processes, including the egress of capsids from the nucleus and the second envelopment from the Golgi, the enveloped virus particles are released from infected cells [2, 18].

### **1.3. Latent infection**

During the HSV-1 latent infection in neuronal ganglia, viral genes named latency associated transcripts (LAT) are expressed to suppress apoptotic cell death by producing miRNA against apoptosis inducing cellular factors such as transforming growth factor-beta1 (TGF- $\beta$ 1), mothers against decapentaplegic homolog 3 also known as (a. k. a.) SMAD family member 3 (SMAD3), trombospondin-1 [19-21], and caspase-8 and caspase-9 [22]. The herpesviral LAT genes can also regulate viral genome replication to establish the latent infection in the infected host [23, 24]. The function of ICP4 is inhibited by neuronal restrictive silencing factor (NRSF) and human repressor element silencing transcription factor (REST) in the latent infection [25, 26]. The latently infected virus can be reactivated by the ICP0 and ICP4 from the dissociation of NRSF in illness or physical stress conditions [27].

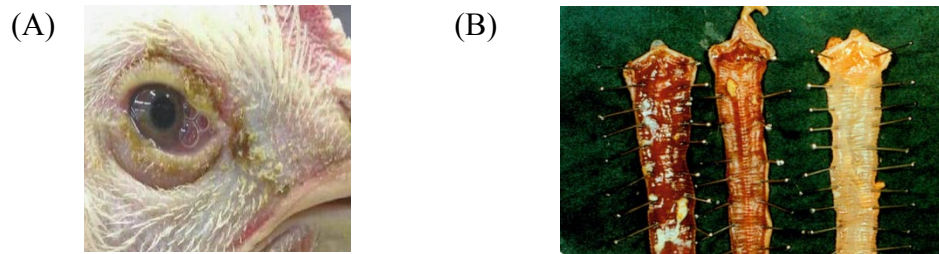
## **2. Infectious laryngotracheitis and infectious laryngotracheitis virus**

### **2.1. Infectious laryngotracheitis**

Infectious laryngotracheitis (ILT) is an acute respiratory disease in the avian species such as chickens [28, 29], pheasants, peafowls [30-32] and turkey [33]. ILT is caused by the infection of ILT virus (ILTV; *Gallad Herpesvirus 1*). Since the first report of fowl ILT in 1925 [34], severe ILT outbreaks have been observed worldwide in Europe, Australia, New Zealand, China, Southeast Asia and the United States where poultry productions are concentrated [35-40].

ILT partially induces the occlusion of the upper larynx and trachea, and intensive inflammation [41]. Chickens in a mild stage of ILT show decreased egg production, watery eyes by conjunctivitis, mild tracheitis, swollen infraorbital sinuses, and a mucoid nasal discharge [42], while a severe stage of ILT induces severe sneezing and coughing, distressed open-mouth

breathing, and bloody mucus [43, 44]. Chickens frequently shake head to remove the bloody mucus since bird's airway and trachea are filled with blood (Figure 1).



**Figure 1.** Clinical signs by ILTV infection.

(A) Swollen, watery eyes (<http://www.worldpoultry.net/background/infectious-laryngotracheitis-28ilt29-targets-broilers-7050.html>). (B) Bloody trachea (left to right: severe, mild and normal) (<http://www.bellsouth.com.au/tech/respiratory%20disease%20in%20breeder%20flocks.html>)

Clinical signs usually appear 6 to 12 days after exposure to ILTV [32, 38]. Though chickens showing breathing difficulty and coughing with bloody mucus usually die within a few days, chickens showing mild clinical signs recover in 10-14 days [45, 46]. The ILTV infection through natural routes can be detected in tracheal tissues from 6 to 12 days, and latent infection in the CNS is achieved at 7 days after an acute infection phase [47-49].

The latent infection in the trigeminal ganglion (TG) in the CNS was established by both vaccine and challenge strains in an experimental study [50]. Though the sporadic ILTV spread is unknown, reactivated ILTV replicates in the respiratory tract and virus can transmit from bird to bird by contact [51]. The reactivation of latently infected ILTV from vaccinated flocks was found in TG post 15 months vaccination [49, 52].

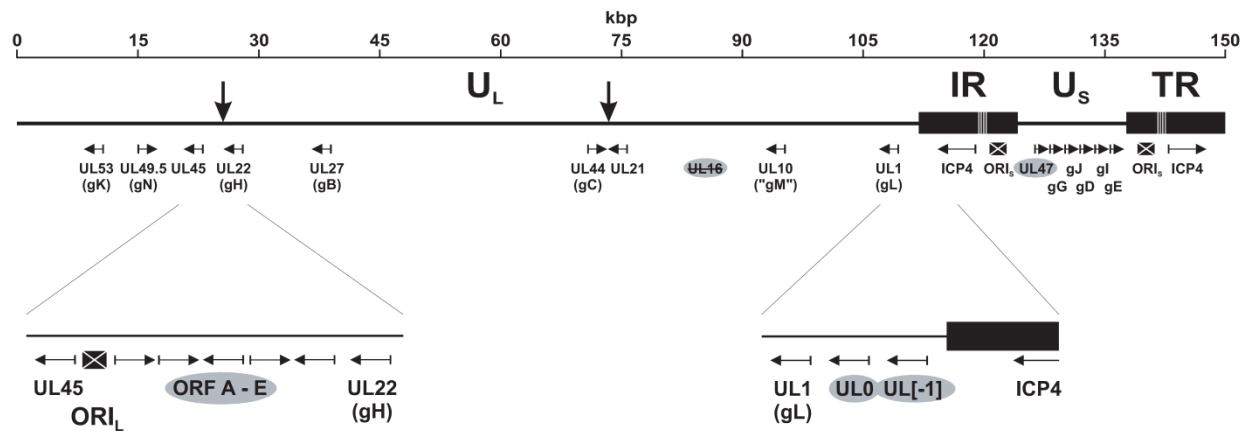
ILT causes high morbidity which is 90-100%, but the mortality varies from 10-20% but can reach up to 70% [38, 45, 46]. The mortality and the decreased egg production by ILTV

infection cause economic losses in the poultry industry. Infected chickens serve as carriers which are the main source of the transmission to uninfected chickens through respiratory routes by coughing and sneezing. People working with ILTV-infected flocks can be candidates to spread ILTV through contaminated materials such as footwear, clothes, vehicles, equipment and even their hands [53].

## 2.2. Infectious laryngotracheitis virus

### 2.2.1. ILTV genome

ILTV is classified as a member of *Iltovirus* genus, *Herpesviridae* family. ILTV has a linearized dsDNA, which is about 150kb in size, consisting of a unique long (UL) and a unique short (US) region flanked by inverted repeat (IR) and terminal repeat (TR) regions (Figure 2) [44, 54-56].



**Figure 2.** Map of ILTV genome [57].

Since partial ILTV sequences were reported in the late 1980s [58], the first full-length ILTV genome sequence was reported in 2006 by the assembly of sequences from six different ILTV strains [59]. The ILTV genome contains unique five open reading frames (ORFs) named



ORF A, B, C, D and E located near a replication origin (ORI<sub>L</sub>) in the UL region that these ORFs have distinct characteristics from other alphaherpesviruses (Figure 2) [60]. The ILTV genome includes three origins for DNA replication as same as shown in other alphaherpesviruses. One Ori<sub>L</sub> is located in the UL region, and two identical ORI<sub>S</sub> are located in the IR and TR sequences (Figure 2) [60]. ORI<sub>S</sub> has not been detected and was excluded in the first known ILTV genome sequence due to its long palindromic sequence characteristic [59]. Of the entire 76 ILTV genes, 63 genes are homologs to those in HSV-1 [61], indicating translational proteins are inferred from the similar structure between ILTV and HSV-1 (Table 1). Eleven conserved ORFs namely UL27, UL44, US6, US8, US4, UL22, US7, US5, UL53, UL1 and UL10 encode glycoproteins of gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM, respectively.

Very recently, the complete genome sequence of a single Australian commercial live attenuated ILTV vaccine strain (Serva) is reported using high-throughput sequencing technology [62]. The Serva ILTV genome encodes 80 predicted ORFs. UL and US regions included 65 and 9 ORFs, respectively, and IR and TR region have three ORFs each. The genome length of the Serva strain was 152,628 bp in size with a G + C content of 48%. The nucleotide sequence is 96.5% identical to the previous mosaic ILTV genome sequence (NC\_006623). Four regions, including 528 bp sequences in the UL29, 594 bp sequences in the UL36, and two 1,563 bp sequences in the repeat regions, which were not identified in the previously known mosaic genome sequence, are newly determined. The size differences of the predicted protein products are also detected in UL54, UL30, UL37 and UL38.

Table 1. Comparison of HSV-1 and ILTV genomes.

<b>HSV</b>	<b>Time</b>	<b>Functions</b>	<b>ILTV (Matched)</b>	<b>ILTV (Unmatched)</b>
ICP34.5	IE			UL-1
ORF-P	IE			UL0
ORF-O	IE			UL3.5
ICP0	IE			sORF4/3
UL1	L	gL	UL1	
UL2	E-L	uracil-DNA glycosylase	UL2	
UL3	L	(P) Nuclear protein	UL3	
UL4	L	(P) Nuclear protein	UL4	
UL5	E	Helicase-Primase complex	UL5, UL8	
UL6	L	portal protein	UL6	
UL7	L	Tegument	UL7	
UL8	E	synthesis of RNA primer		
UL8.5	L	DNA replication		
UL9	E	ATPase and Helicase	UL9	
UL9.5	L			
UL10	L	gM	UL10	
UL11	L	Tegument	UL11	
UL12	E	Alkaline nuclease	UL12	
UL12.5	?			
UL13	L	PK	UL13	
UL14	L	Protein folding	UL14	
UL15	L	DNA cleavage and packing	UL15	
UL15.5	?	(P) Capsid		
UL16	L	(P) virion component		
UL17	L	DNA cleavage and packing	UL17	
UL18	L	VP23 (Capsid)	UL18	
UL19	L	VP5 (Major capsid)	UL19	
UL20	L	(P) Envelop protein	UL20	
UL20.5	L			
UL21	L	Tegument	UL21	
UL22	L	gH	UL22	
UL23	E	TK	UL23	
UL24	L	Nuclear protein	UL24	
UL25	L	Capsid (DNA packaging)	UL25	
UL26	L	Scaffolding protein	UL26	
UL26.5	L	Scaffolding protein	UL26.5	
UL27	L	gB	UL27	
UL27.5	L			
UL28	L	DNA cleavage and packing	UL28	
UL29	E	ICP8	UL29	
UL30	E	DNA polymerase	UL30	
UL31	L	Nuclear phosphoprotein	UL31	
UL32	L	DNA cleavage and packing	UL32	

<b>HSV</b>	<b>Time</b>	<b>Functions</b>	<b>ILTV (Matched)</b>	<b>ILTV (Unmatched)</b>
UL33	L	Capsid (DNA packaging)	UL33	
UL34	L	Nuclear protein	UL34	
UL35	L	VP26	UL35	
UL36	L	Largest tegument	UL36	
UL37	L	Tegument	UL37	
UL38	L	VP19C	UL38	
UL39	E	Ribonucleotide reductase	UL39	
UL40	E	Ribonucleotide kinase	UL40	
UL41	L	VHS	UL41	
UL42	E	DNA polymerase	UL42	
UL43	L	Membrane protein	UL43	
UL43.5	L			
UL44	L	gC	UL44	
UL45	L	Membrane protein	UL45	
UL46	L	VP11 and VP12	UL46	
UL47	L	VP13 and dVP14	UL47	
UL48	L	VP16 and $\alpha$ -TIF	UL48	
UL49	L?	VP22	UL49	
UL49.5	L	Membrane protein (Possible) gN	UL49.5	gN
UL50	E	dUTPase	UL50	
UL51	L	Tegument	UL51	
UL52	E	Helicase-Primase complex	UL52	
UL53	L	gK	UL53	
UL54	IE	ICP27	UL54	
UL55	L			
UL56	L	Membrane protein	UL56	
ICP4	IE	ICP4	ICP4	
ICP22	IE	ICP22		
US1.5	IE			
US2	L	(P) Membrane protein	US2	
US3	L	PK	US3	
US3.5	L			
US4	L	gG	US4	
US5	L	gJ	US5	
US6	L	gD	US6	
US7	L	gI	US7	
US8	L	gE	US8	
US8.5	L			
US9	L	Membrane protein	US9	
US10	L	Tegument	US10	
US11	L			
ICP47	IE	ICP47		
LAT				

The expression time and function of HSV-1 encoding genes were referenced from Fields Virology (5<sup>th</sup> ed.) by David M. Knipe and Peter M. Howley [42].

### **2.2.2. The unique characteristics of ILTV genome**

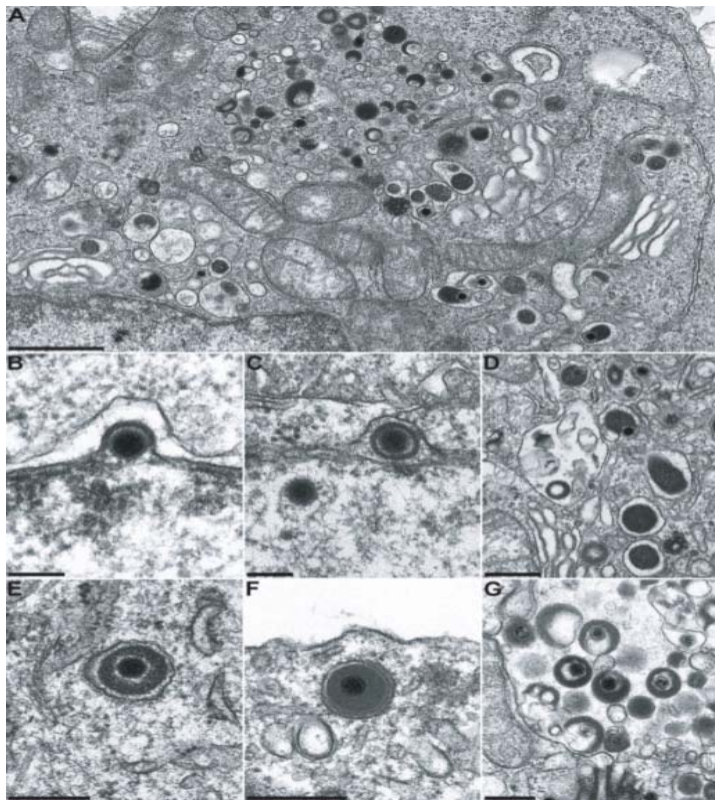
ILTV has unique features and characteristics compared to other herpesvirus species. UL3.5 located in ILTV genome between UL3 and UL4 is not presented in HSV-1, but is conserved in alphaherpesviruses, including BHV-1, BHV-5 and PrV [63-65]. UL16 is not contained in the ILTV genome though it is conserved in all other herpesvirus subfamilies [66, 67]. UL47, which is a major tegument protein located in the UL region of other alphaherpesviruses [61], is present between US3 and US4 on the US region of ILTV genome [68]. A large internal inversion is found uniquely in the UL region of the ILTV genome [60].

ILTV and PsHV-1 were classified as members of the *Iltovirus* genus of the *Herpesviridae* family [59]. Both viruses have an identical inversion of TR region in between UL region and US region, and a translocation of UL47 also has been discovered in both viruses [59]. Genes within the US region [68], and ORF-A to ORF-E localized in the UL region are conserved in both ILTV and PsHV-1 genomes [59, 60]. UL0 and UL(-1) of ILTV show high similarities in deduced amino acid sequence indicating possible ancient duplication [69], while PsHV-1 has only UL(-1), no UL0, in its genome [59]. In genealogy tree, ILTV and PsHV-1 might belong to separate phylogenetic branches in the herpesvirus family, which is confirmed by comparative analyses with the viral amino acid sequences [70, 71]. All these results indicated that ILTV and the mammalian alphaherpesviruses were separated from a common ancestor earlier than the separation into other avian herpesviruses including MDV and HVT [57].

### **2.2.3. ILTV virion structure, morphogenesis and propagation**

Electron micrographs of the reassembly of ILTV particles are very similar to HSV-1 (Figure 3). ILTV virion possesses the hexagonal nucleocapsids which are 80-100 nm in diameter,

and the nucleocapsids are built with icosahedral symmetry and composed of 162 elongated hollow capsomeres [72, 73]. A tegument and an outer envelope membrane containing viral glycoproteins enclose an icosahedral capsid including a DNA-containing core [72, 73].



**Figure 3.** ILTV morphology by electron microscopy [57]. (A)

Micrographs of entire cell and virus micrographs; (B and C) Nuclear egress and primary development; (D and E) Assembly of tegument and secondary envelopment in the *trans*-Golgi; (F and G) Release of virions by exocytosis.

Bars indicate (A) 1  $\mu$ m, (D) 500 nm, (E, F and G) 300 nm and (B and C) 150 nm.

The morphogenesis of ILTV follows that of typical herpesvirus in infected cells (Figure 3) [2, 18, 74]. The capsid formation and viral DNA inclusion occurs in the nucleus (Figure 3A and 3C) and are followed by the nucleocapsids transportation into the cytoplasm by envelopment at the inner and outer leaflets of the nuclear membrane for the first budding (Figure 3B and 3C). The cytoplasmic capsids surrounded by dense tegument are re-enveloped by the second budding into the *trans*-Golgi (Figure 3D and 3E). Matured virions are released by exocytosis (Figure 3F and 3G). A unique feature observed in ILTV infected cells during propagation is to produce

many light (L) particles consisting only tegument and envelope without nucleocapsids, which cannot be replicated (Figure 3D) [18, 74]. The low ILTV titers produce in tissue culture may be due to the excessive production of non-reproducible ghost virion particles.

The in vitro propagation of ILTV was first reported on the choriallantoic membrane of embryonated chicken eggs [75]. ILTV can be propagated in primary chicken embryo kidney (CEK) and liver (CEL) cells [76]. Only one continuously growing cell line, chicken liver tumor cell line (Leghorn male hepatoma; LMH), can propagate ILTV efficiently [77-79]. However, the ILTV titers produced from LMH cells were critically lower than in use of CEK and CEL cells (data not shown).

Cytopathic effects (CPE) showing syncytia and inclusion bodies were detected during ILTV infection in permissive chicken cells at several hours post infection (hpi) [80], and plaques formed by cell lysis were developed 3 to 5 days post infection (dpi). Though the replicative life cycle of ILTV has not been studied sufficiently, it may be similar to that of other alphaherpesviruses such as HSV-1 [67]. The first infectious progeny viruses were detected at 8 to 12 hpi, and maximum number of ILTV replication was reached 24 to 30 hpi [81]. The regulation of ILTV gene expression and DNA replication appeared to follow the way of typical herpesviruse propagation in infected cells [81-83].

#### **2.2.4. Diagnosis of ITLV**

ILTV detection methods include virus neutralization by ILTV-specific antibodies, immunodiffusion, indirect immunofluorescence assay (IFA), enzyme linked immunosorbent assays (ELISA) [48, 76, 84-89]. The gJ- and gC-specific monoclonal antibodies (MAbs) were

developed for the use of diagnostic purposes, such as IFA, immunohistochemistry, immunoelectron microscopy, radioimmunoprecipitations and Western blot analyses [57].

Recently, faster, more accurate and more sensitive methods have been developed to detect ILTV DNA. The methods include dot-blot hybridization using cloned DNA probes [90], in situ hybridization from ILTV infected chickens [91], PCR [88, 92-94], quantitative real-time PCR (qPCR) [95] and restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) [96-99].

### **2.2.5. ILTV proteins and functions**

Generally, herpesviruses produce pathogenically important proteins such as envelop, tegument, capsid, glycoproteins and even non-structural proteins such as thymidine kinase (TK) and transcriptional regulator.

Among 11 glycoproteins encoded in the HSV-1 genome, four glycoproteins, gB, gD, gH and gL which are encoded by UL27, US6, UL22 and UL1, respectively, have critical roles for HSV-1 entry into host cells. The gD binds to TNFRSF14/HVEM, PVRL1 and 3-O-sulfated heparan sulfate, which are entry receptors for HSV-1, and provokes the fusion between host membrane and viral envelop. The gB interacts with a membrane fusion protein, and gH and gL forms heterodimers with gD and gB proteins [100-102]. Moreover, these four glycoproteins are related to the early innate and adaptive immunity of infected host cells [103]. Likewise, ILTV encodes 11 glycoproteins including gL, gM, gH, gB, gC, gK, gG, gJ, gD, gI and gE and a thymidine kinase (TK), and those proteins may critically function in viral virulence and replication.

### **2.2.5.1. Glycoprotein C (gC)**

The gC of alphaherpesviruses is highly conserved and critically functions in virus attachment through the interaction with heparan sulfate proteoglycan (HSPG) chains on the host cell membrane [67, 104]. However, ILTV gC does not bind to heparan sulfate due to the lack of the heparan binding site of about 100 amino acids at the N-terminal end. Thus, ILTV may use different mechanisms from those used by other herpesviruses [105, 106].

### **2.2.5.2. Glycoprotein J (gJ)**

The ILTV gJ, which was previously known as gp60 due to 60,000 D in weight, is encoded at the same position (US5) of HSV-1 [89, 107]. The gJ gene is not found in most other alphaherpesvirus, but EHV-1 has envelope glycoprotein 2 (gp2) which is highly homologous to ILTV gJ sequence [108, 109]. The gJ is processed by N- and O-linked glycosylation and may function in cell-to-cell spread and in the attenuation of ILTV which were shown using  $\Delta$ gJ ILTV, a gJ-deficient form [110].

### **2.2.5.3. Glycoprotein B (gB)**

The gB is highly conserved and functionally similar to that of other herpesviruses [111, 112]. The gB was processed by the addition of N-linked glycosylation and two subunits were produced by proteolytic cleavage [113]. As a fusion protein, gB collaborates with gH and gL for viral entry into the cells [114-117].

### **2.2.5.4. Glycoprotein M (gM) and glycoprotein N (gN)**



In many herpesviruses including ILTV, gM and gN encoded by UL10 and UL49.5 in the ILTV genome, respectively, have to form a heterodimer to be functional [110]. The gN protein of ILTV is O-glycosylated like the gB protein of other herpesviruses, whereas the gM protein of ILTV is not glycosylated, which is different from other herpesviruses [66]. But, both gN and gM proteins are dispensable for viral replication in cultured cells [66, 110].

#### **2.2.5.5. Glycoprotein G (gG)**

The gG, which is encoded by the ILTV US4 gene and conserved among other alphaherpesviruses, is not assembled into virus particles [83] but is secreted from infected cells and possibly has immunomodulating functions [118]. In vivo, gG is a virulence factor of ILTV, but dispensable for virus replication suggesting that gG may become a candidate for ILTV vaccine production [119-121].

#### **2.2.5.6. Glycoprotein E (gE) and glycoprotein I (gI)**

The gE and gI are non-essential proteins and also form a heterodimer for a functional protein. The gE and gI play a role in cell-to-cell spread [122] to the same as those in HSV-1 [123, 124].

#### **2.2.5.7. Glycoprotein L (gL) and glycoprotein H (gH)**

The gL is essential for the replication of ILTV and forms a complex with gH for viral replication [63]. Moreover, gL is not independently anchored with gH in the cell membrane or on the virus envelope [125]. The function of gH in ILTV is not clear. However, based on other

alphaherpesvirus studies, gH of ILTV may be associated with virus entry and cell-to-cell spread and may be nonessential for egress [18, 126].

#### **2.2.5.8. Glycoprotein D (gD) and glycoprotein E (gE)**

The gD and gE of ILTV have not been studied, but it has been shown in DEV-1 infection that gD plays an important function in virus entry and gE is required for cell-to-cell spread [127].

#### **2.2.5.9. Thymidine kinase (TK)**

TK functions in DNA synthesis in living cells through catalyzing deoxythymidine to deoxythymidine 5'-phosphate using adenosine 5'-triphosphate (ATP) [128, 129]. TK encoded from the UL23 gene of ILTV affects the virulence of ILTV in infected cells but not viral replication [130, 131]. TK plays an important role in the reactivation and subsequent replication of alphaherpesviruses from latently infected ganglia [132]. Therefore, TK may become a candidate to study the mechanisms of latent infection, reactivation and virulence of ILTV, and vaccine development.

### **3. Genomics approaches in host-virus interaction**

This thesis mainly focuses on the host-ILTV interactions using microarray and qPCR. Moreover, newly developed bioinformatics programs have been used to analyze extensive data produced from the microarray and qPCR.

#### **3.1. Microarray**

A microarray is a powerful tool to compare the expression of massive number of interesting genes simultaneously. The current microarray concept, which evolved from Southern

blotting method, was first introduced by Maskos in 1992 [133], and the pre-existing approaches for microarray had been reported from 1982 to 1991 [134-137]. Finally, miniaturized microarrays were first utilized in 1995 [138], and a complete genome-wide microarray for eukaryote was developed in 1997 [139]. Cumulative set of DNA probes are utilized on a solid slide, and each probe can be hybridized with a specific target cDNA or cRNA. The expression level is quantified by labeling chemiluminescence dyes such as cyanine 3 (Cy3) and cyanine 5 (Cy5). A microarray slide contains up to hundreds of thousands different probes. Current microarray technology is being used for numerous applications such as gene expression profiling [140], comparative genomic hybridization [141] and SNP detection [142].

### **3.2. Application of microarray in herpesvirus-host interaction**

Microarrays have been used to identify host responses against numerous viruses including herpesviruses such as hCMV and KSHV [143], EBV [144], ILTV [140], VZV [145], MDV [146, 147], HSV-1 [148] and HSV-2 [149].

Host cellular gene expressions in response to herpesvirus infection differed depending on virus types [150]. In case of HVT infection, microarray results showed that cellular gene expression on functions of signal transduction, transcription, scaffolding proteins and the cytoskeleton were regulated differentially in chicken embryo fibroblasts (CEF) [151].

Microarrays on host gene expression with HSV-1 infection reported that IFN-induced antiviral state was blocked through IFN-independent intracellular mechanism [152], and those genes on protein processing, carbohydrate processing, cell adhesion, apoptosis, and host defense and immune response were changed significantly in HSV-1 latent trigeminal ganglia (TG) [153]. In MDV infected CEF cells, data of microarray showed altered host gene expression on

macrophage inflammatory protein, interferon response factor 1, interferon-inducible protein, quiescence-specific protein, thymic shared antigen 1, MHC class I and II, beta-2 microglobulin, clusterin, interleukin (IL) 13 receptor alpha chain, ovotransferrin and a serine/threonine kinase in CEF cells [154]. Also, the critical changes of the gene expression of IL6, IL12 and IFN- $\alpha$  were observed in MDV-infected chicken brain, and IL18 was significantly expressed in only MD-associated transient paralysis (TP) [155]. Differential gene expression of chemokine AH221, B-cell marker BU-1, IgG, IgA, IgM, MHC class II beta chain, granzyme A (GZMA) and signal transducers and activators of transcription 2 (STAT2) genes were found in microarray assay on the resistance or susceptible chickens against MDV infection [156]. The results, found in microarray analysis on EBV infection, showed altered functions in signal transduction, transcription, protein biosynthesis and degradation, cell motility, and shape or adhesion in primary B lymphocytes [157]. Further, EBV infection in alveolar epithelial cells caused the up-regulation of the TGF- $\beta$ 1 pathway resulting in the inhibition of cell proliferation and the increase of caspase 3 and 7 activities [158]. In addition, EBV infection influenced the modulation of DEK, cyclin-dependent kinase (CDK) inhibitor, p53/retinoblastoma (RB) and cascades pathway associated with E2F, activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and STAT in nasopharyngeal carcinoma (NPC) transformation [159]. Microarray assay on hCMV infected human monocyte showed the alteration of host cellular functions such as a unique M1/M2 polarization leading to develop the classical M1 activation phenotype [160].

### **3.3. Bioinformatics**

Bioinformatics has been highlighted as a new approach to manage massive amounts of data from advanced technologies such as microarray or high-throughput DNA sequencing technology. Consequently, the priority of bioinformatics has been to analyze experimental data, to find a prime candidate to predict results and to aid research projects. More powerful and comprehensive analysis program or tools has been required to manage rapid and accurate data analysis [161].

Normally, bioinformatics tools have been categorized into several groups such as homology and similarity analysis, protein function and structure analysis, genomic sequence analysis. The homology and similarity analysis is conducted to identify the evolutionary relationship and divergence of genes among different samples [162]. Protein function analysis is to determine critical motifs and domains, and the secondary structures of predicted protein sequence compared to previous information in databases [163, 164]. The protein structure analysis illustrates a protein's 2D or 3D structures which are essential for the functional study. Genomic sequence analysis programs determine a query sequence thoroughly to identify mutated sequences or regions, evolutionary analysis and compositional bias [165, 166].

### **3.4. Bioinformatics Tools**

The basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) provided from National Center for Biotechnology Information (NCBI) is widely used for comparing gene and protein sequences. To date, several types of BLAST including nucleotide BLAST, protein BLAST, BLASTx, tBLASTn and tBLASTx are available on NCBI website. In NCBI, specialized tools are also available for primer selection, finding conserved domains, gene expression omnibus (GEO), immunoglobulins (igBLAST), transcript and genomic libraries

based on human, animal, plant, microorganism and other genomes. FASTA, which was first introduced by Pearson and Lipman in 1988 using the rapid sequence algorithm [167], have been widely used to search similarity of sequences of either nucleotides or peptides. The European Molecular Biology Open Software Suite (EMBOSS), which is a free open source software for the molecular biology and bioinformatics users [168], contains numerous programs for sequence alignment, codon usage analysis, CpG island detection and analysis, database searching with sequence patterns, protein motif identification and domain analysis, and protein analysis and more. ClustalW is intended for multiple sequence alignment of DNA or proteins (<http://www.clustal.org/>). Biologically meaningful sequences or divergent sequences are determined through the best match of the selected sequences. RasMol is a powerful computer program displaying the structure of DNA, proteins and smaller molecules [169].

The Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>) has been widely used for functional annotation and gene functionality to grasp the meaning of huge number of genes identified by functional genomics data. Using DAVID, gene ontology (GO) terms, functionally related genes and groups, and protein functional domains and motifs can be identified. Moreover, gene pathway maps and related many genes and terms on 2D view can be visualized.

A newly developed program, ingenuity pathway analysis (IPA; [http://www.ingenuity.com/products/pathways\\_analysis.html](http://www.ingenuity.com/products/pathways_analysis.html)) program, helps to understand the complex, dynamic interaction between targeted molecules and surrounding molecules in life science research. To date, IPA has broadly supported various species such as plant, bovine, *C. elegans*, canine, zebrafish, fruit fly, chicken, Rhesus Monkey, chimpanzee and yeast as well as thousands of peer-reviewed journal articles. IPA provides integrated information and insight into

the biological and chemical interactions at various ways with different experimental platforms such as microarray and next-generation sequencing based on the programs own knowledge base system.

#### **4. Host responses against the infection of *herpesvirus* family**

##### **4.1. Host responses by HSV-1 infection**

ILTV genes and genome organization are highly conserved compared to those of HSV-1 indicating close and potential evolutionary relationship between HSV-1 and ILTV [111, 170]. Recently, HSV-1 infection and host responses have been studied using various methods such as microarray [153, 171-174] and qPCR [175-178], and various conditions such as in vitro [179-183] and in vivo [184-186]. The microarray results of the infection of various viruses including HSV-1 provided insights into host virus interactions and further, knowledge obtained from these assays can be compared to systems against infections of other herpesviruses such as ILTV.

HSV-1 infection in neuronally differentiated PC12 cells up-regulated host genes related to proteolytic enzymes for neurite outgrowth/axon remodeling, while the DNA and nucleotide metabolism and apoptosis related host genes were down-regulated suggesting that HSV-1 infection in neuronal cells led to accelerate cell survival and maintain latent infection [187]. In other study, HSV-1 infection leads to the modulation of leukocyte trafficking to inflamed tissues by chemokines, which critically organize the immune response in host cells [188].

In HSV-1 infected cells of the human neuronal cell line, early growth response 1 (EGR-1) is up-regulated, and EGR-1 reduces the HSV-1 LAT gene expression by blocking the immediate downstream site of the TATA box [189, 190]. Furthermore, the mortality of mice was reduced by the depression of EGR-1 expression [191]. In rabbit corneal cells, EGR-1 is rapidly

induced by NF- $\kappa$ B and CREB mediated transactivation by HSV-1 infection [180]. In the mature dendritic cells, tumor necrosis factor (TNF) mRNA is destabilized by the induction of the AU-rich elements (ARE)-binding protein tristetraprolin (TTP) in both the STAT1 and p38-dependent manners [192], and the expression of large multifunctional peptidase 7 (LMP7) mRNA is down-regulated [193]. The expression of chemokine (C-X-C motif) ligand 9 (CXCL9) stimulated by TNF- $\alpha$  and IFN in HSV-1 infected brain is dependent on either TLR2 or TLR9 to induce innate antiviral responses [194]. The recognition of HSV-1 by innate immunity in dendritic cells is occurred in glycoprotein-dependent and TLR2-independent manners [195]. Human leukocyte antigen (HLA)-G which is a nonclassical human major histocompatibility complex class I (MHC-I) and functions in anti-inflammatory responses are up-regulated by HSV-1 infection in human neuroblastoma cells, SK-N-SH and human neurons, NT2-N [196, 197]. Caspase 3 and 7 are activated by HSV-1 infection, and these proteins contribute to HSV-1-dependent apoptosis [198]. Galectin-1 (Gal-1), which is an endogenous lectin functioning in T-cell apoptosis, is up-regulated by HSV-1 infection to avoid from the activation of host immune system by removing activated T-cells [199]. In HSV-1 infection, interferon regulatory factor (IRF), IFN, IL15 and natural killer (NK) cells critically function in host innate immunity according to in vivo study [200]. The data and knowledge on HSV-1-host interaction obtained from microarray studies for HSV-1 could provide the basis on understanding of ILTV-host interaction and host defense mechanisms in ILTV infection for this dissertation.

#### **4.2. The function of HSV-1 viral genes in host responses**

Due to the high genetic and functional conservation between ILTV and HSV-1, the analogical interpretation of the function of ILTV genes can be predicted from previously known



HSV-1 studies. ICP0 protein is expressed in the immediate early stage of HSV-1 infection and promotes the transcription of both viral and cellular genes [201]. During lytic infection, ICP0 acts as an IFN antagonist to block a STAT1-dependent host response involving innate immunity [202], suppresses the expression of SIAH-1, a cellular E3 ubiquitin ligase [203] and deactivates NF- $\kappa$ B and c-JUN N-terminal kinase (JNK) which are the downstream effectors of the TLR signaling pathways [204]. In addition, HSV-1 US7 helps the block of the TLR-mediated NF- $\kappa$ B and JNK through the deubiquitination of TRAF6 and IKK- $\gamma$  [204]. US3 encoding serine/threonine kinase controls the neuronal apoptosis of peripheral nervous region to prevent from virus transmission to the CNS [205].

ICP27 suppresses host gene transcription in addition to cell cycle arrest at the G1 phase and apoptosis [206, 207]. ICP27 is the counterpart to induce the early innate immunity by activating type I IFNs (IFN- $\alpha$  and  $\beta$ ), type III IFNs (IL28 and IL29), TNF- $\alpha$ , CC chemokines ligand (CCL) 5 and CXCL10, and cytokines in macrophages and dendritic cells [208]. ICP27 has critical roles to inhibit STAT/Janus kinase (Jak) pathway by phosphorylation of STAT-1 [209], the secretion of type I interferon-antagonizing protein and IFN signaling by Jak-1 activation [210]. Moreover, ICP27 is related to both a extracellular signal-regulated kinase (ERK) activation and ERK survival activity by producing the AU-rich instability elements (AREs)-containing IEX-1 which is a extracellular signal-regulated kinase (ERK) substrate [211] and is also associated with the stability of the AREs-containing IEX-1 mRNA through the activation of p38 mitogen-activated protein (MAP) kinase (MAPK) pathway [212, 213].

ICP34.5 is associated with viral maturation and egress [214] and acts as a neurovirulence factor for virus growth in the CNS tissue to maintain an appropriate condition for virus replication [215]. Beclin-binding domain (BBD) of ICP34.5 interacts with Beclin 1 (Atg6) to

regulate HSV-1 pathogenesis by the modulation of CD4<sup>+</sup> T-cell responses [216]. The regulation is controlled by interferon regulatory factor (IRF) 3-dependent pathways in cells of the nervous system [217, 218]. ICP34.5 plays a role in the evasion of the host immune system by the dephosphorylation of eukaryotic initiation factor (eIF) 2 $\alpha$  in the cytoplasm [219].

### **4.3. Host responses by MDV**

Marek's disease virus (MDV) serotype 1 is the avian alphaherpesviruses known as *Gallid herpesvirus 2* (GaHV-2) causing viral T cell lymphoma in chickens [220]. MDV-1 has been studied intensively due to the fact that MDV is an oncogenic herpesvirus in cells of the host's immune system. Particularly, the immune responses and viral gene expression during MDV infection have been studied by the microarray analysis [156, 221-223]. The infection of highly oncogenic MDV RB1B strain in the bursa of Fabricius of chickens significantly up-regulates thioredoxin domain-containing protein 5 (TXNDC5), Ras-related protein Rab11A and budding uninhibited by benzimidazoles 3 homolog (BUB3) at 14 and 21 dpi, and IFN- $\alpha$ , IFN- $\gamma$ , inducible nitric oxide synthase (iNOS) and CD4<sup>+</sup> T cells at 4 to 14 days [224, 225]. The up-regulated genes produce tumor-associated proteins functioning in cell metabolism, immune and stress responses, apoptosis and tumorigenesis. In addition, in MDV RB1B strain infection of CEF, MHC class I is significantly decreased, while IFN expression is increased to prevent MHC II-mediated antigen presentation [226, 227]. In contrast, MDV Md11 strain, which is very virulent serotype 1, enhances MHC class II cell surface expressions in CEF cells [228].

### **4.4. The function of MDV viral genes in host responses**

ILTV could be an evolutionary ancient of MDV and HVT. Thus, it suggests that the function of MDV genes may help understand ILTV gene expression comparing to the HSV-1 gene study. The MDV pp38 protein encoded by LORF12 and its splice variants may function in metabolic activity resulting in latency and tumor development [229]. Moreover, the pp38 protein was involved in MDV reactivation [230]. The Meq homodimer, MDV nuclear oncoprotein, is essential to transform T-cell lymphoma by the interplay with c-Jun [231]. The plenty of MDV  $\Delta$ meq lacking domains of the basic leucine zipper (bZIP) and transactivation domains are found in MDV infected cells during apoptosis, while L-meq was highly expressed in MD-derived lymphoblastoid cell lines [232]. Thus, in MDV infected cells, L-meq functions in anti-apoptotic effects, and  $\Delta$ meq acts as a negative regulator in apoptosis.

#### **4.5. Host responses in HVT infection**

In HVT infected CEF cells, the expression of 56 cellular genes involving IFN, signal transduction, transcription, scaffolding proteins and cytoskeleton related proteins are altered [151]. The vnr-13 protein encoded HVT shows 80% homology with cellular Nr-13 which belongs to Bcl-2 family, is an apoptotic inhibitor, and functions to prevent apoptosis and to reduce cellular proliferation [233, 234].

### **5. Control of ILTV outbreak**

#### **5.1. ILTV vaccines**

Commercial attenuated live vaccines have been used to prevent chicken flocks from ILTV outbreaks for many years [42]. Vaccinal laryngotracheitis (VLT) which was caused by the reversion of ILTV vaccine virulence is a current serious issue for the global poultry production.

To overcome this problem, inactivated vaccines and subunit vaccines using a HVT vector containing ILTV glycoproteins such as gI and gD or fowlpox-vectored infectious laryngotracheitis (FP-LT) vaccine have been developed and successfully tested [235, 236]. However, the high production costs and labor intensive immunization method of those approaches have been limited to use in large flocks. Therefore, the development of new vaccines such as genetically engineered live vaccines against ILTV has been demanded continuously.

To find a genetically engineered vaccine candidate, 14 ILTV genes including UL0, ORF-A to E, UL10, UL21, UL23, UL47, UL49.5, UL50, US4 and US5 were tested by individual removal from the ILTV genome. Individual deletions of ILTV genes listed above did not influence to ILTV propagation in cultured cells, indicating those genes are not necessary for virus replication. In turn, ILTV deletion mutants have been tested in vivo animal system as live virus vaccine candidates [44]. The deletion mutants of UL23 (TK gene), US4 (gG), UL47 (tegument protein), US5 (gJ) and UL0 (nuclear protein) completely decreased the virulence of  $\Delta$ ILTV [83, 130, 237-239]. UL50 (dUTPase) deletion mutants might be applied in low doses though the high doses of the mutant caused significant virulence in terms of intratracheal infection [81]. The gJ and possibly gG deleted mutants would serve as marker vaccines for the differentiation of infected from vaccinated animals such as DIVA strategy [238, 240]. These results suggest that a genetically engineered ILTV strain might substitute for current vaccines to a new vaccine candidate.

The objectives of this dissertation research are: 1) host responses against virulent ILTV infection in primary chicken embryo lung cells using microarray assay; 2) host responses against vaccine ILTV infection in primary chicken embryo lung cells using microarray assay; and 3)

expression analysis of whole ILTV encoding genes at different time points using quantitative PCR technique.

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## **CHAPTER 2**

### **Transcriptional Profiling of Host Gene Expression in Chicken Embryo Lung Cells Infected with Laryngotracheitis Virus**

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## 1. Abstract

Infection of infectious laryngotracheitis virus (ILTV; *gallid herpesvirus 1*) causes acute respiratory diseases in chickens, resulting in a high mortality rate. To better understand host-ILTV interactions at the host transcriptional level, a microarray analysis was performed using 4 X 44K Agilent chicken custom oligo microarrays. Microarrays were hybridized by using the two color hybridization method with total RNA extracted from ILTV infected chicken embryo lung cells at 0, 1, 3, 5, and 7 days post infection (dpi). Results showed that 789 genes were highly altered in their expression during the time courses of ILTV infection. The differential expressions include genes responsible for immune responses (cytokines, chemokines, MHC, and NF-kB), cell cycle regulation (cyclin B2, CDK1, and CKI3), matrix metalloproteinases (MMPs) and cellular metabolism. Differential expressions for 20 out of 789 genes were confirmed by quantitative reverse transcription-PCR (qRT-PCR). A bioinformatics tool (Ingenuity Pathway Analysis) was used to analyze biological functions and pathways on the group of 789 genes that exhibited highly altered expression, resulting in that 275 genes were classified into a number of functional groups including cancer, genetic disorder, cellular growth and proliferation, and cell death. Furthermore, 21 possible gene networks indicating the intermolecular connections among 275 functionally identified genes were generated using the gene network analysis. Results provide comprehensive knowledge on global gene expressions, and biological functionalities on differentially expressed genes in the host responses to ILTV infections.

## 2. Introduction

Infectious laryngotracheitis virus (ILTV; *gallid herpesvirus 1*) is the only member of the *Iltovirus* genus of the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family. ILTV includes ~150 kb of linear dsDNA genome consisting of two unique regions (unique long; UL and unique short; US), inverted repeats (IR) and terminal repeats (TR) flanking the US region [15]. About 76 open reading frames (ORFs) have been shown to express viral proteins [53]. The genome structure and gene contents of the ILTV genome clearly proved its classification as an *alphaherpesvirus* [45]. Infection of ILTV causes upper respiratory diseases in chickens during lytic infection, and ILTV can establish latency in the central nervous system. ILTV infection causes severe respiratory diseases such as increased mucus formation in the trachea and tracheal hemorrhage. In acute cases, there has been up to 70% mortality in infected chickens. Currently, live attenuated vaccines, which are developed from chicken embryo or cultured cells, are commercially available to control ILT disease [3]. However, vaccinal laryngotracheitis (VLT), which is caused by the reversion of vaccine virus to virulence by spreading from vaccinated- to unvaccinated birds, is critically associated with live attenuated ILTV vaccines [8, 19].

Microarray methodology was developed as an epochal method to simultaneously analyze enormous data sets for gene expression patterns in various biological conditions [28]. Microarrays have been used to investigate host responses to the infection of various viruses such as Epstein-Barr virus (EBV) [5, 7, 36], varicella-zoster virus (VZV) [25], human cytomegalovirus (HCMV) [6], Marek's disease virus (MDV) [21, 33, 38, 46, 47], herpesvirus of turkey [26], herpes simplex virus-1 (HSV-1) [9, 40], hepatitis virus [41], human immunodeficiency virus (HIV) [10, 16, 50, 54] and coxsackieviruses [52].

ILTV, which is a special type of herpesvirus causing acute respiratory diseases, has not been studied sufficiently in genetic phases of host-virus interactions. Thus, the objective of this study was to understand host responses to ILTV infection in cultured chicken embryo lung cells using microarray analysis. The microarray used in the current study contains 44K chicken genes including functionally identified genes, predicted ORFs, ESTs, genomic contigs, chicken microRNAs and various control spots [32]. Genes were sorted into three groups according to the level of alterations at four different days post infection (dpi) time points. The 20 genes showing the great alterations during the time course of ILTV infection were validated by qRT-PCR.

Microarray data sets for genes expressed differentially can be interpreted further by clustering analysis. Many of the heuristic clustering methods have several shortcomings, including the determination of the number of clusters which generally is unknown when there is no prior knowledge of the number or there no other information about the structure of the data to be clustered. A model-based clustering method can overcome the critical drawback by estimating the number of clusters in clustering analysis, treating a clustering problem as a model selection problem over a variety of candidate models specified by different numbers of clusters and distribution. The best model is selected on the basis of a model selection criterion, providing the optimal number of clusters and assigning cluster membership to observations simultaneously.

Importantly, the functional analysis of differentially expressed genes should be followed by gene discovery research. However, it is difficult to find which gene (or set of genes) is a core regulatory factor or how genes interact with each other in a specific biological mechanism. The Ingenuity Pathway Analysis (IPA) program was developed to analyze large data sets such as microarray data in biological functionalities, gene networks, and physiological pathways [37]. Differentially expressed genes obtained from the microarray analysis of chicken lung cells

infected by ILTV were analyzed by using IPA program to find biological functionalities and molecular interactions.

### **3. Results and discussion**

#### **3.1. Gene expression profile of lung cells infected by ILTV**

Primary chicken embryo lung cells at passage 1 were infected by the USDA reference strain of ILTV and cells were collected at 1, 3, 5, and 7 dpi. Cytopathic effects (CPE) were observed from 3 dpi, which became more severe by 5 dpi, and finally massive cell disruption observed at 7 dpi (Figure 1). Total RNA was isolated from both controls and infected lung cells at each dpi time point and subjected to microarray analysis.

To control dye bias effect, spike-in control mixtures were utilized by mixing with RNA samples according to manufacturer's recommendations, and in the recommend quantity (Methods). The spike-in RNA controls consist of two sets of synthetic RNA mixtures derived from the Adenovirus E1A genes with different concentrations in each set [55]. Agilent chicken 4X44K oligo gene expression array contains 320 spike-in indicating spots to be hybridized with spike-in controls of both A mix, which was hybridized with Cy3, and the B mix hybridized with Cy5 on each array. These spike-in sets were mixed with either uninfected control or infected samples and co-hybridized to arrays. The ratio of signal intensities for all spike-in spots were calculated and evaluated, resulting in that no significant dye effects were detected for all array slides (data not shown) as reported previously [55]. All raw and normalized data were deposited to Gene Expression Omnibus (GEO) and the accession number is GSE20630.

Normalized signal intensities were subjected to statistical analysis to find differentially expressed genes during ILTV infection in cultured lung cells. The 44K array produced 11,491 genes that showed significant signal intensities that were sorted by signal to noise ratio (SNR) >3, meaning that real (forward) signals of the samples were three times greater than background signals. In order to discover the expression patterns over time in the data, a model-based

clustering method [14] is used for clustering the gene expression profiles. A key drawback in heuristic clustering techniques is that they are difficult *a priori* to determine the number of clusters. The method enables the number of clusters to be determined by estimating the number of components in a multivariate normal mixture model from which the data are generated. The clustering analysis results in three clusters, and Figure 2 presents these clusters. 789 genes showed significant differential fold changes in response to ILTV (Group 1), 6,265 genes displayed moderate alterations (Group 2), and 4,437 genes revealed no alterations during ILTV infection at four time points in chicken lung cells (Group 3). Of the 789 genes in Group 1 exhibiting considerable changes in differential expression in response to ILTV (see supplemental Table 1), the top 10 % (79 genes) were sorted by statistical analysis based on the highest value of standard deviations from the mean values of four different time points (Table 1). This is for the purpose to gain insights into genes with more significant alterations during the time course. Out of the 789 genes, 390, 370, 320, and 422 genes were down-regulated, while 399, 419, 469, and 367 genes were up-regulated at 1, 3, 5, and 7 dpi, respectively.

### **3.2. Quantitative reverse transcription-PCR (qRT-PCR)**

To validate the microarray data, 20 of 789 genes were subjected to qRT-PCR with the same RNA samples used in the microarray analysis using gene specific primer pairs (Table 2). Results were analyzed by  $2^{-\Delta\Delta Ct}$  method to determine relative levels of gene expression at each dpi time points compared to uninfected control [34]. There were no differences found between data of microarray or qRT-PCR at any dpi time point (Table 3). However, it should be noted that fold change values for certain genes obtained by qRT-PCR analysis showed much greater expression levels than those observed in the microarray analysis. For example, fold changes for

gene expression for matrix metalloproteinase (MMP) 27, interleukin (IL) 6, fatty acid binding protein (FABP) 4, IL8, and CXC chemokine K60 at 3- or 5 dpi showed much higher levels in qRT-PCR analysis compared to fold changes shown in microarray analysis. This qualitative difference between methodologies may be attributed to the upper detection limits of the fluorescent intensities for the array scanner.

Both quality control data taken with spike-in controls and the results of qRT-PCR indicate that the microarray data sets for differential gene expression are valid to investigate genome-wide differential expression patterns for host responses during ILTV infection.

### **3.3. Expression clustering**

The pattern of differential gene expression over time can provide insights into biologically functional relevance among genes. In the present study, a model-based clustering method [14] was used to cluster alteration patterns for the 789 differentially expressed genes in response to ILTV infection and revealed 7 gene clusters exhibiting distinct expression patterns (Figure 3 and supplemental Table 2). The 287 genes placed in cluster (C) 1 showed only nominal increases at 3 and 5 dpi followed by decreased expression levels at 7 dpi that were similar to those at the onset of the experiment. The C2 representing 97 genes exhibited a dramatic increase in gene expression only at 7 dpi, whereas the expression levels of the 90 genes in C3 progressively declined at 5 and 7 dpi. Three genes in C4 showed higher levels of expression from the early infection stage (1 dpi), sharp increases at 3 and 5 dpi, and slight declines at 7 dpi. Expression patterns of 9 genes in C5 showed slightly lower expression at 1 dpi relative to the other time points, and then dramatically increased in 3 and 5 dpi, followed by decreased expression at 7dpi. The 85 genes in C6 showed lower levels of expression in 1 dpi and then



expression was progressively increased during the later time points, whereas C7 representing 218 genes showed higher expression levels in the 1 dpi and progressively decreasing at 3, 5, and 7 dpi. GenBank accession numbers for genes in each cluster are shown in supplemental Table 2.

Interestingly, the genes in C4 that exhibited the highest expression during ILTV infection include cytokines (IL8 and IL1-beta) and a chemokine (CXC-K60), while in the C5, IL6 showed the most highly expressed. From these findings, it is reasonable to hypothesize that expression of functionally relevant genes such as cytokines and chemokines released in response to an immune challenge may be regulated similarly during a specific biological condition.

### **3.4. Functional clustering**

Recently, new bioinformatics tools have been developed to facilitate efficient analyzing for biological functionalities for large number of differentially expressed gene sets obtained from microarray analysis. By using the IPA program (<http://www.ingenuity.com/>), bioinformatics aspects of differentially expressed genes during ILTV infection were analyzed for the relevance of gene functionalities and gene networks. Since gene functionalities and network analyses used in IPA program are based on the genetic information of mammalian species including human, mouse, and rat, data drawn from the chicken microarray were analyzed depending on mammalian biological pathways. In this study, whereas 789 differentially expressed genes were used as the input number of genes, only 275 have been characterized with specific cellular functions. Also, other bioinformatics tool, Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (<http://david.abcc.ncifcrf.gov/>), was utilized to identify gene functionalities, resulting in that the similar number of genes were identified for their biological functions to the data obtained with IPA program (data not shown). Furthermore, the biologically

functional categories for gene information are also similar to those of IPA analysis (data not shown). Thus, the following bioinformatics results are based on the group of 275 genes obtained from the IPA program.

The group of 275 differentially expressed genes were clustered as to 65 functional groups (see supplemental Table 3) and the top 25 functional groups of genes are displayed in Figure 4. The main categories for gene functionalities include diseases and disorders, molecular and cellular functions, and physiological system developments. Gene information was repeatedly used in multiple clusters of functionalities due to the multi-functional characteristics for designated genes. It can be seen that the cancer related function contains the highest number (140) of genes, while 125 genes were involved in genetic disorders, and 54 genes were clustered as inflammatory responses.

### **3.5. Gene network analysis**

In addition to functional clustering analysis, gene network analysis was performed using the IPA program, resulting in that top 6 network groups were generated from differentially expressed genes (supplemental Table 4). The network analysis represents the intermolecular connections among interacting genes based on the functional knowledge inputs contained within the IPA program. Independent networks were generated in each dpi sample group with considering alteration levels of expression, and 21 possible gene networks for all dpi groups were obtained from the 275 functional input genes. Of the 21 potential gene networks, only top 6 identical networks were observed across all dpi time points. It might be caused by different sets of focus molecules, which were generated based on different p-values calculated by the IPA algorithms with the altered fold change values in each dpi group. Dynamics of alterations in gene

expression for a subset of genes during the time course of ILTV infection can provide insights into biologically interacting genes within a network displaying functional similarities. The most strongly interactive network (network #1) is presented in Figure 5 whereas the other networks are shown in S1 in the supplemental materials.

The network #1 is closely associated with a signaling pathway of IL6, which is a cytokine known to be involved in cell proliferation and inflammatory responses [27]. The top functions related with genes in network #1 involve cancer, gastrointestinal disease, and the cell cycle. Interestingly, expression of certain genes in network #1 such as CDC20 (cell division cycle 20 homolog), PTTG1 (pituitary tumor transforming 1), CDC2, and Cyclin B, which function for cellular proliferation by the cell cycle progression, appeared to be inversely regulated with the time course response of IL6 expression to ILTV infection. When considered functionalities of genes in network #1, the dynamics of alterations in gene expression over time during ILTV infection suggests that ILTV infection elevates IL6 expression followed by the inhibition of cellular proliferation. In contrast, expression patterns of HPGD (hydroxyprostaglandin dehydrogenase 15-NAD), SOCS (suppressors of cytokine signaling), JAK (Janus kinase 1), and NASP (nuclear autoantigenic sperm protein) exhibited expression patterns that were independent to the IL6 expression pattern (Figure 5A, 5B and S1. A). The consistent downregulation of JAK, which is known to enhance cellular proliferation through signal transducer and activator of transcription (STAT) pathway that can be suppressed by IL6 signaling [22], supports the repression of cellular proliferation by ILTV infection. The top functions of genes in network #2 are involved with cellular compromise, connective tissue disorders, and post-translational modifications. Several heat shock proteins (HSP) were focused in this network (see S1. B). Heat shock proteins, especially HSP70 family as molecular chaperones, are known to interact with

viral early immediate genes in HSV-1 genomic DNA replication [31]. Interestingly, the expression of several HSPs in the network #2 are decreased consistently except in the 7 dpi, suggesting that lowered level of HSPs until 5 dpi may lead to production of erroneous structures of ILTV virions, resulting in the low titers of ILTV in tissue culture, which barely exceed on infectious unit per cell [15, 18]. Network #3 contains genes for growth factors and matrix metalloproteinases (MMPs), and the genes have top functions associated with endocrine system development and function, carbohydrate metabolism, and digestive system development and function (see S1. C). Levels of expression for growth factors and MMPs are increased from 3dpi and maintained higher levels until 7dpi. Infection of herpesviruses, such as HSV and HCMV, lead to an increase of the expression of growth factors and MMPs for extracellular remodeling, tissue invasion and angiogenesis [20, 35, 48]. Networks #4 and #5 contain genes of the cytokines (IFN beta and IL1B), chemokines (CCL20 and CCL4), and NF-kB families (NF-kB and NFIB) and the top functions involve organism injury and abnormalities, antigen presentation, cell mediated immune responses, lipid metabolism, small molecule biochemistry, and molecular transport (see S1. D and E). Finally, genes in network 6 contain IL1, NF-kB, and ID1 that function in cardiac inflammation, cardiovascular disease and inflammatory response (see S1. F). Interacting molecules found in networks #4, 5, and 6 are mostly focused on the host immune responses against pathogenic inflammations.

The network analysis suggests that a large number of biological pathways, regulated by various sets of genes, closely interact each other in host responsiveness during ILTV infection. More detailed interactions among genes showing altered expression levels in each network are currently under investigation to find unknown host-response mechanisms in addition to general immunological reactions during ILTV infections.

The altered fold changes for interesting molecules that were associated with cellular immune response, cell signaling, MMP molecules, cytokines, chemokines, and cell proliferation are plotted individually (Figure 6) since those molecules are noteworthy on clarifying the interaction of host lung cell and ILTV. Four matrix metalloproteinases (MMPs) including MMP 7, 13, 16, and 27 were differentially expressed during ILTV infections. Previously, MMP 1, 2, and 9 were shown to function in cell invasions of primary human endothelial cell in Kaposi's sarcoma-associated herpesvirus (KSHV) pathogenesis [42]. The viral oncoprotein meq in MDV is known to activate MMP3 transcription [2]. Furthermore, in HCMV infection, the balance of MMP9 and tissue inhibitor of metalloproteinases 1 (TIMP1) was changed in human macrophages, and the MMP9 activity was declined in infected cells. It could be proven that HCMV infection may affect atherogenesis in mice through the control of MMP9 expression. [48]. Taken together, these results suggest that MMPs generally play a role in herpesvirus pathogenesis, but different isoforms of MMPs may be capable of responding to specific herpesviruses. Furthermore, in the present study with chicken lung cells, expression of surfactant protein A (SFTPA-1; GenBank accession - AF411083; Table 1 and 3) was downregulated by ILTV infection. When considering that surfactant protein A is a transcriptional indicator of EGFR (epidermal growth hormone receptor) signaling pathway [11], EGFR signaling pathway was suppressed during ILTV infections. This result is consistent with the downregulation of EGFR functions in hCMV infected human lung [11] and foreskin fibroblastic cells [23]. Also, our result is same as the mRNA expression of SFTPA-1 that was decreased after inoculation of influenza A virus H9N2 into chicken lung cells [44]. Ovotransferrin (Otrf; GenBank accession- X02009; Table 1 and 3) is related with both iron transport and antibacterial activities. In the present study, the expression level of Otrf was increased in ILTV infected cells (Figure 6A). The

finding may lead to similar results that the upregulation of Otrf in MDV infection could protect the spread of MDV in chicken embryonic fibroblast [17]. Otrf accelerates the expression of immune response gene such as IFN- $\gamma$  against MDV infection [12]. Furthermore, lactoferrin which is homologous form of transferrin in mammals showed antiviral activity against canine herpesvirus [51]. It has been hypothesized that the anti-viral activity of Otrf may be similar to the anti-HSV capability of mammalian transferrins [24]. IL6 plays a role in both pro-inflammatory and anti-inflammatory responses [27]. The elevation of IL6 expression during virus infection is one of well-characterized immune responses in the pathogenesis of various viruses, such as Dengue virus in human [30], or HSV-1 in mice [29]. Also, the expression of IL6 is increased by KHSV-encoding miRNA [43]. Similarly, expressions of IL8 and IL1B were upregulated in ILTV infected cells, and gene expression pattern were observed in MDV infected chicken lung [1]. These are consistent with previous findings of NF-kB activation via IL8 signaling pathway by human herpesvirus (HHV)-8 infection [49] and HHV-6 infection in peripheral blood mononuclear cell cultures [13], respectively.

In addition to the well-characterized host-virus interactions, a variety of unique responses were drawn from the microarray analysis of ILTV infection. For instance, the expression level of vasoactive intestinal peptide (VIP; GenBank accession number- U09350; Table 1 and 3) was decreased dramatically in ILTV infection, but the functional aspects were not determined. Likewise, genes related to various metabolic enzyme functions such as acyl-CoA synthetase long chain-1 (ACSL1; GenBank Accession number- AJ851480; Table 1 and 3) were differentially expressed in ILTV infected cells, but the precise mechanisms have not been verified. Therefore, further investigations are being performed to identify unique and more deeply involved interactions between host and ILTV.

## **4. Materials and methods**

### **4.1. Cell culture and ILTV infection**

Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Chicken embryo lungs were isolated from 19 day old specific-pathogen free (SPF) chicken embryos (Charles River Laboratories, North Franklin, CT, USA). Lung tissues were homogenized and incubated in a 0.125% trypsin solution for 30 min at room temperature (25°C). Cells dissociated from lung tissues were suspended in a 1:1 ratio of mammary epithelial growth media (MEGM; Lonza, Rockland, ME, USA) and Dulbecco's Modified Eagle's Medium (DMEM, 0.45% glucose) plus 2% fetal bovine serum (FBS), 100units/ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamine in 10 cm tissue culture dishes (Sarstedt Inc., Newton, NC, USA) pretreated with 0.5% gelatin in PBS to improve cell adhesion. Cultured cells were grown at 39°C containing 5% CO<sub>2</sub> until cells reached confluent monolayers (2 to 4 days). The USDA reference strain of ILTV (National Veterinary Services Laboratories, Ames, IA, USA) was used to infect the chicken embryonic lung cells at a multiplicity of infection (m.o.i.) of 0.1. Infected cells were incubated at 37°C for 1hr with rocking gently every 15 min. After the incubation, 10ml of media, 1:1 MEGM/DMEM, were added to each culture dish, and the cells were incubated at 37°C in 5% CO<sub>2</sub> for up to 7 days. This research was performed under the permitted protocol approved by both the Institutional Biosafety Committee (IBC; permit number: 10007) of University of Arkansas and the Animal and Plant Health Inspection Service (APHIS; permit number: 102743) of United States Department of Agriculture (USDA).

### **4.2. Total RNA extraction**

Total RNA was extracted from uninfected- or ILTV infected chicken embryonic lung cells at 1, 3, 5, and 7 dpi using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was treated with DNase I (New England BioLabs Inc., Ipswich, MA, USA), and RNA was re-purified by the TRIzol reagent. The quality of RNA was checked by fractionation on an agarose gel (data not shown).

#### **4.3. Probe labeling and microarray hybridization**

A two color labeling microarray system was used to compare uninfected- and ILTV infected embryonic lung cells at 1, 3, 5, and 7 dpi. Fluorescently labeled complementary RNA (cRNA) probes were generated by using the Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA, USA) and following the manufacturer's instructions. RNA spike-in controls were used to adjust possible dye effects following manufacturer's instructions. The Spike-in controls represent two sets of ten synthesized RNA mixtures derived from the Adenovirus E1A transcriptome with different concentrations in each set [55]. These spike-in sets were mixed with either uninfected control or infected samples and co-hybridized to arrays. Briefly, 2 µg of total RNA were mixed with Spike-ins and converted to cDNA using reverse transcriptase and oligo dT primers in which T7 promoter sequences were added. T7 RNA polymerase was used for the synthesis and labeling of cRNA with either Cy3 dye for the uninfected control or Cy5 dye for the ILTV infected samples. The fluorescently labeled cRNA probes were purified using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), and the concentration, fluorescent intensities, and quality of labeled cRNA probes were determined using a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). An equal amount (825ng) of Cy3 and Cy5 labeled cRNA probes were hybridized on a 4X44K Agilent



custom chicken oligo microarray (array ID: 017698). The hybridized slides were washed using a commercial kit package (Agilent Technologies, Palo Alto, CA, USA) and then scanned using a Genepix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA) with the tolerance of saturation setting of 0.005%. Three biological replicates were conducted.

#### **4.4. Microarray data collection and analysis**

Background-corrected red and green intensities for each spot were used in the subsequent analysis. Global normalization based on local polynomial regression (loess) was applied to the intensities to remove effects that were due to undesirable systematic variations in microarray experiments rather than biological differences. The average values of the resulting normalized expression values in replicate hybridization sets were considered in the subsequent analysis. In order to identify a set of genes with high alterations over time, a model-based clustering method [14] was employed, and the genes in the cluster were considered as differentially expressed over the time period. All analytic techniques were implemented in R (<http://www.R-project.org>).

#### **4.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Reverse transcription was performed with 3  $\mu$ g of total RNA using Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) with oligo dT<sub>12-18</sub> primers (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The reverse-transcribed products were diluted by 1:10 ratio and a portion (1  $\mu$ l) of each product was subjected to qPCR under the following conditions: 40 cycles of 95°C for 30 s, gene-specific annealing temperature for 62°C for 1 min, extension for 30 s at 72°C, and a final extension at 72°C for 10 min. A non-template control and endogenous control (chicken GAPDH) were used

for the relative quantification. The differential expression levels for the ILTV infected group were compared by the  $2^{-\Delta\Delta CT}$  method against the uninfected controls [34]. Primers for qRT-PCR were designed using Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>) with these parameters: amplicon length, 95–100 bp; primer length, 18–27 nucleotides; primer melting temperature, 60–64°C; primer and amplicon GC content, 20–80%; difference in melting temperature between forward and reverse primers, 1–2°C. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer information is listed in Table 2.

#### **4.6. Bioinformatics**

Functional interpretation of differentially expressed genes was analyzed in the context of gene ontology and molecular networks using the Ingenuity Pathways Analysis (IPA) 6.5 software (Ingenuity Systems®; [www.ingenuity.com](http://www.ingenuity.com)). The differentially expressed genes were categorized, compared to genetic categories in the IPA database, and ranked according to p-values. [39]. The IPA analysis determined the subcategories within each category which is supplied with an appropriate p-value and the number of genes identified. Since the size of the created network could potentially be enormous, the IPA software limited the number of molecules in the network to 35, leaving only the most important ones based on the number of connections for each focus gene (focus genes= a subset of uploaded significant genes having direct interactions with other genes in the database) to other significant genes [4].

## **5. Authors' contributions**

JYL and BWK designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. JJS contributed in statistical analysis of microarray data, and AW participated in virus preparation and helped analysis of the qRT-PCR data. XL and HZ contributed the analysis of microarray data. All authors read and approved the final manuscript.

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**Table 1.** Top 10% (79 out of 789 genes) of the most highly differentially expressed.

Accession #	Gene Symbol	Fold Change			
		Day 1	Day 3	Day 5	Day 7
Y14971	CXC chemokine K60	3.7	17.3	19.7	11.3
X65459	FABP7	1.1	0.3	0.8	0.2
X16881	CDC2	1.9	0.4	0.7	0.4
X03509	CKB	0.9	4.7	5.5	2.0
X02009	LTF	0.7	2.7	3.6	3.2
U62026	CENPF	3.3	0.7	1.4	0.6
U12438	RFC2	1.9	0.8	1.0	0.3
U09350	VIP	2.0	0.2	0.3	0.2
M16199	IL8	3.7	22.4	26.8	18.5
CR733296	LIPG	1.0	0.7	0.6	0.1
CR523746	TMEM196	1.0	2.3	2.8	0.7
CR406543	SELO	1.0	0.9	1.1	4.1
CR406252	Prematurely terminated mRNA decay factor-like	1.2	1.1	1.4	4.7
CR391404	ITGA8	0.7	0.8	0.5	0.2
CR391234	LL	1.5	0.3	0.6	0.2
CR387914	CHAD	1.1	0.8	0.7	0.2
CR385491	IDI1	1.3	0.8	0.9	0.2
CR385166	MYCN	0.6	1.0	1.1	5.7
CR385124	DHCR7	1.3	0.9	1.0	0.3
CR382435	HDGFRP3	0.8	0.9	0.9	5.1
CR352395	OSTN	0.8	0.9	0.9	4.2
CO635775	HSP90AA1	0.8	0.5	0.5	2.6
CN218923.1	ARHGEF9	1.1	1.3	0.9	5.5
CF250950	ALDH1A3	0.6	2.1	2.3	3.6
CD763113	FDPS	1.1	0.6	0.7	0.2
BX936026	AURKA	2.4	0.5	0.7	1.0
BX935864	XBP1	0.9	0.8	0.7	3.4
BX935550	AKR1D1	0.6	2.9	2.7	1.2
BX935026	MAT1A	0.7	1.0	0.7	3.5
BX934121	TFPI2	0.9	1.5	2.2	5.5
BX932212	PTTG1	2.1	0.7	0.8	0.3
BX931971	SPON2	1.6	2.8	4.0	12.1
BX931663	ROPN1L	0.7	2.7	1.1	5.6
BU456021	SNAI1	1.9	1.0	0.7	0.4
BU409770	HMG_COA_S	2.5	1.4	1.2	0.3
BU200000	TNFAIP6	1.0	1.9	4.0	0.4
BU138507	CYP51	1.0	0.7	0.7	0.2
AJ851480	ACSL1	1.2	7.3	8.7	7.3
AJ721110	VNN2	0.5	3.3	6.5	2.5
AJ721107	SLA	0.8	1.1	1.4	5.0
AJ720861	LSS	1.3	1.1	1.3	0.2
AJ720657	DNAJB9	0.9	0.9	0.8	3.8
AJ720217	STARD4	1.8	0.9	1.0	0.3

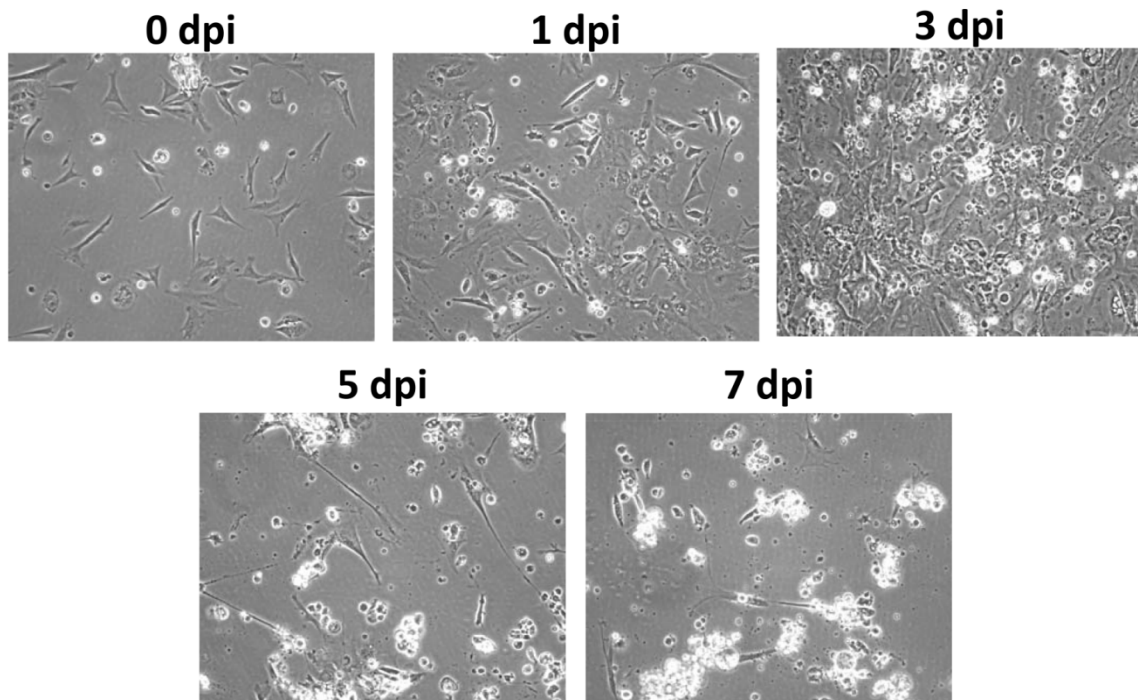
AJ719858	ITFG1	1.3	1.0	1.2	4.8
AJ719718	SC4MOL	1.1	0.8	1.1	0.2
AJ719295	INSIG1	1.5	0.9	0.9	0.2
AJ443395	TRIP13	2.1	0.6	0.7	0.4
AJ393939	ITPR3	1.5	1.4	1.8	0.4
AJ309540	IL6	0.9	4.8	7.2	3.1
AJ004940	HSPA8	0.9	0.5	0.6	2.7
AF432506	FABP4	1.0	5.3	7.1	14.9
AF411083	SFTPA1	1.7	0.4	0.9	0.4
AF070478	MMP-13	0.7	3.4	2.9	0.7
AF062392	MMP27	0.8	4.1	6.6	3.5
AB031398	LEFTY2	1.2	1.7	1.8	5.8
BU306841	NSDHL	1.5	0.6	0.8	0.1
BU144940	ATF3	0.6	0.9	2.0	3.2
BU106686	MKI67	2.6	0.8	1.1	0.5
BU336892	HSPH1	1.0	0.5	0.5	3.2
CR385186	PREDICTED: similar to CUG2	2.2	0.5	0.8	0.4
CR387761	PREDICTED: similar to Gap junction alpha-7 protein	1.2	0.7	0.8	0.2
BU456843	PREDICTED: similar to Cancer susceptibility candidate 5	2.9	0.7	1.2	0.6
BU468099	PREDICTED: similar to Histone protein Hist2h3c1	2.5	0.9	0.9	0.4
BX950657	PREDICTED: chemokine (C-C motif) receptor-like 1 isoform 1	1.1	1.3	2.5	0.3
CR390562	PREDICTED: hypothetical protein	1.2	1.2	1.3	6.2
CR388632	Unknown	0.7	1.4	0.8	0.2
BU212825	Unknown	0.7	0.6	0.6	0.2
BU281664	Unknown	1.1	0.8	0.9	0.2
BU377399	Unknown	0.9	1.4	1.2	5.3
BU420694	Unknown	0.6	18.2	26.2	3.1
BU433279	Unknown	0.7	1.2	2.4	3.5
CR385201	Unknown	0.6	5.0	7.0	0.6
CR385678	Unknown	1.1	1.3	1.8	5.5
CR386845	Unknown	1.0	0.8	0.7	3.1
CR389767	Unknown	1.2	3.2	3.2	0.5
CR389813	Unknown	1.0	1.3	1.2	5.9
CR390519	Unknown	1.4	1.5	1.8	8.3
CR391100	Unknown	0.3	3.0	4.1	0.4
DR431104	Unknown	1.2	1.7	1.9	0.3

**Table 2.** Primers used for qRT-PCR.

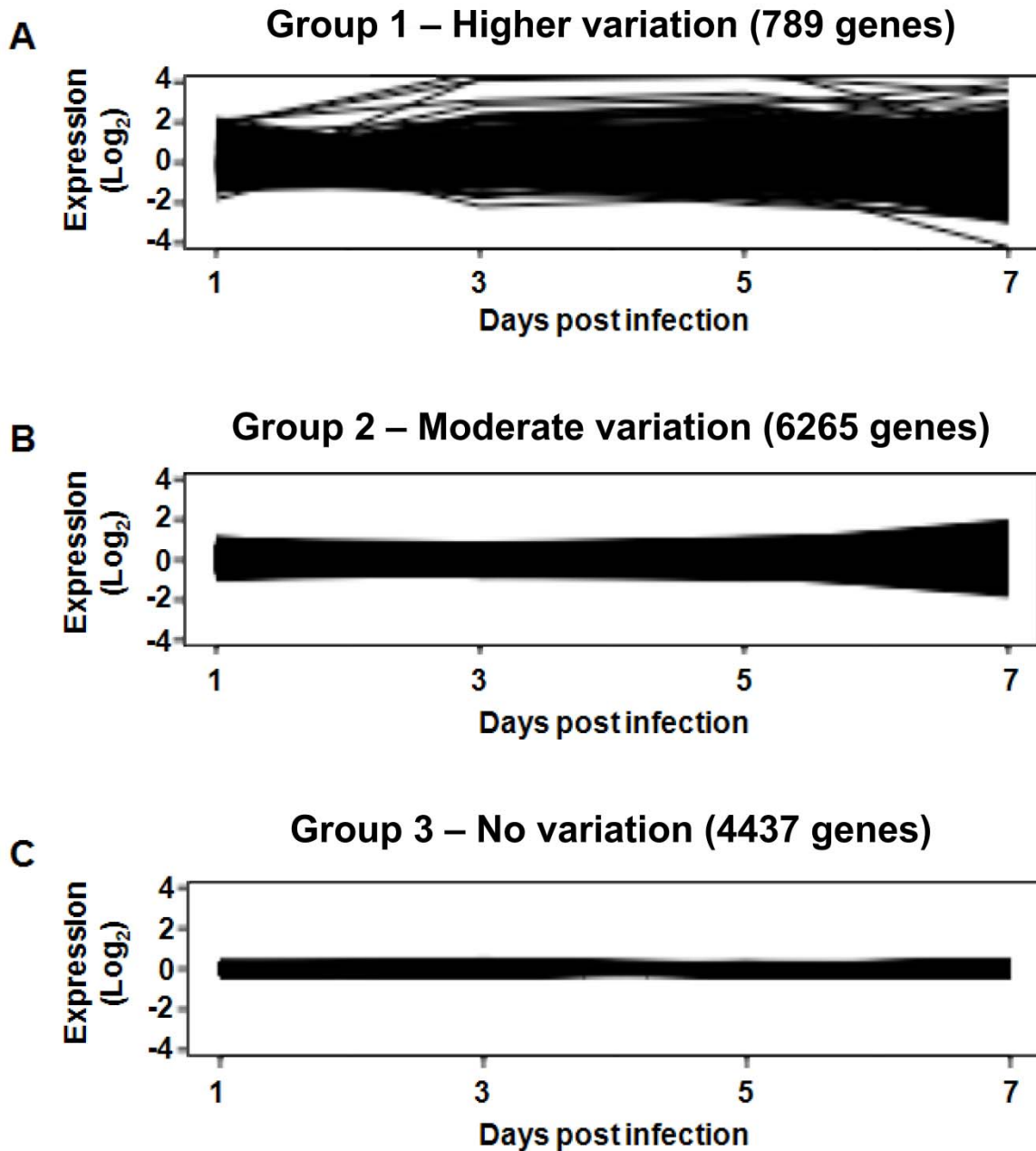
<b>Accession #</b>	<b>Forward Primer Reverse Primer</b>	<b>Gene Symbol</b>
AJ711110	TGGTGGCTCGTTACCACAAG TTCCCAAAGGGAGTCTCGAA	VNN2
AF070478	TCCCAAACGCCAGAGAAAT TCGCCAGAAAAACCTGTCCT	MMP-13
AF062392	CAGCCCCAGTGAATTTCTC GACGGTTGGCCTTTTACCTG	MMP27
X03509	CCAGGGGTATCTGGCACAAT TCATGTTGCCACCTTTCTGC	CKB
AJ309540	CCTGTTCGCCTTTCAGACCT GCCAGGTGCTTTGTGCTGTA	IL6
AF432506	CCTGTTCGCCTTTCAGACCT GCCAGGTGCTTTGTGCTGTA	FABP4
X65459	GGACAGCCACAACCTTTGACG GCTGCTGATGATCACTGTGG	FABP7
AJ851480	TGATGCAAGCACACGACTTG ACCCACCAGGGTATTTGTCG	ACSL1
AJ720861	AGGTTACCCAGATCCCAGA CCACAGTCCCGTGTGCTAAA	LSS
AJ719295	CTGTTTCCCGACGAGCTCAT GGTACAGCAGGCCAACAACA	INSIG1
AJ719718	GGCAGTGAACGACAGCGTTA TAAATGGCTGCTGCAGAGGA	SC4MOL
U09350	CGAAAGCAAATGGCTGTGAA TGCTTCACCTCGAAGTTTGG	VIP
U62026	GAATGCTGGCACCAGGAAA TCCGGAAAGGTTCCATCATC	CENPF
M16199	CGCTGGTAAAGATGGGGAAT CTTGGCGTCAGCTTCACATC	IL8
X02009	GATAGCGGCTGTGTGTTTCG GAGGTCCCTGAGGTTGTTGC	LTF
AJ004940	CTGAATTCAAGCGCAAGCAC TGACAGGGTACGCTTTGCAC	HSPA8
AF411083	GTTGCTTTGCTAACGCCTTG AGAGCTCCCAGACCAAGCAG	SFTPA1
X16881	TTCCACGGGGACTCAGAGAT TGCAAGGATTCCACATCAGG	CDC2
U12438	GTCAGCAGGCTGGAGGTCTT AGCAGAGGATGCTCCTCCTT	RFC2
Y14971	GGCTGTAGCTGCTGTCATGG TATGCACTGGCATCGGAGTT	CXC chemokine K60
NM_204305	GGCACTGTCAAGGCTGAGAA TGCATCTGCCATTTGATGT	chGAPDH

**Table 3.** Comparison of fold changes between microarray and qRT-PCR.

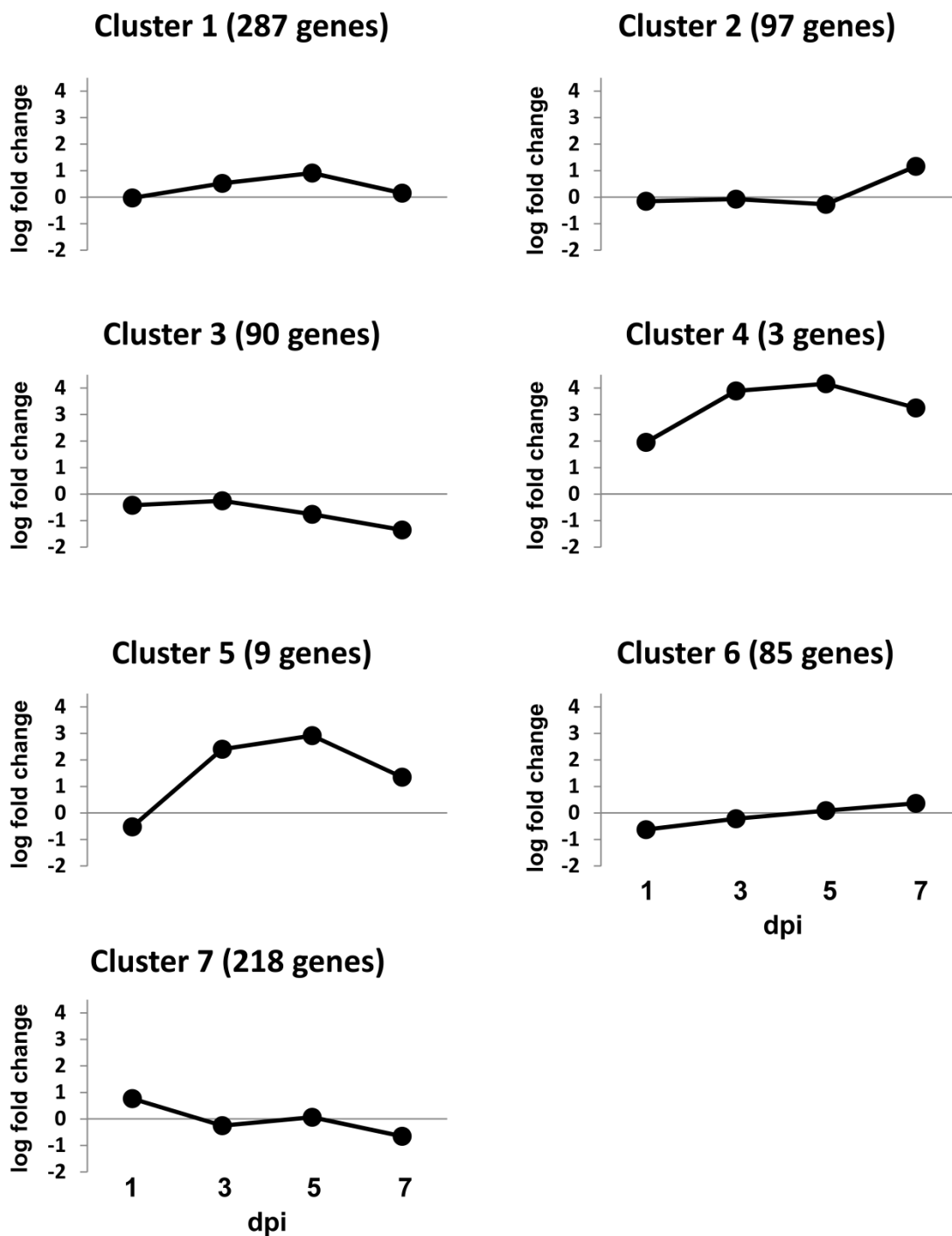
Gene Accession #	Gene Symbol	1 Day		3 Day		5 Day		7 Day	
		Micro array	RT- PCR	Micro array	RT- PCR	Micro array	RT- PCR	Micro array	RT- PCR
AJ721110	VNN2	0.5	0.3	3.3	3.5	6.5	8.7	2.5	2.6
AF070478	MMP13	0.7	0.8	3.4	3.7	2.9	3.8	0.7	0.5
AF062392	MMP27	0.8	0.8	4.1	7.8	6.6	23.7	3.5	7.9
X03509	CKB	0.9	1.4	4.7	6.8	5.5	11.0	2.0	3.3
AJ309540	IL6	0.9	1.1	4.8	7.7	7.2	37.1	3.1	5.7
AF432506	FABP4	1.0	1.2	5.3	7.8	7.1	33.4	15.0	10.0
X65459	FABP7	1.1	1.5	0.3	0.8	0.8	1.1	0.2	0.3
AJ851480	ACSL1	1.2	0.9	7.3	6.0	8.7	8.0	7.3	7.6
AJ720861	LSS	1.3	1.1	1.1	0.7	1.3	1.1	0.2	0.1
AJ719295	Insulin induced gene 1	1.5	1.3	1.0	0.7	0.9	0.9	0.2	0.1
AJ719718	SC4MOL	1.1	1.7	0.8	0.9	1.1	1.3	0.2	0.2
U09350	VIP	2.0	2.4	0.2	0.1	0.3	0.2	0.2	0.2
U62026	CENPF	3.3	9.9	0.7	0.7	1.4	2.2	0.6	0.6
M16199	IL8	3.7	3.0	22.4	43.4	26.8	172.7	18.5	39.0
X02009	LTF	0.7	0.5	2.7	2.3	3.6	5.2	3.2	4.1
AJ004940	HSPA8	0.9	1.1	0.5	0.4	0.6	0.5	2.7	3.2
AF411083	SFTPA1	1.7	2.5	0.4	0.7	0.9	0.9	0.4	0.3
X16881	CDC2	1.9	2.2	0.4	0.3	0.7	0.6	0.4	0.4
U12438	RFC2	1.9	2.0	0.8	0.6	1.0	0.9	0.3	0.2
Y14971	CXC chemokine K60	3.7	7.9	17.3	60.3	19.7	206.4	11.3	38.2



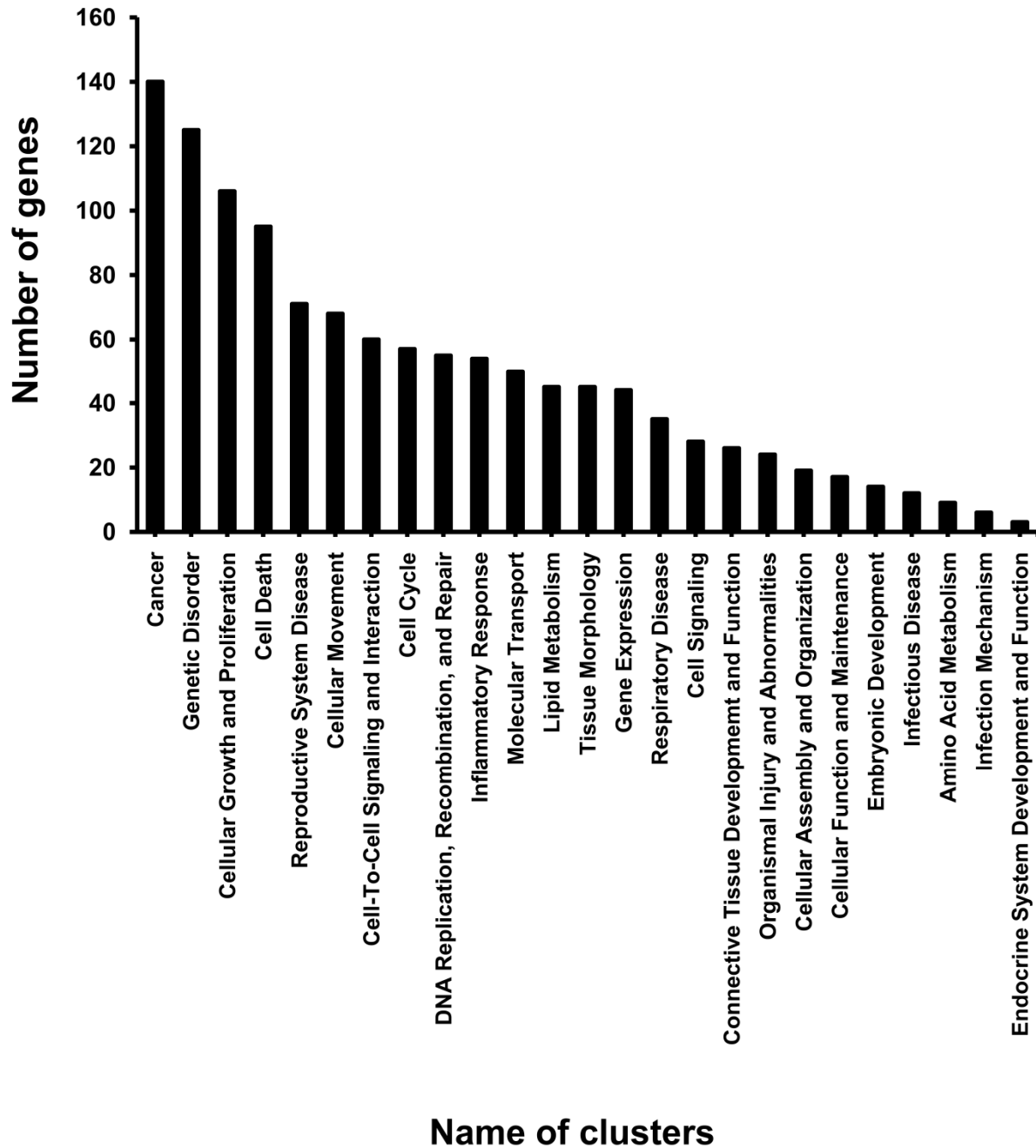
**Figure 1.** ILTV infection in chicken embryonic lung cells. The chicken embryo lung cells were infected with ILTV at a MOI of 0.1. The infected cells were visualized at 0, 1, 3, 5, and 7 dpi, respectively, using a phase contrast microscope at 200X magnification.



**Figure 2.** Groups of differentially expressed genes in the time course of ILTV infection. The 11,491 genes showing a signal to noise ratio (SNR) > 3 were sorted into three groups based on alterations of fold changes at each dpi time point. The Y-axis represents log<sub>2</sub> values of fold changes and the X-axis indicates dpi time points.



**Figure 3.** Patterns of differential expression. The mean value of each cluster was independently plotted in the graph. The closed circle displays dpi time points. The Y-axis indicates fold changes by  $\log_2$  value.

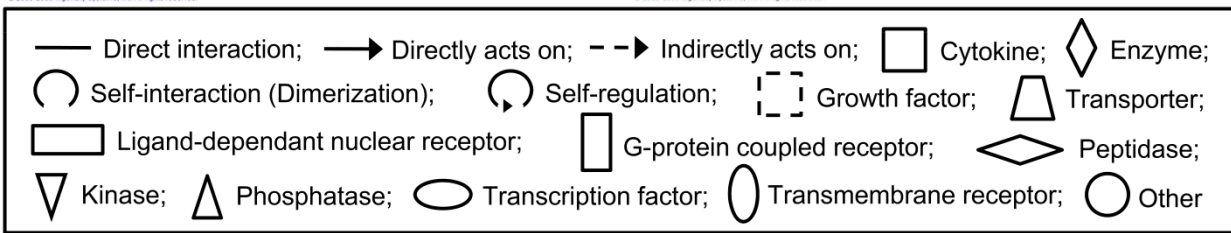
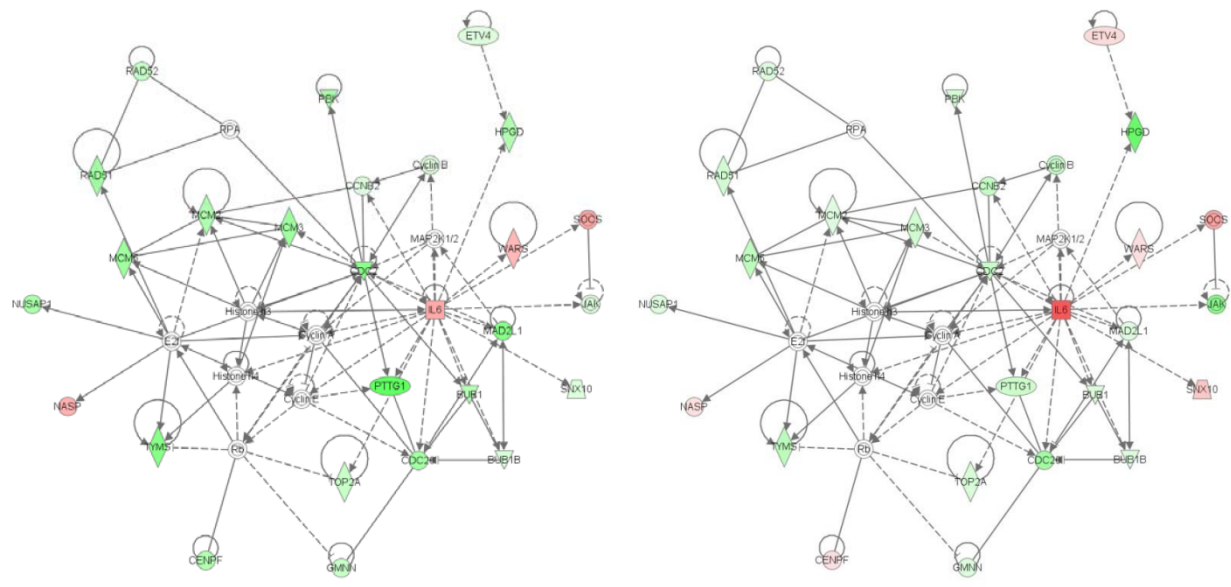
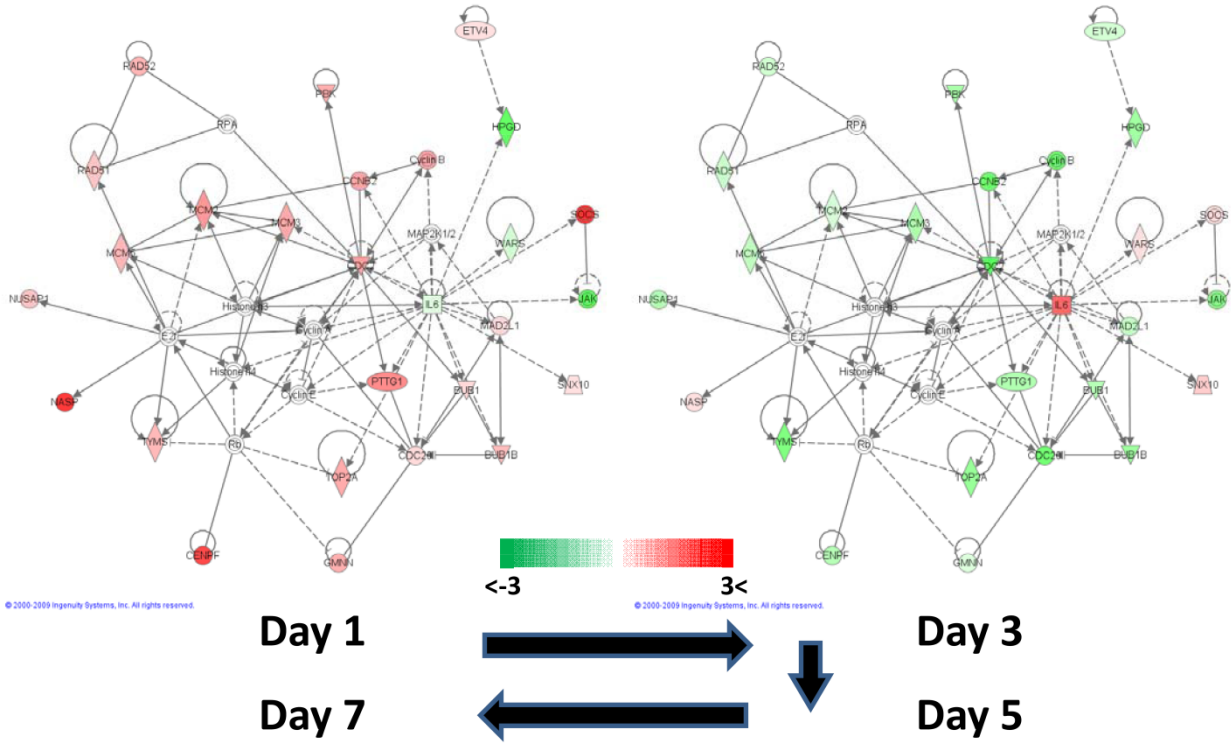


**Figure 4.** Functional gene Ontology (GO) for differentially expressed genes. The 789 genes were categorized into functional groups by the IPA program. Bars represent the number of genes for each cluster. The Y-axis shows the total number of genes, and the X-axis indicates name of clusters.



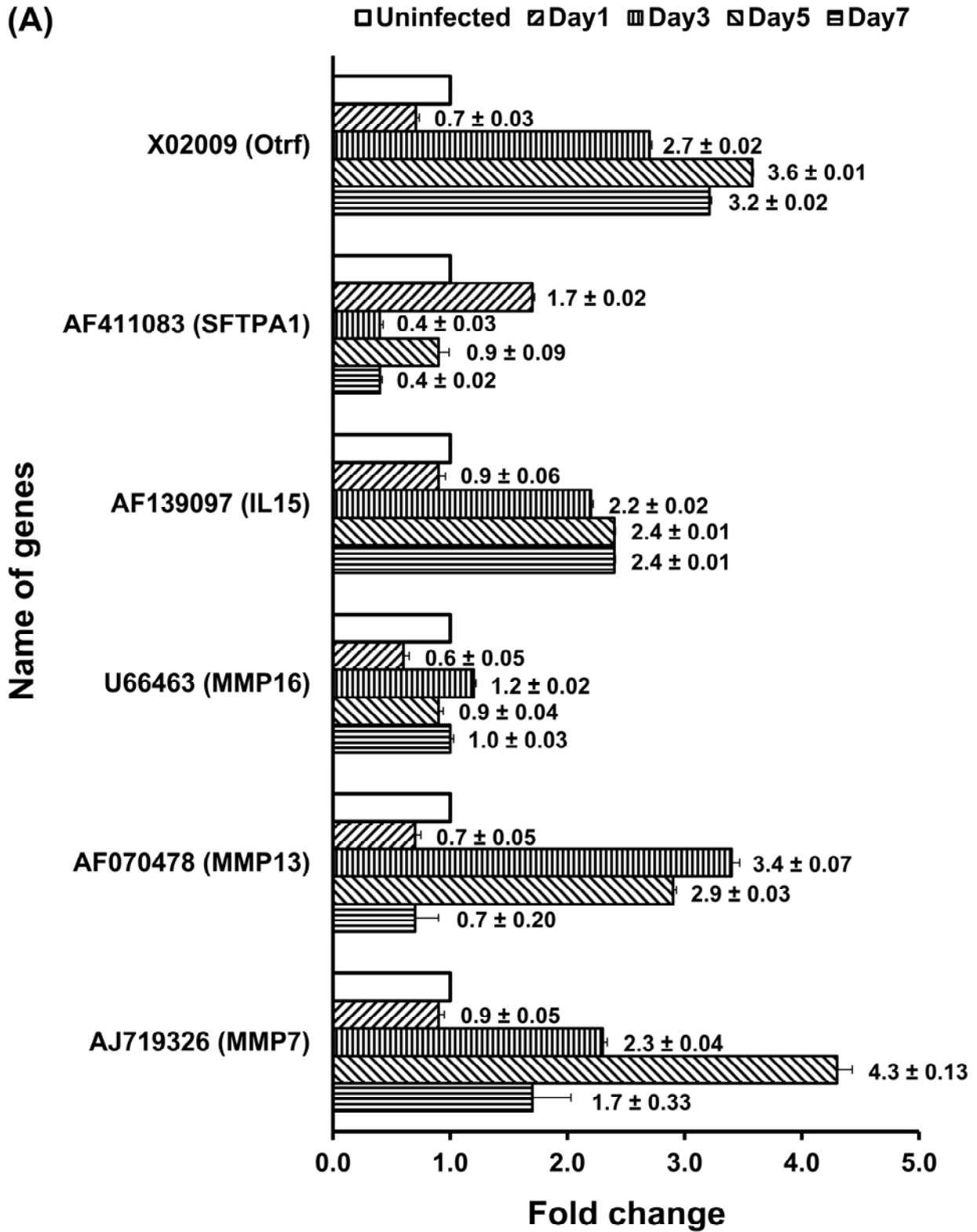
# Network 1

# Fig. 5

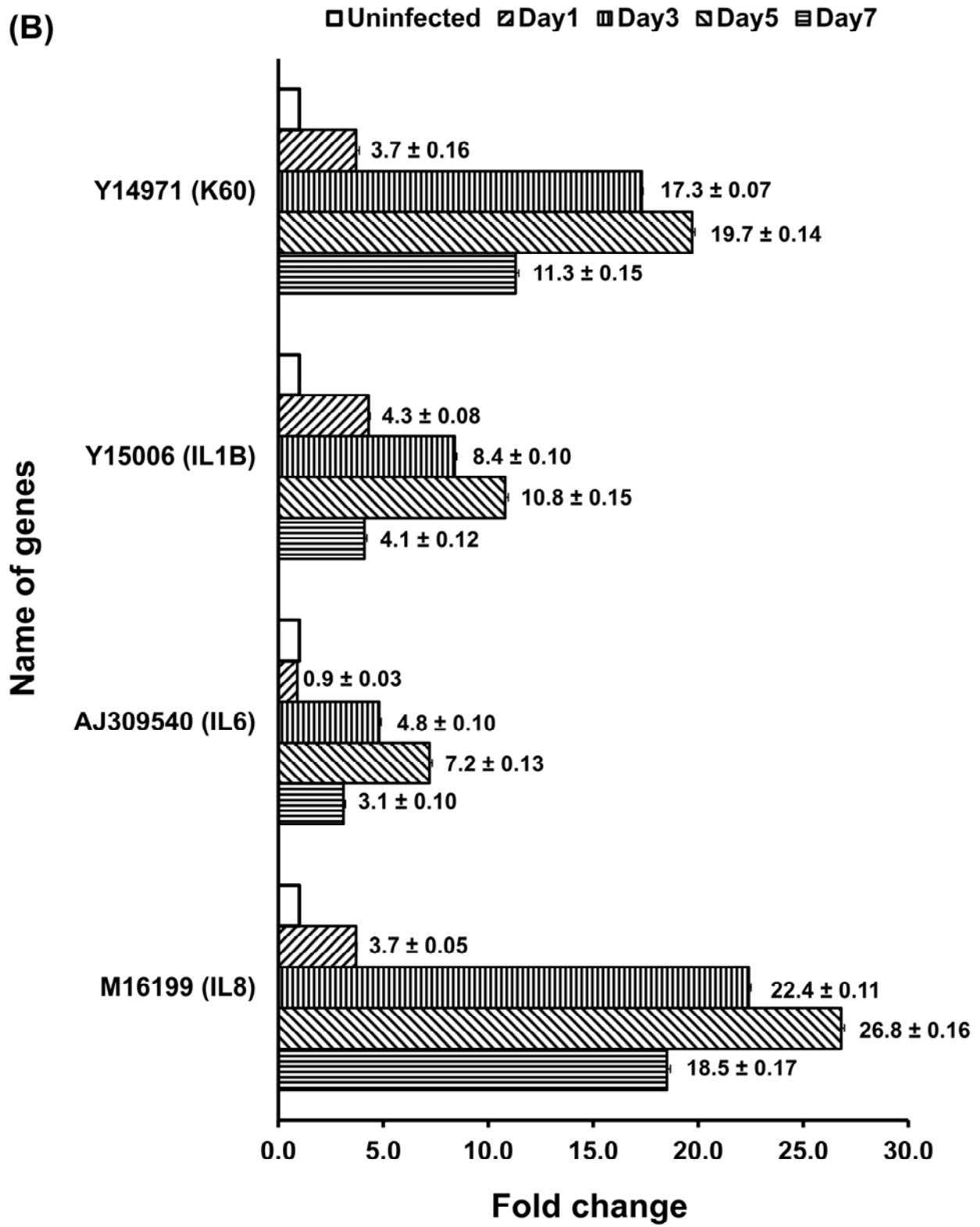


**Figure 5.** Network 1 of gene network analysis. Molecular interactions among important focus molecules are displayed at each dpi time points. Green represents down-regulation while red depicts up-regulation. White symbols depict neighboring genes. The intensity of color represents the average of log fold change in a given population. The numbers below the color change bar denote  $\log_2$  values. Symbols for each molecule are present according to molecular functions and type of interactions.

(A)



(B)



**Figure 6.** Genes of interest in the time course of ILTV infection. The expression levels for genes of interest at each time point displayed by the bar graphs; Genes showing lower range of alteration levels are displayed in (A), whereas genes ranging higher alteration levels displayed in (B). The open, upward diagonal, vertical, downward diagonal and horizontal bars reflect 0, 1, 3, 5, and 7 dpi, respectively. Graphs show the mean  $\pm$  SE.

## **CHAPTER 3**

**An integrated transcriptome analysis of host responses against infectious laryngotracheitis  
virus vaccine infected chicken embryo lung cells**

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**Submitted to BMC Genomics. 2011.**

## 1. Abstract

Infectious laryngotracheitis virus (ILTV; gallid herpesvirus 1) infection causes high mortality and huge economic losses in the poultry industry. To protect chickens against ILTV infection, chicken-embryo origin (CEO) and tissue-culture origin (TCO) vaccines have been used. However, the transmission of vaccine ILTV to unvaccinated chickens can cause severe respiratory disease. Previously, host cell responses against wild-type ILTV infections were determined by microarray analysis. In this study, a microarray analysis was performed to understand host-vaccine ILTV interactions at the host gene transcription level. The 44 K chicken oligo microarrays were used, and the results were compared to those found in wild-type ILTV infection. Total RNAs extracted from vaccine ILTV infected chicken embryo lung cells at 1, 2, 3 and 4 days post infection (dpi), compared to 0 dpi, were subjected to microarray assay using the two color hybridization method. Data analysis using JMP Genomics 5.0 and the Ingenuity Pathway Analysis (IPA) program showed that 306 differentially expressed genes could be grouped into a number of functional categories including cellular metabolism, immune response, cancer, genetic disorder and cellular proliferation. Moreover, 10 possible gene networks were created by the IPA program to show intermolecular connections. Interestingly, of 306 differentially expressed genes, 31 genes were commonly found in both wild-type ILTV and vaccine ILTV infections. Eight of these commonly expressed genes, F2RL1, BMP2, IKBIP, TYMS, C8orf79, F10, PTGS2 and NPY were expressed oppositely between the vaccine and the wild-type ILTV infection. Intensive knowledge of gene expression and biological functionalities by comparing host cell responses to either wild-type or vaccine ILTV infection can provide insight into the pathogenesis and immune responses during ILTV infections.

## 2. Introduction

Avian infectious laryngotracheitis virus (ILTV), named as Gallid herpesvirus 1, is a member of the Iltovirus genus, Alpharherpesvirinae subfamily and Herpesviridae family. ILTV has a linearized dsDNA genome of about 150kb in size which contains unique long (UL), unique short (US) sequences flanked by inverted repeat (IR) and terminal repeat (TR) sequences [1, 2]. The genome encodes 80 predicted viral protein open reading frames (ORFs). ILTV infection causes respiratory disease symptoms in chickens, pheasants, partridges and peafowl [3, 4] that includes clinical signs of the extension of the neck, gasping, gurgling, rattling and coughing of clotted blood [5]. ILTV usually causes the reduction of egg production and variable mortality ranging from 5 to 70%, resulting in the severe economic losses in the poultry industry [6]. Two types of commercial live attenuated vaccines, chicken embryo origin (CEO) and tissue culture origin (TCO) have been widely used to immunize chicken flocks against ILTV outbreak [6, 7]. However, it was found that live vaccines infect the nervous system similar to that exhibited in the wild-type ILTV infections, and could revert to become a causative agent to induce vaccinal laryngotracheitis (VLT) by transmission to unvaccinated birds [8-10]. Moreover, global ILTV outbreaks are mostly associated with CEO vaccines [11-13], and the genomic- and antigenic characteristics between wild-type and vaccine ILTV are very similar [6].

Microarray analysis has become popular, along with the recent development of a RNA-seq (RNA sequencing) technique using next-generation sequencing, to analyze massive gene expression in different biological conditions. Microarrays have been performed intensively to investigate host gene transcriptional responses to infections of various viruses such as hepatitis C virus (HCV) [14], rice dwarf virus (RDV) [15], influenza virus [16], herpesvirus saimiri (HVS) [17], human immunodeficiency virus (HIV) [18, 19], Japanese encephalitis virus (JEV) [20, 21],



chicken anemia virus (CAV) [22], human cytomegalovirus (hCMV) [23], Epstein-Barr virus (EBV) [24], infectious laryngotracheitis virus (ILTV) [25], varicella-zoster virus (VZV) [26], alphaherpesvirus [27], Marek's disease virus (MDV) [28] and herpes simplex virus type 1 (HSV-1) [29], and in even vaccine strains including recombinant flavivirus [30], west nile/dengue 4 virus [31] and dengue virus [32].

Previously, we studied the differential gene expression of host responses against wild-type ILTV infection in cultured primary chicken embryo lung cells using microarray analysis [25]. To compare and contrast host responses to the infection of either wild-type or vaccine ILTV, primary chicken embryo lung cells were infected with live attenuated CEO vaccines and host gene expression during a four days period post infection was determined using 44K chicken oligo microarrays in the present study. Interestingly, genes showing opposite expression patterns in vaccine ILTV infection were identified compared to wild-type ILTV infection. Results in this study provide knowledge of differential regulation of host pathogenic and immunologic responses against either wild-type or vaccine ILTV.

### **3. Results and discussion**

#### **3.1. Profiling of differentially expressed host genes in vaccine ILTV infection**

Primary chicken embryo lung cells at passage 1 were infected with 3 vaccination doses of a live fowl laryngotracheitis vaccine, which is widely used in the poultry industry, and infected cells were subjected to analysis for cytopathic effect (CPE) and virus infection validation at 1, 2, 3 and 4 days post infection (dpi). Although weak CPE, such as cell rounding, aggregation and syncytia were observed at certain locations of plates at 1 and 2 dpi, infected cells began to recover with healthy cells at 3 dpi, and no CPEs were observed at 4 dpi (Fig. 1A). To verify the infection of vaccine ILTV, the expression of ILTV viral RNA was determined, and genes of UL35 encoding small capsid protein and US5 encoding envelop glycoprotein J (gJ) were shown to progressively increase their expression post infection though US5 expression began to be detected from 2 dpi (Fig. 1B).

In the microarray assay using 44K genes, two approaches to avoid possibly hidden dye effects were used: the use of RNA Spike-in controls synthesized from the Adenovirus E1A transcriptomes containing different concentrations of dye in each set [33], and the dye-swap in two replicates of four total replicates. No significant dye effects were detected in all microarray slides (data not shown). The subsequent data analysis revealed that 1305 differentially expressed genes at four dpi time points of vaccine ILTV infection were selected by one-way ANOVA test using the JMP Genomics 5.0 program (Additional file 2). The 1035 differentially expressed genes were subjected to the bioinformatics study using Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)) to generate the gene networks and functional annotations, resulted in 306 genes that recognized as mapped IDs (functionally known genes) by

IPA program (Additional file 3). The list of 306 genes was analyzed for further bioinformatics studies.

### **3.2. Quantitative reverse transcription-PCR (qPCR)**

To validate the microarray results, expression of 18 genes of the 1,305 differentially expressed genes were subjected to qPCR using the same RNA samples as those used in the microarray and gene specific primer sets (Table 1 and Additional file 1). Of the 18 genes tested, the expression pattern for 12 genes completely matched the microarray data at four time points. The expression pattern for the remaining 6 genes also qualitatively matched to microarray data, though they were not quantitatively matched (Table 1). With the comparison of the spike-in controls, qPCR results indicated that the microarray data in this experiment were valid to determine host gene expression responses against vaccine ILTV infection.

### **3.3. Top 10 differentially expressed genes.**

Out of 306 mapped IDs, the top 10 most differentially expressed genes in ILTV vaccine infection were selected by differences of standard deviation (SD) among all four dpi time points (Table 2A). The general functions and possible roles in vaccine ILTV infection of the selected genes are described briefly in Table 2B. These top 10 genes listed are involved in functions of immune responses, inflammation, cell proliferation, apoptosis and cell-to-cell interactions.

### **3.4. Biological functions and gene networks of differentially expressed genes**

Using the IPA program, 306 mapped genes were categorized into 75 biologically functional groups, and the top 20 groups associated with greater numbers of genes, are shown in

Fig. 2. The 20 functional groups are mostly related to cellular growth and proliferation, cellular and tissue development, cell or tissue survival and interaction, diseases and disorders, and host immune activation and inflammatory responses.

Network analysis by IPA to draw the connection of focus molecules displayed relationships between the interacting genes. Of the 10 networks that were generated, the top 4 networks were identical among all four dpi time points, which may be due to the fact that the algorithms of the IPA program generate a network by considering fold change values and p-values of focus molecules in addition to their biological functions. The lists, top functions and the main focused molecules of each network are shown in Additional file 4, and drawings of interacting molecules in each network during the time course of all dpi time points are displayed in Additional file 5.

### **3.5. Common genes found in both wild-type and vaccine ILTV infection**

Previously, we reported 273 differentially expressed chicken genes mapped by the IPA program for the wild-type ILTV infection, and the analysis of the functions and molecular networks of these genes [25]. To find commonly involved host cellular mechanisms against both wild-type and vaccine ILTV infection, the 306 differentially expressed genes in response to vaccine ILTV infection were compared to the 273 differentially expressed genes found in the previous wild-type ILTV infection study. Result showed that 31 genes were altered in their expression in both wild-type and vaccine ILTV infections. Of these 31 genes, 23 genes showed a similar differential expression pattern as found in the wild-type ILTV infection at 1 and 3 dpi, and the GenBank accession, gene symbol and gene name are listed in Table 3. Interestingly, eight of 31 genes were regulated in the opposite direction for expression patterns at 1 or 3 dpi

between wild-type and vaccine ILTV (Fig.3 and Table 4). Those include coagulation factor II (thrombin) receptor-like 1 (F2RL1, AJ851370), bone morphogenetic protein 2 (BMP2, AY237249), inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (IKBKB) interacting protein (IKBIP, BX931418), thymidylate synthetase (TYMS, BX932834), chromosome 8 open reading frame 79 (C8orf79, CR390951), coagulation factor X (F10, D00844), prostaglandin-endoperoxide synthase 2 (PTGS2, M64990) and neuropeptide Y (NPY, M87294).

Bone morphogenetic protein 2 (BMP2) was not altered in its expression in wild-type ILTV infection at 3 dpi, while it was increased in vaccine ILTV infection at 3 dpi. The expression of BMP2 was related to both the inhibition of tumor cell growth in carcino-embryonic antigen (CEA) expressing cells and the induction of the differentiation of non-committed stem cells to bone [34]. The functional role of BMP2 in herpesvirus (including ILTV) propagation has not been studied, but the differential expression pattern of BMP2 in between wild-type and vaccine ILTV suggests that the up-regulation of BMP2 in vaccine ILTV infection may support the recovery of cellular structure at later phase of the infection.

Thymidylate synthase (TYMS) catalyzes the conversion of deoxyuridine 5'-monophosphate (dUMP) to deoxythymidine 5'-monophosphate (dTMP), which is one of the four essential components for DNA synthesis [35, 36]. Herpesviruses such as hCMV or murine CMV (mCMV), that do not contain its own thymidine phosphorylase (TYMP), uses host TYMS to replicate viral DNA [37, 38]. At 3 dpi, the expression of TYMS in wild-type ILTV infection was decreased about 2 fold, while the expression of TYMS in vaccine ILTV infection was similar between 1 and 3 dpi, which is 1.5 fold higher than that of uninfected control. The down-regulation of TYMS in wild-type ILTV propagation may be due to the massive cell disruption

generated by severe CPE as observed in the previous report [25]. However, the expression of TYMS in vaccine ILTV infected cells was slightly elevated until 3dpi, which would support continuous cell growth even during the time course of vaccine ILTV propagation. According to a previous report, hCMV immediate early gene activates the expression of the TYMS gene to efficiently replicate viral DNA genome [39]; similar expression patterns were observed at 1dpi for both wild-type and vaccine ILTV replication. TYMS gene in wild-type ILTV infection might be related to cell death due to the suppression of DNA replication of the host cell, and the severe CPE production in wild-type ILTV infection might be caused by the down-regulation of TYMS [25]. However, in vaccine ILTV infection, maintaining expression level of TYMS gene may be a result of activation of the immune system by vaccine virus infection, resulting in cells that recovered and proliferated with the marginally increased expression of TYMS gene.

Prostaglandin synthetase-2 (PTGS2), as known as (a. k. a.) cyclooxygenase-2 (COX2), is responsible for prostanoid biosynthesis, and its functional roles are related to inflammation and mitogenic stimuli [40]. Several reports have shown that viruses and viral products, such as HSV-1 [41], HSV-2 [42] and Kaposi's sarcoma-associated herpes virus (KSHV) [43, 44] induce COX2 expression and prostaglandin production in various cell types and the induction of COX2 is associated with viral latency, inflammation and immune response. In the present study, vaccine ILTV infection caused slight down-regulation of COX2 gene which may play a role in mild inflammatory response found in vaccine ILTV infection compared to that wild-type ILTV infection induced COX2 expression and led to massive CPE production. The transduction of HSV-1 thymidine kinase has been known to enhance COX2 expression and enzymatic activity in vitro and in vivo [45], suggesting that differential thymidine kinase activity between wild-type and vaccine type ILTV may regulate the opposite pattern of COX2 gene expression during either

wild-type or vaccine ILTV infection. Furthermore, the down-regulation of COX2 genes by inhibitors blocked the cell-to-cell spread of hCMV [46]. These observations suggest that COX2 in avian host cells and thymidine kinase gene in ILTV can be potential targets to reduce the risk of ILTV infection.

Neuropeptide Y (NPY) involves multiple cellular mechanisms related to both virus entry into the central nervous system (CNS) and virus-induced neurological diseases. NPY protects the nervous system from murine retrovirus-induced neurological disease [47]. The up-regulation of NPY was observed in the reactivation of VZV from a latent infection in human sensory trigeminal and dorsal ganglia, which are both sensory neurons [48]. It suggests that the up-regulation of NPY in wild-type ILTV infection at 3 dpi may facilitate the latent infection in the nervous system. In contrast, the continuous down-regulation of NPY in vaccine ILTV infection might delay the latent infection by the up-regulation of immune responses to protect the host from diseases.

Inhibitor of NF- $\kappa$ B (I $\kappa$ B) kinase subunit beta (IKBK $\beta$ ) interacting protein (IKBIP) interacts with IKBK $\beta$  triggering immune responses through the cytokine-activated intracellular signaling pathway in various herpesviruses infections including HSV-1 [49], KSHV [50, 51], EBV [52], hCMV [53], and gammaherpesvirus human herpesvirus 8 (HHV-8) [54]. The hCMV infection induces the prevention of viral genome replication by the activation of NF- $\kappa$ B [55]. The activation of IKK- $\beta$  causes the degradation of I $\kappa$ B by ubiquitination of phosphorylated I $\kappa$ B followed by the activation of NF- $\kappa$ B [56]. In addition, the free NF- $\kappa$ B enters the nucleus and activates genes related to inflammation and immune responses. From this, it would appear that the relative up-regulation of IKBIP in vaccine ILTV infection may cause the increased activity of

NF- $\kappa$ B, and cause higher immune response levels, compared to those in the down-regulated IKBIP gene in the wild-type ILTV infection .

F2RL1 and F10 are coagulation factors, and C8orf79 is an ORF region on chicken chromosome 8. The functional roles in virus infection of the genes have not been studied yet.

#### **4. Conclusion**

In this study, we examined host gene responses by vaccine ILTV infection. Unlike our previous research of wild-type ILTV infection, vaccine ILTV infection showed weak CPEs and cellular recovery, little massive cell death, and activated immune responses and non-pathogenic inflammatory responses. Moreover, eight host genes were significantly modulated by vaccine ILTV infection, and could be important regulators in non-pathogenic host immune responses. Results in this study provide crucial insights into host immunologic and pathogenic responses against vaccine ILTV infection.



## **5. Materials and methods**

### **5.1. Cell culture and vaccine ILTV infection**

Primary chicken embryo lung cells were prepared as previously described [25]. All cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator in 10 cm culture dishes by passaging every 3-4 days in 10ml growth medium consisting Dulbecco's Modified Eagle Medium (DMEM, 0.45% glucose) plus 10% fetal bovine serum (FBS), 100units/ml penicillin, 100µg/ml streptomycin, and 2mM L-glutamine. A modified live ILTV commercial vaccine, LT-Blen (Merial Limited, Duluth, GA, USA), was used to infect the chicken embryonic lung cells by the amount equal to 3 vaccination doses. After incubation of infected cells for 1hr with gentle rocking every 15 min, growth medium was added to each culture dish. The cells were incubated for up to 4 days. The protocols used in this study were approved by both the Institutional Biosafety Committee (IBC; permit number: 10007) of University of Arkansas and the Animal and Plant Health Inspection Service (APHIS; permit number: 102743) of United States Department of Agriculture (USDA).

### **5.2. Total RNA extraction**

TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from uninfected- or vaccine ILTV infected chicken embryonic lung cells from 1 to 4 days post infection (dpi). Total RNA treated by DNase I (New England BioLabs Inc., Ipswich, MA, USA) was re-purified by TRIzol reagent and quantified by Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and qualified quality assessed by agarose gel (data not shown). To validate vaccine ILTV infection, expression of UL35 and US5 genes, in addition to chicken GAPDH (a host gene expression control), were determined by end-

point reverse transcription PCR with gene specific primers. PCR amplicons were analyzed by agarose gel electrophoresis and images were obtained using the GelDoc system (Biorad, Hercules, CA, USA).

### **5.3. Probe labeling and microarray hybridization**

Initially, 2  $\mu\text{g}$  of total RNA was used to synthesize Cy3 or Cy5 labeled complementary RNA (cRNA) using the Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA, USA) following the manufacturer's instructions and as described previously [25]. To avoid possible dye effects, RNA Spike-in controls, which were synthesized from the Adenovirus E1A transcriptomes containing different concentrations of dye in each set [33], were added to RNA samples as Spike-in A for Cy3 and Spike-in B for Cy5 and mixed with un-infected control and vaccine ILTV infected samples at each dpi time point, respectively. For the additional control of dye effects, the dyes were swapped in two replicates of four total replicates to confirm further hidden dye effects. Each 825ng of Cy3 and Cy5 labeled cRNA probes were co-hybridized on a 4X44K Agilent chicken oligo microarray (array ID: 015068). After washing and drying, the slides were scanned using a Genepix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA) with the tolerance of saturation at 0.005%.

### **5.4. Microarray data analysis**

Background-corrected red and green intensities for each spot were used in subsequent analyses. Global normalization based on locally weighted linear regression (LOWESS) was applied to the intensities by removing effects which arise from undesirable systematic variations in microarray experiment. The ratio of signal intensities of corresponding spots to all Spike-ins

used were compared to reference ratios reported previously [33]. All normalized data were deposited in the Gene Expression Omnibus (GEO; accession number: GSE30269). Genes showing both signal to noise ratio (SNR) of  $>3$  (meaning foreground signals are three times greater than background signals), and foreground intensity of  $> 100$  at all time points were considered as reliable signals. To identify differentially expressed genes throughout the four dpi time points, normalized fold change values were subjected to statistical analysis by one-way ANOVA test in JMP Genomics 5.0 (<http://www.jmp.com/software/genomics/>) licensed to the Cell and Molecular Biology (CEMB) program of the University of Arkansas. Fold change values representing differential expression were displayed as  $\log_2$  conversion.

### **5.5. Quantitative reverse transcription-polymerase chain reaction (qPCR)**

To verify the microarray data, qPCR was performed with 18 genes using gene specific primer sets designed by Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>), and the primer sets were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer information is shown in Additional file 1. Three  $\mu\text{g}$  of total RNA, which was used for the microarray analysis, was converted to cDNA and qPCR was performed by following conditions: 40 cycles of denaturing  $95^\circ\text{C}$  for 30 s, annealing at  $58 - 62^\circ\text{C}$  for 1 min, extending at  $72^\circ\text{C}$  for 30 s, and finally extending at  $72^\circ\text{C}$  for 10 min. A non-template control (NTC) and an endogenous loading control (chicken GAPDH) were used for the relative quantification. The fold change values for the vaccine ILTV infected groups comparing to uninfected control were determined by the  $-\Delta\Delta\text{CT}$  method, which is comparable to  $\log_2$  values in microarray [57].

### **5.6. Bioinformatics**

The Ingenuity Pathways Analysis (IPA) software version 9.0 (Ingenuity Systems®; [www.ingenuity.com](http://www.ingenuity.com)) was used to study biological functions and molecular interactions among differentially expressed genes. IPA analyzes various bioinformatics tools including functional annotation, clustering, and network discovery based on Ingenuity Knowledge Base, which is the core technology of all IPA systems and the p-value developed from Right-tailed Fisher's exact test were mainly considered to interpret the interaction and functions of the differentially expressed genes [58, 59]. For the network analysis, 10 networks and 35 molecules in each network were limited to concentrate on the closest interacting focus molecules (focus genes= a subset of uploaded significant genes having direct interactions with other genes in the database) within the differentially expressed genes [60].

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## **7. Authors' contributions**

JYL designed and performed entire experiments, analyzed the data, and wrote the manuscript. WB contributed the bioinformatics analysis using the IPA program and manuscript editing. BWK supervised entire processes of the research, initial data analysis and manuscript editing. All authors read and approved the final manuscript.

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**Table 1.** Validation of gene expression between microarray and qPCR.

GenBank	Symbol	Fold Change (Microarray / qPCR)			
		Day 1	Day 2	Day 3	Day 4
BX932962	SLC37A2	1.2 / 1.3	2.1 / 2.6	3.3 / 3.7	3.9 / 4.5
M60853	THBS2	1.5 / 1.8	2.0 / 2.5	2.8 / 2.8	3.3 / 3.3
BX933888	C1QTNF3	-0.2 / 0.1	1.2 / 2.0	1.9 / 2.5	2.7 / 3.4
BX933728	CAPSL	2.3 / 3.0	2.6 / 3.7	3.2 / 4.3	3.5 / 4.4
BX931297	CYTL1	1.0 / 1.7	1.4 / 2.8	2.7 / 4.2	2.9 / 4.4
CR352775	ALDOB	0.7 / 1.1	1.0 / 1.7	2.1 / 2.2	2.7 / 2.5
BX933478	MXRA5	0.8 / 1.5	1.7 / 3.0	2.5 / 3.7	3.0 / 4.4
BX935456	EGLN3	-2.7 / -2.0	-2.3 / -1.9	-1.7 / -1.4	-1.3 / -0.9
BX931599	VIPR2	1.7 / 2.0	2.3 / 2.9	2.9 / 3.6	3.1 / 5.1
CR385566	CLEC3B	0.2 / 1.0	0.4 / 2.2	1.3 / 2.4	1.4 / 3.1
M87294	NPY	-0.4 / -0.3	-0.9 / -0.6	-1.5 / -0.9	-1.7 / -1.3
M80584	LUM	1.2 / 1.6	1.5 / 2.3	1.9 / 2.5	2.5 / 3.2
M64990	PTGS2	-0.6 / -0.6	-0.8 / -0.3	-0.2 / -0.4	0.7 / 1.2
AB109635	HMGCR	0.4 / 0.4	0.2 / 0.6	-0.5 / -0.3	-0.9 / -0.6
BX936211	TMEM116	0.0 / 0.0	-0.3 / 0.3	-0.3 / -0.1	-1.3 / -0.7
X87609	FST	1.3 / 1.0	0.6 / 1.2	0.6 / 0.6	-0.3 / 0.2
D87992	ANPEP	-0.1 / -1.2	-0.5 / 0.5	-0.6 / 0.4	-1.4 / -0.2
AF505881	SCX	1.1 / 0.2	1.4 / -0.6	1.8 / 0.1	2.3 / -0.6

The gene expression levels of microarray were presented by  $\log_2$  fold changes, whereas those of qPCR were indicated by  $-\Delta\Delta Ct$  that are comparable to the  $\log_2$  fold change values in microarray.

**Table 2.** The 10 most differentially expressed genes in ILTV vaccine infection.

**A. The gene list and fold changes at each day post infection (dpi)**

GenBank	Symbol	1 dpi	2 dpi	3 dpi	4 dpi	Mean	Std
BX933888	C1QTNF3	-0.2	1.2	2.0	2.7	1.4	1.2
AJ829443	AQP5	1.7	-0.4	-0.8	-0.6	-0.1	1.1
AJ251273	CCK	0.2	-0.5	-1.1	-2.2	-0.9	1.0
J00902	SPINK5	-0.4	-1.4	-2.1	-2.5	-1.6	0.9
M60853	THBS2	1.5	2.0	2.8	3.3	2.4	0.8
AJ719394	PPIF	0.1	-0.1	-0.8	-1.7	-0.7	0.8
CR390466	NMU	-0.1	-0.7	-1.3	-1.8	-1.0	0.8
X80207	VLDLR	-0.4	-0.6	-1.0	-2.0	-1.0	0.7
AJ851685	ADAM28	-1.1	-1.2	-1.8	-2.5	-1.7	0.7
X87609	FST	1.3	0.6	0.6	-0.3	0.6	0.7

**B. Gene functions**

Symbol	Functions
C1QTNF3 (CTRP3)	<ul style="list-style-type: none"> <li>• C1q and tumor necrosis factor related protein 3, a. k. a. CTRP3.</li> <li>• Regulates angiogenesis by stimulating ERK1/2 and p38 MAPK [61].</li> <li>• Cartonectin, an adipokine of the CTRP3 reduced IL6 and TNF production and caused anti-inflammatory function by the suppression of NF-kB signaling [62-64].</li> <li>• IL6 among cytomegalovirus secreted cytokines enhances angiogenesis and survival of endothelial cells through impediment of apoptosis by blocking caspase 3 and 7 [65].</li> <li>• C1qTNF3 could accelerate embryonic growth and energy utilization for the development of high feed efficiency (FE) broiler line [66].</li> <li>• During ILTV vaccine infection, continuous increase of CTRP3 expression until 4 dpi except 1 dpi, suggested to prevent inflammation responses, but promote immune responses without massive cell death through cell recovery.</li> </ul>
AQP5	<ul style="list-style-type: none"> <li>• Aquaporin 5, a water channel protein.</li> <li>• Deficiency of Aquaporin 5 (AQP5) exacerbated lung injury by the infection of <i>pseudomonas aeruginosa</i> [67].</li> <li>• Down-regulation of AQP5 affects the increases of the proliferation and migration of human corneal epithelial (CEP117) cell line through indirect activation of ERK pathway [68].</li> <li>• Adenovirus infection to mouse lung caused the down-regulation of AQP5 gene expression [69].</li> <li>• The down-regulation of AQP5 gene expression in vaccine ILTV infection might be associated to the reduced CPE and cell recovery in later dpi time points.</li> </ul>

CCK	<ul style="list-style-type: none"> <li>• Cholecystokinin, a peptide hormone of the gastrointestinal system to stimulate the digestion of fat and protein.</li> <li>• Adenovirus suppresses CCK mediated activation of c-Jun kinase (JNK) inducing apoptosis or inflammatory responses on pancreatic acinar cells [70].</li> <li>• Down-regulation of CCK expression in vaccine ILTV infected cells may result in suppression of apoptotic cell death or inflammatory responses induced by JNK pathway activation.</li> </ul>
SPINK5	<ul style="list-style-type: none"> <li>• Serine peptidase inhibitor, Kazal type 5 or chicken ovomucoid.</li> <li>• Lymph-epithelial Kazal-type-related inhibitor (LEKTI).</li> <li>• Inhibits immune and inflammatory responses in human primary keratinocytes (HK) [71].</li> <li>• Down-regulation of SPINK5 at all dpi time points may support the induction of immune responses caused by vaccine ILTV infection.</li> </ul>
THBS2	<ul style="list-style-type: none"> <li>• Thrombospondin 2, a potent inhibitor of tumor growth and angiogenesis and a matricellular glycoprotein to mediate cell-to-cell interaction [72].</li> <li>• Functions in angiogenesis in patients with early-stage non-small cell lung cancer [73], and wound healing and development of exuberant granulation tissue in horse [74].</li> <li>• Up-regulation of THBS2 in vaccine ILTV infection may function in virus spreads in infected cells.</li> </ul>
PPIF	<ul style="list-style-type: none"> <li>• Peptidylprolyl isomerase F, one of the peptidyl-prolyl cis-trans isomerase (PPIase) family proteins and a member of the mitochondrial permeability transition (PT) pore in the inner mitochondrial membrane.</li> <li>• Stimulates the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerate the folding of proteins [75, 76].</li> <li>• Apoptosis and necrosis of cells were induced by the activation of the PT pore [77-79].</li> <li>• Down-regulation of PPIF genes in vaccine ILTV infection may play a role in cell death and recovery of cells.</li> </ul>
NMU	<ul style="list-style-type: none"> <li>• Neuromedin U, a multifunctional neuropeptide.</li> <li>• Functions in conditions of pain and stress, the metabolism and homeostasis of feeding and energy in body, immune and inflammatory diseases, smooth muscle contraction, and the control of blood flow and pressure [80, 81].</li> <li>• Deeply related to innate and adaptive immunity according to previous reports regarding NMU and neuromedin U receptor 1 (NMU-R1).</li> <li>• Expressed in antigen presenting cells (APCs) such as dendritic cells, monocytes and B cells, and its receptor, NMU-R1, is fluently expressed in immune cells such as natural killer cells, T cells, and monocytes [82].</li> <li>• Induces early-phase inflammation through the degranulation in mast cells in which NMU-R1 is highly expressed [83].</li> <li>• Acts as an inflammatory mediator via the acceleration of IL-6 production in macrophage [84].</li> <li>• Down-regulation of NMU may inhibit inflammatory responses and elevate immune responses.</li> </ul>



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VLDVR	<ul style="list-style-type: none"> <li>• Very low density lipoprotein / vitellogenin receptor.</li> <li>• Binds to baculovirus surface membrane to inhibit ligand-receptor interaction in viral infection of HeLa cells [85].</li> <li>• The meaning of down-regulation of VLDLR in ILTV vaccine infection in addition to in other herpesvirus infection, is unknown.</li> </ul>
ADAM28	<ul style="list-style-type: none"> <li>• A disintegrin and metalloproteinase (ADAM) domain 28.</li> <li>• Functions to cell-to-cell and cell-to-matrix interaction on the cell surface for cancer cell proliferation, invasion and metastasis [86, 87].</li> <li>• Up-regulated at carcinoma cells and functions the proliferation and progression of human lung and breast cancer cells [88, 89].</li> <li>• Acts as an inhibitor against human dental pulp stem cells (HDPSCs) proliferation and an inducer of apoptosis of HDPSCs through the stimulation of alkaline phosphatase (ALP) secretion and dentin sialophosphoprotein (DSPP) [90].</li> <li>• Degrades Insulin-like growth factor (IGF) binding protein 3 (IGFBP3) [91].</li> <li>• The decreased expression of ADAM28 in vaccine ILTV infection, may suppress the active induction of apoptosis.</li> </ul>
FST	<ul style="list-style-type: none"> <li>• Follistatin.</li> <li>• Inhibits follicle-stimulating hormone [92].</li> <li>• Binds and neutralizes activin, a paracrine hormone of TGF-<math>\beta</math> superfamily, which is related to the regulation of cell proliferation, apoptosis, and carcinogenesis [93, 94].</li> <li>• A member of fibrotic and wound healing response genes and cellular proliferation genes and plays a role in muscle growth and strength in nonhuman primates and liver proliferation. Moreover, the small plaque mutant of VZV down-regulates FST [95].</li> <li>• Up-regulation of FST at early phase (1 dpi) of vaccine ILTV infection may play a role in the initiation of CPE.</li> </ul>

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**Table 3.** The 23 genes showing a similar expression pattern in both wild-type and vaccine ILTV infection.

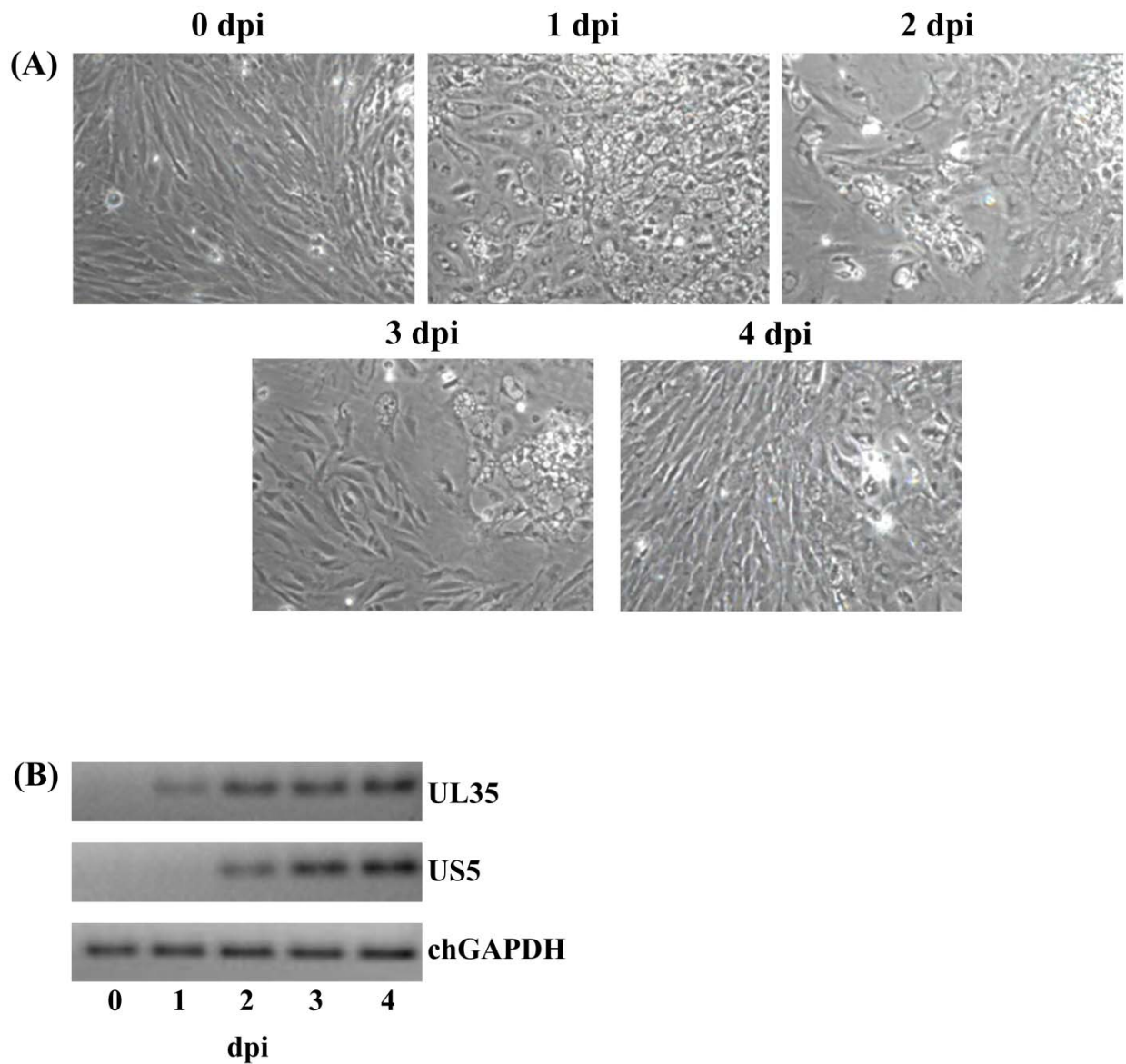
<b>GenBank</b>	<b>Symbol</b>	<b>Name</b>
AB055783	CENPH	centromere protein H
AB105812	GEM	GTP binding protein overexpressed in skeletal muscle
AF051399	FBLN1	fibulin 1
AJ309540	IL6	interleukin 6
AJ719326	MMP7	matrix metalloproteinase 7
AJ719339	NASP	nuclear autoantigenic sperm protein (histone-binding)
AJ720813	HNRNPD	heterogeneous nuclear ribonucleoprotein D or AU-rich element RNA binding protein 1, 37kDa
AJ720861	LSS	lanosterol synthase or 2,3-oxidosqualene-lanostrol cyclase
AY265159	LHFPL5	lipoma HMGIC fusion partner-like 5
BX930381	EMP1	epithelial membrane protein 1
BX931297	CYTL1	cytokine-like 1
BX932426	LRRC6	leucine rich repeat containing 6
BX932427	BATF3	basic leucine zipper transcription factor, ATF-like 3
BX933215	SOCS1	suppressor of cytokine signaling 1
BX933888	C1QTNF3	C1q and tumor necrosis factor related protein 3
CR353484	C9orf91	chromosome 9 open reading frame 91
CR387407	LOC424161	similar to LOC129881 protein
D16187	MAFK	V-maf musculoaponeurotic fibrosarcoma oncogene homolog K
M61145	PRNP	prion protein p27-30
M80584	LUM	lumican
U34977	FMOD	fibromodulin
X91638	SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
Y09235	GLRX	glutaredoxin or thioltransferase

The JMP Genomics 5.0 was used to compare the expression values at 1 and 3 dpi between wild-type and vaccine ILTV infection. The expression levels can be found in supplemental file 1 in chapter 2 and supplemental file 3 in chapter 3.

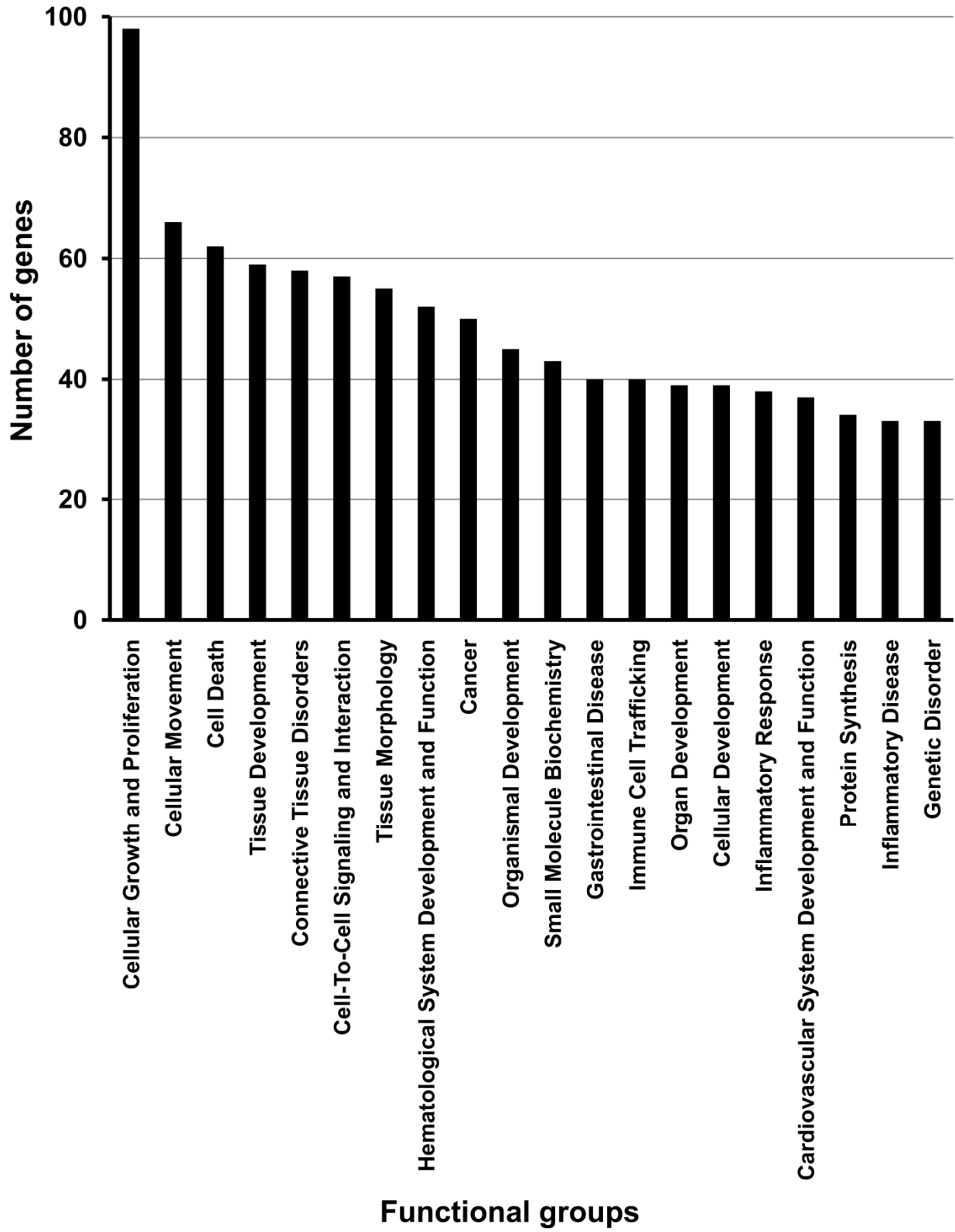
**Table 4.** The comparison of expression levels for eight genes showing opposite expression pattern in between wild-type and vaccine ILTV infection.

<b>ID</b>	<b>Wild-type ILTV Day1</b>	<b>Wild-type ILTV Day3</b>	<b>Vaccine ILTV Day1</b>	<b>Vaccine ILTV Day3</b>
F2RL1	0.703	-0.051	0.924	0.614
BMP2	1.152	-0.047	1.425	1.011
IKBIP	-0.326	-0.262	0.445	0.185
TYMS	0.665	-0.989	0.678	0.622
C8orf79	0.544	-0.636	1.663	2.015
F10	-0.161	0.553	2.472	2.220
PTGS2	-0.236	1.370	-0.641	-0.223
NPY	-0.437	0.368	-0.400	-1.456

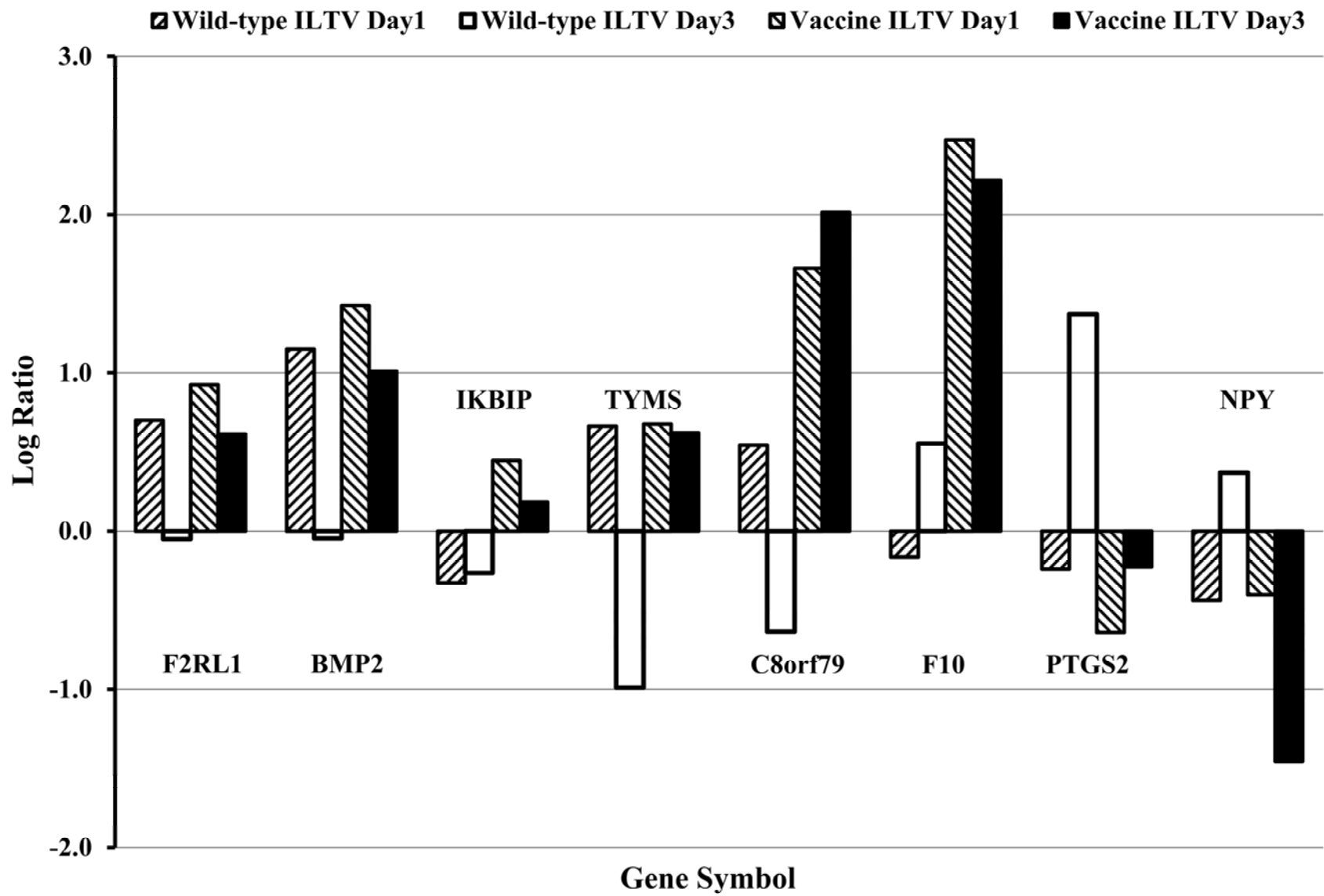
The values are presented by log<sub>2</sub> ratio.



**Figure 1.** Morphology of chicken embryo lung cells infected by vaccine ILTV and the expression of ILTV genes. (A) Cell morphology and CPE development at 0, 1, 2, 3 and 4 dpi. Phase contrast microscopic images are displayed at 200× magnification. (B) ILTV gene expression (UL35 and US5) at 0, 1, 2, 3 and 4 dpi by RT-PCR. Expression of chicken GAPDH (chGAPDH) was used as an endogenous control.



**Figure 2.** Functional groups of differentially expressed genes. Out of 75 functional groups, the top 20 groups considered by gene numbers related to each category were displayed.



**Figure 3.** Eight differently expressed genes in both infections. Relative expressions were compared for eight genes in between wild-type and vaccine ILTV infection. The upward diagonal, open, downward diagonal and close bar represent ILTV infected samples for wild-type at 1 dpi, wild-type at 3 dpi, vaccine at 1 dpi, and vaccine at 3 dpi, respectively.



## **CHAPTER 4**

### **Kinetic analysis of infectious laryngotracheitis virus (ILTV) gene expression during the lytic replication phase in cultured cells**

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## 1. Abstract

Avian infectious laryngotracheitis virus (ILTV; gallid herpesvirus 1) is a member of the *Iltovirus* genus of the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family. ILTV encodes 80 predicted open reading frames (ORFs) in about 150kb linearized dsDNA that contains UL region and US region flanked by IR and TR regions. The regulatory mechanisms of ILTV gene expression in infected cells are still unclear. In this study, the expression levels of all 76 genes known to be expressed in the ILTV genome were determined by quantitative PCR (qPCR) using SYBR green staining method with total RNA extracted from ILTV infected cells at 1, 3, 5 and 7 dpi. It was observed that ICP4, UL21 and UL42 showed unique expression patterns at all dpi time points, whereby ICP4 showed the highest expression levels at all time points. UL21 showed similarly high expression levels between 5 and 7 dpi after an initial steadily increase, and UL42 showed the fluctuations of expression level at all time points. UL17, UL28, UL29, UL50 and UL52 showed decreased expression at 3 dpi compared to 1 dpi, and increased again until 7 dpi. Twenty nine ILTV genes including UL-1, UL0, UL1, UL2, UL3, UL3.5, UL6, UL9, UL11, UL12, UL15, UL24, UL25, UL26, UL26.5, UL27, UL38, UL44, UL45, UL49, UL49.5, ORFA, ORFB, US2, US4, US5, US6, US7 and sORF1 indicated progressively increased with days post infection (dpi) time points. The relative expression levels of the remaining, 39 ILTV genes including UL4, UL5, UL7, UL8, UL10, UL13, UL14, UL18, UL19, UL20, UL22, UL23, UL30, UL31, UL32, UL33, UL34, UL35, UL36, UL37, UL39, UL40, UL41, UL43, UL46, UL48, UL51, UL53, UL54, UL56, ORFC, ORFD, ORFE, ORFF, US3, US8, US8A, US10, sORF4/3, are not different at 1 and 3 dpi, and then increased expression by 7 dpi. These results may provide insights into the molecular mechanisms of ILTV pathogenesis during the cytolytic infection phase.

## 2. Introduction

ILT as chicken respiratory disease is an economically important disease for the poultry industry worldwide. ILT causes a severe illness resulting in weight loss, reduction of egg production and high mortality rates, up to 70%. Currently, chicken embryo-origin (CEO) and tissue culture-origin (TCO) vaccines, which are live attenuated vaccines, have been broadly used to prevent ILT outbreaks in flocks, but the reversion of vaccine ILTV into virulent virus during passage in chickens became a current threat of ILT outbreak called vaccinal laryngotracheitis (VLT) [1-3]. ILTV is a causative agent for ILTV and ILTV Infected chickens or vaccinated chickens can transmit latently infected ILTV to uninfected chickens via respiratory route by coughing and sneezing or by bird-to-bird contact.

ILT virus (ILTV), which is a member of the *Iltovirus* genus of the *Alphaherpesvirinae*, is a causative agent of ILT. The dsDNA ILTV genome, which is about 150kb in size, includes one unique long region (UL), one unique short region (US), one inverted repeat (IR) and one terminal repeat (TR) flanking the US region [4, 5]. The ILTV genome encodes 80 predicted open reading frames (ORFs) expressing functional or structural proteins [6]. Previously, a mosaic ILTV genome sequence combined with partial sequences from 6 different ILTV strains has been used for ILTV research since 2006 [7]. Recently, the complete genome sequence of a single Australian commercial live attenuated ILTV vaccine strain (Serva) is reported in 2011[5]. Taken together, discriminatively, 5 ORFs named ORF A, B, C, D and E are located in near ORI<sub>L</sub> in the UL region in the ILTV genome [8]. Also, like typical herpesvirus, three DNA replication origins, one Ori<sub>L</sub> in the UL region and two identical ORI<sub>S</sub> in the IR and TR regions, are included in the ILTV genome. Furthermore, 63 genes in the ILTV genome show homologies with the HSV-1 genome [9], and it could be inferred that those proteins in both ILTV and HSV-1 genomes have

similar structure and function. Of 63 genes, 11 genes encode glycoproteins namely gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM, which function immunogenic and antigenic activity in ILTV infected cells.

Normally, herpesvirus gene expression can be classified into three stages; immediate-early (IE), early (E) and late (L). Usually, proteins expressed in the IE stage function as viral gene transcription regulators, E stage proteins play roles in viral genome replication, and finally L proteins are produced for structure proteins of virus particles [10]. However, kinetic expression patterns of ILTV during lytic infection have not been determined. This study uses a time course approach to gain insight into ILTV gene expression in cultured chicken embryo lung cells using qPCR and to help fully understand ILTV pathogenesis and the control mechanism of ILTV genes.

### **3. Materials and methods**

#### **3.1. Primary cell culture and ILTV infection**

Primary chicken embryo lung cells were prepared from 19 days old SPF chicken embryos as previously described [11]. ILTV purchased from the National Veterinary Services Laboratory (NVSL) was used to infect chicken embryo lung cells. The infected cells were incubated at a 37°C and 5% CO<sub>2</sub> incubator with gently rocking every 15 min for 1h. Ten ml of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA) were added in infected cells. Cells were incubated at 37°C and 5% CO<sub>2</sub> for up to 7 days. The protocol used for this study was approved by both the Institutional Biosafety Committee (IBC; permit number: 10007) of University of Arkansas and the Animal and Plant Health Inspection Service (APHIS; permit number: 102743) of United States Department of Agriculture (USDA).

#### **3.2. Total RNA extraction**

The total RNA was extracted from uninfected or ILTV infected primary chicken embryo lung cells at 1, 3, 5 and 7 days post infection (dpi) using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following manufacturer's instructions. After treating DNaseI (New England BioLabs Inc., Ipswich, MA, USA), the total RNA was re-extracted by the TRIzol reagent. The quantification of the total RNA was determined by NanoDrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the quality was determined by the fractionation of 18S and 28S RNAs on an agarose gel (data not shown).

### 3.3. qPCR

qPCR was performed for all known 76 genes encoded in the ILTV genome using gene specific primer sets. Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>) was used to design primers synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer information is provided in Table 1. Briefly, 3 µg of the total RNA were initially used for reverse transcription and the qPCR was performed under the following conditions: total 40 cycles, denaturation at 95°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 30 s, and one cycle of final extension at 72°C for 10 min. A non-template control (NTC) and endogenous loading control (reference; chicken GAPDH) were used for the relative quantification. The relative expression levels of ILTV infection were calculated with Cp value which is  $Ct^{ref} - Ct^{sample}$  adding 24 as a specific constant in order to revise negative values to all positive value [12].

### 3.4. Data analysis

All gene expression values were subject to one-way ANOVA test using JMP Genomics 5.0 (<http://www.jmp.com/software/genomics/>) licensed for Cell and Molecular Biology (CEMB) of the University of Arkansas. The significant differences of the ILTV gene expression levels at each time point were considered by  $p < 0.05$ .

## **4. Results and discussion**

### **4.1. Highest, fluctuation and invariable expression patterns in ILTV genes**

Of the viral genes determined, UL21, UL42 and ICP4 showed a distinct gene expression pattern during ILTV propagation (Figure 1).

Of these three genes, ICP4 consistently expressed the highest level compared to other genes during all dpi time points with Cp values ranging from 17.15 to 17.67. ICP4 of herpes simplex virus was also highly expressed in the murine trigeminal ganglia until 7 dpi during lytic infection [13]. ICP4 as an immediate early gene is known to regulate many early and late genes [14-16]. ICP4 localizes in intranuclear distribution or in globular compartments at early or late stage of infection, respectively [17].

The expression of UL21, a tegument protein functioning in virion morphogenesis, increased from 1 dpi to 5 dpi and remained elevated on 7 dpi. Specifically, Cp expression levels were 1.33, 3.75, 7.09, and 8.06 at 1, 3, 5 and 7 dpi, respectively. The UL21 gene, which is conserved among mammalian herpesviruses [18], is known to function in herpesvirus virulence and virus propagation in cultured cells during infections of HSV-1 [19] and PrV [20-23]. In addition, the UL21 protein in HSV-1 infection is associated with virus transportation through microtubules [23]. Also, UL21 in PrV infection is required for package of UL46, UL49 and US3 into mature virions [24] and is also related to retrograde and neuronal invasiveness in vitro and in vivo [25]. Thus, ILTV UL21 may play critical roles in virus propagation and ILTV virulence by progressively increasing its protein amount during lytic infection phase.

Expression levels of UL42, which codes for a DNA polymerase processivity subunit that functions in DNA replication [26], fluctuated between time points determined. The Cp values were 8.23, 12.43, 7.92, and 14.93 on 1, 3, 5 and 7 dpi, respectively. UL42 has been shown for

other herpesviruses to play a role in viral DNA replication [27, 28]. Additionally, in HSV-1 infections, it appears to be also important in DNA replication for lytic viral growth [29]. The UL42 gene is expressed early under the regulation of immediate-early IE63 (ICP4) and can be detected at as early as 3 hpi in HSV-1 infection [30-32]. Likewise, ILTV UL42 examined here was expressed already on early lytic infection stages, 1 dpi, presumably to drive DNA replication. The reason for the biphasic nature of UL42 expression exhibiting a decrease at 5 dpi is unknown.

#### **4.2. Genes showing decreased expression at 3 dpi**

The similar levels of expressions of UL17, UL28, UL29, UL50 and UL52 observed at 1 dpi remained at the same levels on 3 dpi and increased further by 7 dpi (Figure 2). The Cp expression levels of UL17, DNA packing tegument protein related to DNA encapsidation and capsid transport, were 6.84, 5.89, 10.60 and 12.80 at 1, 3, 5 and 7 dpi, respectively. The UL17 in HSV-1 and PrV shown to be involved in viral capsid cleavage and packaging by association of immature B-type capsids [18, 33]. In Marek's disease virus type 1 (MDV-1) infection, UL17 was formed to have a fundamental role for virus growth in the nucleus [34]. Likewise, ILTV UL17 expression is increased at the later stages of lytic infection presumably because of its function in virion packaging. UL28, which codes for a DNA packaging terminase subunit during DNA encapsidation, showed Cp expression as of 4.45, 2.49, 6.56 and 8.59 at 1, 3, 5 and 7 dpi, respectively. In HSV-1 infection, UL28 play an important role in the maturation of capsid protein [35], DNA cleavage and encapsidation of viral gene through interacting with UL33 [36, 37]. Previously, the homologues of UL28 were reported as a conserved region between herpesviruses such as MDV-2, bovine herpesvirus 1 (BHV-1) and green turtle herpesvirus



(GTHV) [38-40]. Similar to UL17 shown above, the functional role of ILTV UL28 in virion maturation is correlated to its increased expression at the late phase of lytic infection.

The UL29, single-stranded DNA-binding protein in DNA replication [29] showed Cp expression of 5.82, 4.56, 9.91 and 12.42 at 1, 3, 5 and 7 dpi, respectively. The Cp expressions of the UL50, deoxyuridine triphosphatase nucleotide metabolism, were 8.17, 7.61, 11.72 and 13.75 at 1, 3, 5 and 7 dpi, respectively. UL50 is closely conserved in herpesviruses such as BHV-1, equine herpesvirus type 1 (EHV-1), HSV-1 and varicella zoster virus (VZV) [41-45]. UL50 deletion mutant of ILTV resulted in decreased cell-to-cell spread in vitro and attenuation in vivo and in efficient replication in the respiratory tract of infected hosts [46], demonstrating the importance of the virus gene in viral replication and virulence. The expression levels of UL52, helicase-primase subunit DNA replication, were 4.84, 3.02, 6.88 and 10.58 at 1, 3, 5 and 7 dpi, respectively. In HSV-1, UL52 is a subunit of a three polypeptide complex consisting of UL5, UL8 and UL52 proteins, the functions as a DNA helicase-primase [47]. Particularly, UL52 has the primase activity within the helicase-primase complex and the function is conserved among different herpesviruses such as VZV, Epstein-Barr virus (EBV), human cytomegalovirus (hCMV) and EHV-1 [48]. Generally, the viral gene transcription machinery is activated earlier than viral genomic DNA replication pathways. Therefore, the large increase of expression of UL29, UL50 and UL52, which are responsible mainly in DNA replication, may be correlated to the later onset of ILTV genome replication.

#### **4.3. The typical expression patterns among ILTV genes.**

Most of the ILTV genes were expressed in similar patterns showing continuous increase from 1 through 7 dpi or 3 through 7 dpi (Table 2 and 3). In total, 29 genes out of 76 ILTV genes

showed the progressive increase of gene expression until 7 dpi (Table 2). The mean values of Cp expression for each dpi time point were  $7.51 \pm 0.25$ ,  $8.88 \pm 0.18$ ,  $12.52 \pm 0.10$  and  $15.15 \pm 0.15$  at 1, 3, 5 and 7 dpi, respectively. Among the 29 genes, 23 and 6 genes were in the UL (UL-1, 0, 1, 2, 3, 3.5, 6, 9, 11, 12, 15, 24, 25, 26, 26.5, 27, 38, 44, 45, 49, ORF-A and OEF-B) and US (US2, 4, 5, 6, 7 and sORF1) region, respectively. In contrast, 39 genes showed no significant changes of gene expression between 1 dpi ( $6.46 \pm 0.34$  Cp) and 3 dpi ( $6.52 \pm 0.33$  Cp), but their expression increased further at 5 dpi ( $10.51 \pm 0.17$  Cp) and 7 dpi ( $13.20 \pm 0.11$  Cp) (Table 3). Among 39 genes, 34 genes (UL4, 5, 7, 8, 10, 13, 14, 18, 19, 20, 22, 23, 30, 31, 32, 33, 34, 35, 36, 37, 39, 40, 41, 43, 46, 48, 51, 53, 54, 56, ORF-C, ORF-D, ORF-E and ORF-F) and 5 genes (US3, 8, 8A, 10 and sORF4/3) were in the UL and US region, respectively.

Kinetic analysis of ILTV gene expression showed critical patterns compared to other herpesviruses. In the case of human herpesvirus (HHV)-6B, most HHV-6B genes were actively expressed until 8 hours post infection (hpi). Late genes slowly increased until 48 hpi [49], and after this time only late genes and few early and delayed-early genes were expressed until 60 hpi [50]. Most of the genes encoding HHV-8, which is also known as KSHV, were highly expressed within 48 hpi, and the expression level of most genes decreased after 48 hours [51]. In infected NIH 3T3 fibroblast cells with mCMV, late genes were detected at 48 hpi [52]. The expressions of murine herpesvirus (MHV)-68 genes in infected baby hamster kidney (BHK)-21 cells peaked until 32 hpi and then gradually decreased or remained at similar level until 5 dpi [53]. Among the genes, the expression of IE and E genes were reached to a peak by 8 to 12 hpi. However, most of ILTV genes were continuously up-regulated until 7 dpi.

## **5. Conclusion**

In this study, expression of ILTV genes was determined at 1, 3, 5 and 7 dpi by qPCR using SYBR. Though most of ILTV genes continuously increased during lytic infection period, some genes such as ICP4, UL21 and UL42 showed unique expression patterns. Also, the observation of elevated ILTV gene expression until 7 dpi was not previously reported for other herpesviruses. Therefore, the findings of ILTV gene expression may provide information on ILTV life cycle and pathogenesis.

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**Table 1.** The primer sets for qPCR in ILTV infected chicken embryo lung cells.

<b>Gene Name</b>	<b>Forward Primer (5' → 3')</b>	<b>Reverse Primer (5' → 3')</b>
UL-1	TGT GCG ATG CTC CAA ATA GC	ACA ATT CCT GCG ACA CTC CA
UL0	CTT GAC GTC CGT GCT GTC AT	CCA CTC ACG GTG GAT TTG AA
UL1	TCC GTG TTT ACG CAC AGA GG	AGA TAA CGG TGC GCG AAT TT
UL2	CAC CCC CTA GGG ACA CTG AA	ACT GCA ATC CGC AAG ATG TG
UL3	TTC GTT TTG ACA TGC CCT TG	TTG ACT GTG TTT CCG GAT CG
UL3.5	GTG AGG AAA ATC GGG TCT CG	CGG CTG CGT TAC AGG GTA AT
UL4	TGG AGG AAT GCG GTT TAT TTG	ATC ATT GTT GCG ACG GGA TT
UL5	AAC AAC GTT CGT TGG CAG AA	ACC GGA GAT GCC GTG TCT AT
UL6	ATT TCG AAC CCT CTG GCA AA	TGG CAG TGA TCT CGG AAA TG
UL7	ATC ATC TAG GCG CAC ACA CG	ATC CAG ATG GAT GGG GAA AA
UL8	CCT GGG CTT CGA GAA GAG AA	TAG CGG TGC TTA CGG TGT TG
UL9	TTT CAA ACG TCG TTG CGT TC	CGC CCC CAG TTA GTC GTA TC
UL10	GCC CTT AGC AGT CCA GGT TG	TTA GTC CGC AAG CAG GGT TT
UL11	TCT GTG GAC ACC TCG GAG AAT	TAG GGA CAA GCG CTC TCC AG
UL12	GGC AGA GAA GCG TTC GAG TT	CGC AGG ACT TTG AAC AGC AG
UL13	GCG CAT CGA TCT ACG AGA AA	TCC GCA TCC CAG TGA CAT AG
UL14	AGG TGA CCC TGA TCG CAG AG	GCA GTA GCG TCG GAG GAT TT
UL15	TAG TGC GGC ATC GGT ATC TG	CTA TTT CGC GTG CGT TTT CA
UL17	GGC AAA AAC CGG CAA TAT GT	TAG CCT GTT TGG CTG ACG TG
UL18	CAG AAG CTT GCG TGG GAC TA	TTC CAT GGG CGA AAA ATG AT
UL19	CAC TTT TTC CGC CCA TGA TT	GCT CTC TCT CCG AAG GTG GA
UL20	CCA ATT CAA TCC GCC TCT CT	TCA CCA TCA CGT TCT TTT TCG
UL21	AGT TTC GGG GCT CTG GAT TT	GCT CGG GCA CTA TCC TTG TC
UL22	GAC AGC AGT TCC GTG TGG TC	ACT GTT AGC CGG TGC CAA CT
UL23	GAC GGC AAC CTC TCC AAT TT	CGC CGC GTT GTA CTT CTT TA
UL24	CTC GGA GCA AAA CTG CAC AC	AGC GAT TAT GGC AAC GGA TT
UL25	GAT CTG GAA AGG CGG AAC AG	AGT TTG CAA TGC CGA TGA GA
UL26	GAC CTG CCG AGA GGA CTG TT	GGC ATT CGC AAA AAG AGC AT
UL26.5	ACG GAG CAG CAG CTA TGT GA	CCG GCA ACC TCT TTT TGA AG
UL27	TAG CCT GGC GCT TAC AGG TT	TCA GAA CCG CCA ACT GAT TG
UL28	TTT CCC GTA CCC AAG CGT AG	AGA GCG TGC TCC GAA ATA GC
UL29	ACG CGC TCC ATT TGA GTT TT	TCT GTC TCC TGC GCT CCA TA
UL30	CCA AAT TCG AGA CCC AGG AC	TCT GGC CAC GAG ACA TTG TT
UL31	CGT ACC AGC GAA TAC GTC CA	AAA CAG AGC ACG CCA CTT CA
UL32	TCG AAG AGC ATG CCA GTG TT	GGC GTC TAT TTT GCC TCA CC
UL33	TTA GCG GAA AGG CCA CTC TC	CCG GAG GCG ATA GGT CAT AA
UL34	GAA CGA CAG CGA GTC TGG TG	TGG GGG ACC GTC AAG TTT AG

UL35	AAA CCG AGC TCC AAT GCT TC	GCG TTG TCT CAC TCC AAA CG
UL36	GCG GCT TTG GCT ACT TTG AT	AAA ACG AGC TCG AGG GAG TG
UL37	CGC TTT CAG ACA CCC CTA CC	ATC CGC GGA CGC TAC AGT AT
UL38	ACG AGC CCA GGG AAA TTC TT	GCG ATG GCC CTT GTA TCT TC
UL39	AGA GCG GGA ACC ACA GGT TA	GCC CTC AAC GCT AGT CAT CC
UL40	TTT TTC GTG GCG TCC TTT G	TTC ATC GCG ACT GAT GAG GT
UL41	AGG ATC CTG AGG GCG GTA AT	CCT TTG CCT GGA CGC TAT GT
UL42	GGA GGA GCG CCT CAT ACA AC	GGT GGT GGG ATG GGC TAA TA
UL43	CTG GCA TTC CAT TTG GTG TC	GTG TAT GGG TGG CCG TTT TT
UL44	AGC TCG GTG ACC CCA TTC TA	ACT AAG CTG GCT GGG TGT CG
UL45	GGG CAC GGC AAG AAA GTT TA	CGC CTG ACT GGA TTC AAC AT
UL46	TCT CGA TGT CCC ATT TGG TG	TGT CCG GGA AAA CCC AGT AG
UL48	TGA GGA TGA TGC GAC TGA CC	GTA CTT TGG CGC ATT GAC CA
UL49	ATT CGA ACA CCC CTG GTC TG	GAT GGC CGT AAT CCC CTT CT
UL49.5	GCC GTG GGA AAG GAG TTT TA	CAT TCC GTG TCC TGG TGT CA
UL50	TAG CTG TGC CAC TCG GTC AC	GAC CCG ATA CAT GTG CAG GA
UL51	GCA GAC AGG ACG ATG ATG CT	CCA TGC CGA GTA GTT CCA AA
UL52	CCA GCA TGT CAG CCA AGT GT	TTT TTG TCG TTC CGC ATT TG
UL53	GCA CGG GTC AGC AAG TAA CA	CGT CAT GGA AGT GGC AGA CT
UL54	ACG TTT CGC AAC TCC TTC GT	TTG GGA AAG AAA TCG GGA TG
UL56	TGG GTT GGA GTG GCC TTA AT	GGA TGC GGA TAC AAC CTT CC
US2	CGC TCC ACT CGG GAC TTA CT	AGT TTC CGG GGA GCT GTT TT
US3	CAG CTC ATG TCA GCG GTC TC	TGT CTT GCC GTC GCA GTT TA
US4	CCG GGA TAC CTG ATT TCG AC	AGG GAT CCA TTG CCA GAC AC
US5	TCC AGC TAA TGT GCC TGT CG	CTG GCG CGA TAT CTT TTT CC
US6	GGA ATG TGG CGA CGT ACA AC	TTC GCG ATA CAA GGG TGC TA
US7	TGC GTG CGA AAT AAA CAA GG	TTA TTG AGT CGG GCG AGC TT
US8	AGA CGA GAA GGC TCC AGC AC	CAT GAA ACG GCA GCA TTT GT
US8A	GGC ACC GGT AAG AAA ACT GC	CGC GTG GTT GTC AGA TTC C
US10	GCA ACG GGT TGG GAG AAT TA	TAC GGC TCT CGT CCT CGA A
sORF1	ACT GCC GAG GTC CTT TCT CA	GGG GTC GGT TTG GAC TCA TA
sORF4/3	GAA CCT TCG GGG AGG TCT TC	GGC GAT AAG GAT AGG CAT GG
ICP4	ACA GGG GAT GGA CCA TGA AC	GCC AAT CCG AAA TCA GCT TC
ORFA	TCT CCT GGG ATC TGC GTT CT	TCC TTC GCC TAT CTC GAA GC
ORFB	TTG CAG GTT GGA GTG CCT AA	AGC ACA CTG CAG GAT CCA AA
ORFC	AAT CTG GCG TTG GTC CAC TT	CGC AAA CTT GAG ATG GGA CA
ORFD	AAT GTG AAC GCA CTG CCA AG	ATC TTT CCA GCT CCG ACG AA
ORFE	CTA TCT CAT CGG GGG TCC AA	ATG TGC GGC AAG TTT CTG TG
ORFF	GAG AAT AGC TGC CCC TGC TG	CCT GGG TCC TCT TCA CGT TC

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**Table 2.** The list of virus genes continuously increased at all time points in ILTV infected chicken embryo lung cells.

Genes	1 dpi	3 dpi	5 dpi	7 dpi	Protein <sup>§</sup> Functions <sup>§</sup>
	Cp <sup>†</sup> SE <sup>‡</sup>	Cp SE	Cp SE	Cp SE	
UL-1	9.41 <sup>a</sup>	10.64 <sup>b</sup>	13.48 <sup>c</sup>	15.28 <sup>d</sup>	Protein LORF2
	0.16	0.12	0.11	0.09	Unknown
UL0	5.63 <sup>a</sup>	7.21 <sup>b</sup>	11.49 <sup>c</sup>	14.60 <sup>d</sup>	Protein UL0
	0.12	0.22	0.07	0.04	Unknown
UL1	7.35 <sup>a</sup>	9.71 <sup>b</sup>	13.60 <sup>c</sup>	16.33 <sup>d</sup>	Envelope glycoprotein L
	0.14	0.14	0.08	0.12	Cell entry: cell-to-cell spread
UL2	7.06 <sup>a</sup>	8.73 <sup>b</sup>	12.62 <sup>c</sup>	15.74 <sup>d</sup>	Uracil-DNA glycosylase
	0.11	0.13	0.13	0.26	DNA repair
UL3	6.99 <sup>a</sup>	7.94 <sup>b</sup>	11.28 <sup>c</sup>	13.33 <sup>d</sup>	Nuclear protein UL3
	0.25	0.19	0.06	0.14	Unknown
UL3.5	8.13 <sup>a</sup>	9.29 <sup>b</sup>	13.03 <sup>c</sup>	17.00 <sup>d</sup>	Protein V57
	0.17	0.14	0.08	0.01	Possibly virion morphogenesis
UL6	4.19 <sup>a</sup>	6.14 <sup>b</sup>	8.85 <sup>c</sup>	12.01 <sup>d</sup>	Capsid portal protein
	0.48	0.40	0.21	0.23	DNA encapsidation
UL9	1.65 <sup>a</sup>	3.08 <sup>b</sup>	5.51 <sup>c</sup>	7.14 <sup>d</sup>	DNA replication origin-binding helicase
	0.39	0.46	0.34	0.03	DNA replication
UL11	8.93 <sup>a</sup>	9.98 <sup>b</sup>	13.99 <sup>c</sup>	16.76 <sup>d</sup>	Myristylated tegument protein
	0.06	0.24	0.04	0.13	Virion morphogenesis
UL12	9.34 <sup>a</sup>	10.72 <sup>b</sup>	14.27 <sup>c</sup>	17.19 <sup>d</sup>	Deoxyribonuclease
	0.10	0.05	0.09	0.10	DNA processing
UL15	5.87 <sup>a</sup>	6.70 <sup>b</sup>	9.63 <sup>c</sup>	11.88 <sup>d</sup>	DNA packing terminase subunit 1
	0.35	0.18	0.11	0.17	DNA encapsidation
UL24	4.76 <sup>a</sup>	5.55 <sup>b</sup>	9.55 <sup>c</sup>	13.22 <sup>d</sup>	Nuclear protein UL24
	0.31	0.26	0.03	0.12	Unknown

UL25	5.79 <sup>a</sup>	7.02 <sup>b</sup>	10.46 <sup>c</sup>	14.02 <sup>d</sup>	DNA packaging tegument protein UL25
	0.50	0.12	0.12	0.07	DNA encapsidation
UL26	5.37 <sup>a</sup>	6.43 <sup>b</sup>	10.33 <sup>c</sup>	13.34 <sup>d</sup>	Capsid maturation protease
	0.10	0.06	0.16	0.15	Capsid morphogenesis
UL26.5	9.43 <sup>a</sup>	10.26 <sup>b</sup>	14.22 <sup>c</sup>	17.16 <sup>d</sup>	Capsid scaffold protein
	0.20	0.14	0.08	0.23	Capsid morphogenesis
UL27	6.74 <sup>a</sup>	7.54 <sup>b</sup>	11.68 <sup>c</sup>	14.46 <sup>d</sup>	Envelope glycoprotein B
	0.11	0.14	0.12	0.18	Cell entry: cell-to-cell spread
UL38	4.64 <sup>a</sup>	6.10 <sup>b</sup>	10.21 <sup>c</sup>	13.85 <sup>d</sup>	Capsid triplex subunit 1
	0.53	0.23	0.03	0.03	Capsid morphogenesis
UL44	8.78 <sup>a</sup>	9.84 <sup>b</sup>	13.50 <sup>c</sup>	15.91 <sup>d</sup>	Envelope glycoprotein C
	0.08	0.14	0.02	0.06	Cell attachment
UL45	6.67 <sup>a</sup>	9.29 <sup>b</sup>	12.94 <sup>c</sup>	15.28 <sup>d</sup>	Membrane protein UL45
	0.36	0.09	0.11	0.16	Possibly membrane fusion
UL49	10.15 <sup>a</sup>	11.44 <sup>b</sup>	15.05 <sup>c</sup>	18.00 <sup>d</sup>	Tegument protein VP22
	0.29	0.16	0.07	0.10	RNA transport to uninfected cells
UL49.5	7.63 <sup>a</sup>	8.67 <sup>b</sup>	12.23 <sup>c</sup>	14.71 <sup>d</sup>	Envelope glycoprotein N
	0.41	0.27	0.05	0.19	Membrane fusion
ORF-A	10.90 <sup>a</sup>	12.35 <sup>b</sup>	15.91 <sup>c</sup>	18.48 <sup>d</sup>	Protein IA
	0.11	0.13	0.17	0.35	Unknown
ORF-B	10.38 <sup>a</sup>	12.17 <sup>b</sup>	15.72 <sup>c</sup>	18.19 <sup>d</sup>	Protein IB
	0.17	0.12	0.10	0.16	Unknown
US2	4.58 <sup>a</sup>	6.32 <sup>b</sup>	9.57 <sup>c</sup>	10.93 <sup>d</sup>	Virion protein US2
	0.31	0.26	0.05	0.01	Possibly interacts with cytokeratin 18
US4	12.06 <sup>a</sup>	12.44 <sup>b</sup>	16.68 <sup>c</sup>	19.48 <sup>d</sup>	Envelope glycoprotein G
	0.08	0.10	0.12	0.14	Cell-to-cell

US5	8.38 <sup>a</sup>	10.15 <sup>b</sup>	13.95 <sup>c</sup>	15.37 <sup>d</sup>	Envelope glycoprotein J
	0.59	0.24	0.17	0.14	Unknown
US6	9.34 <sup>a</sup>	11.22 <sup>b</sup>	14.81 <sup>c</sup>	16.74 <sup>d</sup>	Envelope glycoprotein D
	0.21	0.15	0.02	0.02	Cell attachment
US7	10.63 <sup>a</sup>	11.97 <sup>b</sup>	15.86 <sup>c</sup>	18.11 <sup>d</sup>	Envelope glycoprotein I
	0.16	0.18	0.19	0.08	Cell-to-cell spread
sORF1	7.15 <sup>a</sup>	8.74 <sup>b</sup>	12.69 <sup>c</sup>	14.72 <sup>d</sup>	Tegument protein VP13/14
	0.27	0.26	0.10	0.25	Possibly gene regulation

<sup>†</sup>The expression levels of ILTV genes were presented to Cp indicated by  $-\Delta Ct + 24$ .

<sup>‡</sup>a, b, c and d indicate differential expression, and they were deduced by ANOVA test in JMP Genomics 5.0.

<sup>§</sup>The productive proteins and their functions were referenced from the nucleotide information of Gallid herpesvirus 1 (NC\_006623) in NCBI.

**Table 3.** The list of virus genes which were no differences of gene expression at 1 and 3 dpi in ILTV infected chicken embryo lung cells.

Genes	1 dpi	3 dpi	5 dpi	7 dpi	Protein <sup>§</sup> Functions <sup>§</sup>
	Cp <sup>†</sup> SE <sup>‡</sup>	Cp SE	Cp SE	Cp SE	
UL4	5.80 <sup>a</sup>	5.43 <sup>a</sup>	10.40 <sup>b</sup>	13.10 <sup>c</sup>	Nuclear protein UL4
	0.27	0.36	0.01	0.05	Unknown
UL5	4.19 <sup>a</sup>	4.50 <sup>a</sup>	7.40 <sup>b</sup>	10.28 <sup>c</sup>	Helicase-primase helicase subunit
	0.43	0.57	0.12	0.03	DNA replication
UL7	2.83 <sup>a</sup>	3.78 <sup>a</sup>	6.65 <sup>b</sup>	12.37 <sup>c</sup>	Tegument protein UL7
	0.73	0.56	1.59	0.47	Virion morphogenesis
UL8	4.98 <sup>a</sup>	5.38 <sup>a</sup>	9.36 <sup>b</sup>	10.84 <sup>c</sup>	Helicase-primase subunit
	0.49	0.35	0.18	0.26	DNA replication
UL10	8.49 <sup>a</sup>	8.77 <sup>a</sup>	11.89 <sup>b</sup>	14.48 <sup>c</sup>	Envelope glycoprotein M
	0.24	0.29	0.12	0.01	Membrane fusion
UL13	8.01 <sup>a</sup>	8.32 <sup>a</sup>	12.52 <sup>b</sup>	15.35 <sup>c</sup>	Tegument serine/threonine protein kinase
	0.25	0.10	0.07	0.16	Protein phosphorylation: PK family
UL14	7.29 <sup>a</sup>	7.69 <sup>a</sup>	11.99 <sup>b</sup>	14.81 <sup>c</sup>	Tegument protein UL14
	0.53	0.22	0.11	0.26	Virion morphogenesis
UL18	7.21 <sup>a</sup>	7.48 <sup>a</sup>	12.35 <sup>b</sup>	14.67 <sup>c</sup>	Capsis triplex subunit 2
	0.29	0.43	0.21	0.18	Capsis morphogenesis
UL19	6.79 <sup>a</sup>	7.36 <sup>a</sup>	11.87 <sup>b</sup>	13.54 <sup>c</sup>	Major capsid protein
	0.36	0.32	0.20	0.12	Capsid morphogenesis
UL20	4.03 <sup>a</sup>	4.43 <sup>a</sup>	8.42 <sup>b</sup>	11.75 <sup>c</sup>	Envelope protein UL20
	0.38	0.38	0.13	0.07	Membrane fusion
UL22	7.40 <sup>a</sup>	6.32 <sup>a</sup>	10.42 <sup>b</sup>	13.05 <sup>c</sup>	Envelope glycoprotein H
	0.79	0.38	0.11	0.10	Cell entry: cell-to-cell spread
UL23	3.72 <sup>a</sup>	3.17 <sup>a</sup>	7.62 <sup>b</sup>	11.09 <sup>c</sup>	Thymidine kinase
	0.41	0.26	0.11	0.04	Nucleotide metabolism
UL30	5.46 <sup>a</sup>	6.08 <sup>a</sup>	8.83 <sup>b</sup>	10.56 <sup>c</sup>	DNA polymerase catalytic subunit
	0.53	0.47	0.23	0.17	DNA replication

UL31	7.42 <sup>a</sup> 0.08	7.38 <sup>a</sup> 0.43	11.25 <sup>b</sup> 0.06	13.97 <sup>c</sup> 0.15	Nuclear egress lamina protein Nuclear egress
UL32	5.93 <sup>a</sup> 0.44	5.76 <sup>a</sup> 0.22	9.41 <sup>b</sup> 0.08	11.31 <sup>c</sup> 0.35	DNA packaging protein UL32 DNA encapsidation
UL33	5.35 <sup>a</sup> 0.04	5.54 <sup>a</sup> 0.13	9.77 <sup>b</sup> 0.23	11.75 <sup>c</sup> 0.20	DNA packaging protein UL33 DNA encapsidation
UL34	8.67 <sup>a</sup> 0.16	8.50 <sup>a</sup> 0.22	12.81 <sup>b</sup> 0.02	15.64 <sup>c</sup> 0.01	Nuclear egress membrane protein Nuclear egress
UL35	8.38 <sup>a</sup> 0.07	8.24 <sup>a</sup> 0.16	13.08 <sup>b</sup> 0.02	16.43 <sup>c</sup> 0.03	Small capsid protein Capsid morphogenesis
UL36	5.31 <sup>a</sup> 0.74	5.27 <sup>a</sup> 1.13	9.59 <sup>b</sup> 0.27	12.10 <sup>c</sup> 0.13	Large tegument protein Capsid transport
UL37	6.46 <sup>a</sup> 0.26	5.76 <sup>a</sup> 0.54	10.97 <sup>b</sup> 0.19	13.25 <sup>c</sup> 0.13	Tegument protein UL37 Virion morphogenesis
UL39	6.96 <sup>a</sup> 0.08	6.54 <sup>a</sup> 0.45	11.09 <sup>b</sup> 0.06	12.51 <sup>c</sup> 0.27	Ribonucleotide reductase subunit 1 Nucleotide metabolism
UL40	8.51 <sup>a</sup> 0.45	8.24 <sup>a</sup> 0.36	12.40 <sup>b</sup> 0.15	14.98 <sup>c</sup> 0.03	Ribonucleotide reductase subunit 2 Nucleotide metabolism
UL41	5.44 <sup>a</sup> 0.20	5.55 <sup>a</sup> 0.28	10.02 <sup>b</sup> 0.09	13.03 <sup>c</sup> 0.10	Tegument host shutoff protein Cellular mRNA degradation
UL43	8.75 <sup>a</sup> 0.23	8.23 <sup>a</sup> 0.19	13.29 <sup>b</sup> 0.07	15.95 <sup>c</sup> 0.03	Envelope protein UL43 Possibly membrane fusion
UL46	8.35 <sup>a</sup> 0.22	8.39 <sup>a</sup> 0.08	12.32 <sup>b</sup> 0.18	14.66 <sup>c</sup> 0.05	Tegument protein VP11/12 Possibly gene regulation
UL48	6.46 <sup>a</sup> 0.22	6.57 <sup>a</sup> 0.11	10.58 <sup>b</sup> 0.11	13.09 <sup>c</sup> 0.16	Transactivating tegument protein VP16 Transactivates immediate early genes
UL51	7.55 <sup>a</sup> 0.10	7.06 <sup>a</sup> 0.37	11.30 <sup>b</sup> 0.07	14.93 <sup>c</sup> 0.02	Tegument protein UL51 Virion morphogenesis
UL53	6.14 <sup>a</sup> 0.32	6.77 <sup>a</sup> 0.61	9.90 <sup>b</sup> 0.14	12.11 <sup>c</sup> 0.04	Envelope glycoprotein K Membrane fusion

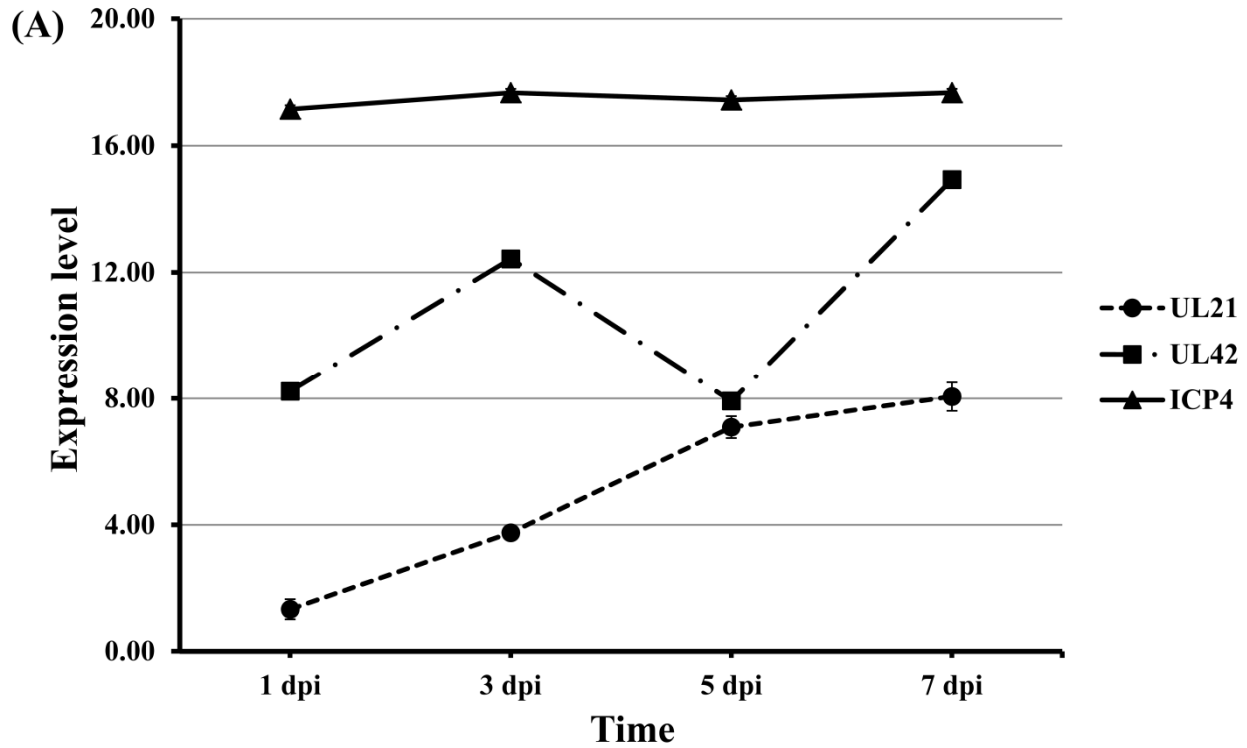
UL54	6.08 <sup>a</sup> 0.24	6.57 <sup>a</sup> 0.34	10.62 <sup>b</sup> 0.10	12.89 <sup>c</sup> 0.13	Multifunctional expression regulator RNA metabolism and transport
UL56	6.78 <sup>a</sup> 0.24	6.98 <sup>a</sup> 0.01	9.39 <sup>b</sup> 0.15	11.94 <sup>c</sup> 0.08	Unknown Possibly vesicular trafficking
ORF-C	8.07 <sup>a</sup> 0.37	8.13 <sup>a</sup> 0.06	11.16 <sup>b</sup> 0.07	14.23 <sup>c</sup> 0.02	Protein IC Unknown
ORF-D	5.39 <sup>a</sup> 0.73	5.83 <sup>a</sup> 0.44	9.80 <sup>b</sup> 0.08	13.29 <sup>c</sup> 0.08	Protein ID Unknown
ORF-E	6.48 <sup>a</sup> 0.14	6.13 <sup>a</sup> 0.21	10.56 <sup>b</sup> 0.08	13.59 <sup>c</sup> 0.07	Protein IE Unknown
OEF-F	4.55 <sup>a</sup> 0.53	5.27 <sup>a</sup> 0.31	8.14 <sup>b</sup> 0.09	11.84 <sup>c</sup> 0.01	Protein IF Unknown
US3	7.48 <sup>a</sup> 0.13	7.32 <sup>a</sup> 0.30	11.89 <sup>b</sup> 0.06	14.61 <sup>c</sup> 0.06	Serine/threonine protein kinase US3 Protein phosphorylation: apoptosis
US8	6.90 <sup>a</sup> 0.21	7.19 <sup>a</sup> 0.18	11.27 <sup>b</sup> 0.14	13.16 <sup>c</sup> 0.20	Envelope glycoprotein E Cell-to-cell spread
US8A	7.46 <sup>a</sup> 0.25	7.77 <sup>a</sup> 0.23	12.32 <sup>b</sup> 0.61	14.36 <sup>c</sup> 0.07	Membrane protein US8A Unknown
US10	5.44 <sup>a</sup> 0.93	5.58 <sup>a</sup> 0.30	10.26 <sup>b</sup> 0.20	13.29 <sup>c</sup> 0.02	Virion protein US10 Unknown
sORF4/3	5.32 <sup>a</sup> 0.22	5.02 <sup>a</sup> 0.41	6.94 <sup>b</sup> 0.11	9.96 <sup>c</sup> 0.05	Protein sORF3 Unknown

<sup>†</sup>The expression levels of ILTV genes were presented to Cp indicated by  $-\Delta Ct + 24$ .

<sup>‡</sup>a, b, c and d indicate differential expression, and they were deducted by ANOVA test in JMP Genomics 5.0.

<sup>§</sup>The productive proteins and their functions were referenced from the nucleotide information of Gallid herpesvirus 1 (NC\_006623) in NCBI.

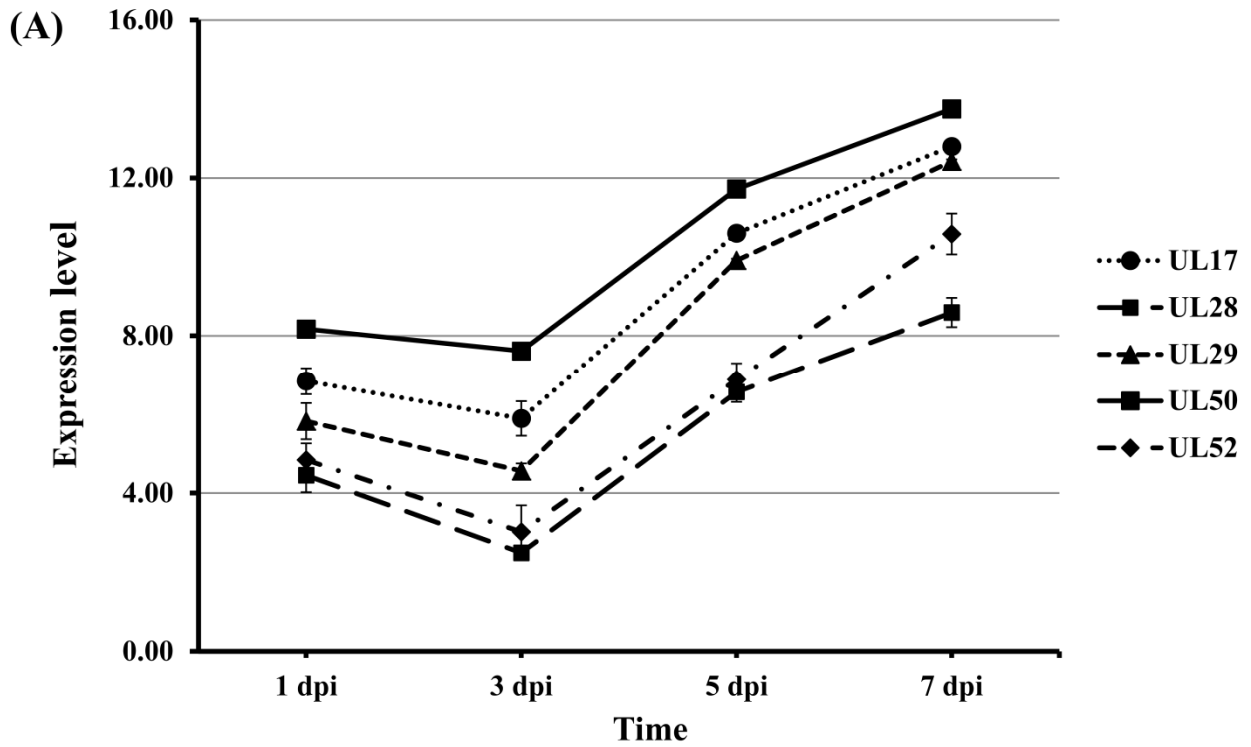




(B)

Genes	1 dpi	3 dpi	5 dpi	7 dpi	Protein Functions
	Cp SE	Cp SE	Cp SE	Cp SE	
UL21	1.33 <sup>a</sup>	3.75 <sup>b</sup>	7.09 <sup>c</sup>	8.06 <sup>c</sup>	Tegument protein UL21
	0.32	0.19	0.35	0.45	Virion morphogenesis
UL42	8.23 <sup>a</sup>	12.43 <sup>b</sup>	7.92 <sup>a</sup>	14.93 <sup>c</sup>	DNA polymerase processivity subunit
	0.13	0.14	0.22	0.03	DNA replication
ICP4	17.15 <sup>a</sup>	17.67 <sup>b</sup>	17.44 <sup>abc</sup>	17.67 <sup>bc</sup>	Transcriptional regulator ICP4
	0.13	0.02	0.05	0.12	Gene regulation

**Figure 1.** The virus genes indicating the unique pattern changes of gene expression at all time points in ILTV infected chicken embryo lung cells. (A) The three genes including UL21, UL42 and ICP4 among 76 ILTV genes brought out a distinctive style at all time points. The blue line with the closed circle, the red line with the closed square and the green line with the closed triangle are shown UL21, UL42 and ICP4, respectively. The X-axis appears dpi as a time point, and the Y-axis exhibits Cp values as an expression level of each gene. (B) The definite numbers involving expression levels and standard error (SE) of each gene at each time point, expressing proteins and protein functions are denoted. The Cp and SE values were calculated from triplicates of biological samples, and the superscripts were drawn from ANOVA test.



(B)

Genes	1 dpi	3 dpi	5 dpi	7 dpi	Protein Functions
	Cp SE	Cp SE	Cp SE	Cp SE	
UL17	6.84 <sup>a</sup>	5.89 <sup>b</sup>	10.60 <sup>c</sup>	12.80 <sup>d</sup>	DNA packing tegument protein UL17
	0.33	0.44	0.10	0.05	DNA encapsidation: capsid transport
UL28	4.45 <sup>a</sup>	2.49 <sup>b</sup>	6.56 <sup>c</sup>	8.59 <sup>d</sup>	DNA packaging terminase subunit
	0.43	0.13	0.25	0.37	DNA encapsidation
UL29	5.82 <sup>a</sup>	4.56 <sup>b</sup>	9.91 <sup>c</sup>	12.42 <sup>d</sup>	Single-stranded DNA-binding protein
	0.46	0.19	0.04	0.05	DNA replication
UL50	8.17 <sup>a</sup>	7.61 <sup>b</sup>	11.72 <sup>c</sup>	13.75 <sup>d</sup>	Deoxyuridine triphosphatase
	0.08	0.16	0.12	0.03	Nucleotide metabolism
UL52	4.84 <sup>a</sup>	3.02 <sup>b</sup>	6.88 <sup>c</sup>	10.58 <sup>d</sup>	Helicase-primase primase subunit
	0.42	0.67	0.42	0.52	DNA replication

**Figure 2.** The five virus genes expressing down-regulation of gene expression at 3 dpi in ILTV infected chicken embryo lung cells. (A) The expressions of the five genes named UL17, UL28, UL29, UL50 and UL52 were once down-regulated at 3 dpi than those of 1 dpi and then continually up-regulated until 7 dpi. The red line with the closed square, the light blue line with the closed diamond, the green line with the closed triangle, the blue line with the closed circle, and the purple line with the asterisk stand for UL28, UL52, UL29, UL17 and UL50, respectively. The X-axis represents dpi as a time point, and the Y-axis signifies Cp value as an expression level. (B) Overall, all expression levels, SE values, the names of functional proteins and the functions of the proteins were shown up. The Cp and SE values were resulted from triplicates of biological samples, and the superscripts were come up with ANOVA test.

## **Appendix**

### **Establishment of Immortalized Chicken Embryo Liver Derived Cell Lines**

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## 1. Abstract

A continuously growing immortal cell substrate can be utilized for viral vaccine production based on a cell culture. The aim of this study was to establish an immortal chicken cell line for efficient propagation of avian infectious viruses including ILTV causing acute upper respiratory disease in chickens. Various ectopic expression plasmids containing cell cycle stimulating genes and small interfering RNA (siRNA) expression systems targeting cell cycle inhibitory genes were transfected into primary chicken embryo liver (CEL) cells. As results, three immortalized chicken embryo liver (CELi) cell lines named CELi-si-p53, CELi-im and CELi-vector were established. The CELi-im was spontaneously immortalized with no transfection, while the CELi-si-p53 and CELi-vector were immortalized with transfection of the expression construct for siRNA against p53 and the expression vector control, respectively. All three CELi cell lines are permissive to ILTV infection, but low ILTV titers (~10 plaque forming units/ml) were produced. In addition, newly immortal cell lines were permissive to MDV and avian metapneumovirus (AMPV). To identify genetic alterations in immortal CELi cell lines, mRNA expressions for cell cycle regulatory genes were determined during the immortalizing progresses. Compared to those in the primary CEL counterpart, the mRNA expressions of p53, Mdm2 and p16<sup>INK4a</sup> were down-regulated in all three CELi cell lines, while those of RB, E2F-1, p19<sup>ARF</sup> and c-myc were up-regulated. The p21<sup>WAF1</sup> showed up-regulation in qPCR in contrast to down-regulation in RT-PCR results. These results are closely similar to genetic alterations found in previously established immortal chicken embryo fibroblast (CEFi) cell lines showing efficient propagation of MDV. Therefore, newly established CELi cell lines can serve as alternative cell lines for vaccine production against infectious avian viruses.

## 2. Introduction

The primary chicken embryo kidney (CEK), chicken embryo fibroblast (CEF) and CEL have been preferred for virus replication, propagation, detection and even vaccine production [1-6]. However, the primary cultured cells directly prepared from live organ tissues have limitations for virus propagation such as the limited life-span, time consuming and labor intensive preparation, heterogeneous cell populations, and potential microbial contamination. Thus, continuously growing immortal cell lines can serve as an alternative cell substrate for virus propagation. Two immortalized avian cell lines, DF-1 derived from chicken embryo fibroblast [7] and LMH chemically induced chicken hepatocellular carcinoma cell line [8], have been widely used for the propagation of avian infectious herpesviruses including avian influenza, avian broncheitis virus, MDV, AMPV and ILTV [9]. For virus propagation and vaccine production, immortalized cell lines should not carry existing endogenous and exogenous viral genomes, should supply homogenous cells and should overcome a disadvantage found in the use of primary cells such as virus titer fluctuation.

To date, only two immortal CEF cell lines named DF-1 and SC-1 have been established spontaneously without the use of tumorigenic viruses or oncogenic chemicals [7, 10]. In addition, other CEFi cell lines such as breast CEFi (BCEFi) and heart CEFi (HCEFi) were established, but not spontaneously [11]. Although many chicken embryo cell lines have been established and reported, various cellular characteristics including cellular growth rate (rapid vs slow), morphology (piling-up vs contact inhibition) and the steady state expression levels (up- vs down-regulation) for various cell cycle regulatory genes are critical to determine whether the established cell line is a tumor line or not, and in turn, whether a cell line can serve as a suitable substrate for the vaccine production.

Of a variety of cell cycle regulatory factors, key regulatory factors including p53, Rb, Mdm2, E2F-1, p21<sup>WAF1</sup>, p19<sup>ARF</sup>, p16<sup>INK4a</sup> and c-myc in chicken cells were determined previously [10-13], and those factors have been well-characterized for cell cycle regulation in mammalian species in addition to model organisms. The tumor suppressor p53 functions in cell cycle arrest [14], and p21<sup>WAF1</sup> (CDK inhibitor) and Mdm2 (ubiquitin ligase) are transcriptionally up-regulated by p53 protein [15-18]. Rb protein inhibits premature G1/S phase transition by binding to E2Fs, which activate G1/S transition when E2Fs were released from phosphorylated Rb [19-22]. Both p16<sup>INK4a</sup> and p19<sup>ARF</sup> were encoded at INK4a/ARF locus called CDKN2A [23]. ARF (CDK inhibitor) acts as a tumor suppressor or to active p53-dependent cell cycle arrest [24], and INK4a (CDK inhibitor) is also tumor suppressor regulating the cell cycle [25]. Myc (c-myc) is a well-known proto-oncogene and transcriptional regulator controlling cell proliferation, cell growth, differentiation and apoptosis [26, 27].

In this study, we established three immortal chicken liver (CELi) cell lines mainly targeting the propagation of ILTV causing acute upper respiratory disease in chickens in addition to other avian viruses. Furthermore, the mRNA expression levels of cell cycle regulatory genes including p53, Mdm2 and p21<sup>WAF1</sup> (transcriptional target of p53 and CDK inhibitor), p16<sup>INK4a</sup> and p19<sup>ARF</sup> (CDK inhibitor), Rb and E2F-1 (cell cycle progression), and c-myc (proto-oncogene), were determined at various passages during the progression of immortalization in CELi cell lines.



### **3. Materials and methods**

#### **3.1. Isolation and culture of chicken liver cells**

The primary chicken embryo liver (CEL1<sup>o</sup>) cells were isolated from 15 days old chicken embryos. Embryonic liver tissues were treated with VT solution (1:1 of 0.25% trypsin and PBS) for 30 minutes. For cell growth and transfection, cells were plated into 100 mm tissue culture dishes (Sarstedt, Newton, NC, USA) coated with 0.5% gelatin in PBS. The CEL cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, 0.45% glucose with 10% of FBS, 1% of penicillin-streptomycin and 1% of L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA) at 39°C and 5% CO<sub>2</sub> in a humidified incubator. The 6x10<sup>5</sup> cells were transferred to a new tissue culture dishes, the medium was changed every 2 days, the cells were passaged every 4-5 days after establishing immortal stage, and cell numbers were counted at every passage.

#### **3.2. Transfection and selection**

Lipofectamin2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for the transfection using manufacturer's instruction. After transfection, the 100 µg/ml concentration of hygromycinB (EMD, Darmstadt, Germany) was used to select out untransfected cells. After selection for 15 days, surviving cells were transferred into a new 100 mm tissue culture dish.

#### **3.3. RNA extraction**

The RNA samples were prepared at passages of 20, 50, 70 and 90 for CELi-im, passages of 35, 50, 70 and 90 for both CELi-si-p53 and CELi-vector. Total RNA was extracted from all three CELi cells at designated passages in addition to every tenth passgae cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following manufacturer's

instruction. The total RNA was treated with DNase I (New England BioLabs Inc., Ipswich, MA, USA) and re-purified by TRIzol reagent. Quantity of the re-purified total RNA was measured by Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and quality was assessed by agarose gel electrophoresis (data not shown).

### **3.4. End-point reverse-transcription (RT)-PCR and quantitative RT-PCR (qPCR)**

RT reactions were performed with SuperScript II RTase (Invitrogen Life Technologies, Carlsbad, CA, USA) using 3 µg of total RNA, and the RT products were diluted to 1:10 with DEPC-water. PCR reactions were performed by the following condition: denaturing 95°C for 30 s, annealing at 62°C for 1 min, extending at 72°C for 30 s and finally extending at 72°C for 10 min in 40 cycles and 18 cycles for chicken GAPDH (chGAPDH) which was used as an endogenous loading control. The same RT products used in end-point RT-PCR were used for qPCR with dyes of SYBR and reference ROX dye (Invitrogen Life Technologies, Carlsbad, CA, USA). The qPCR reactions were performed by the following condition: 40 cycles of denaturing 95°C for 30 s, annealing at 62°C for 1 min, extending at 72°C for 30 s. A non-template control (NTC) and chGAPDH were used for the relative quantification. The fold change values for target gene groups comparing to the samples of CEL1° were determined by the  $-\Delta\Delta CT$  method [28]. Moreover, gene specific primer sets for 9 genes were designed by Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>) and were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer sequences are shown in Table 1.

## **4. Results and discussion**

### **4.1. Cellular proliferation during immortalizing processes**

Various ectopic expression constructs for cell cycle stimulating genes and siRNA expression systems against cell cycle inhibiting genes were introduced into CEL1° cells, which were freshly isolated from chicken embryo liver and three embryo liver cell lines were established as immortalized cell lines. The cell lines were generated by the transfection with no construct (spontaneous), siRNA expression vector against p53 (siRNA-p53) and vector control (pcDNA3.1-hygro), and cell lines were named as CELi-im, CELi-si-p53 and CELi-vector, respectively. Cells were passaged every 4-5 days until passage # 100 for more than 1 year. The rate of population doubling (PD) per day for each cell lines was determined at every passage (Figure 1). The CELi-si-p53 exhibited a low PD for the first 4 months until passage 30. Between passage 31 and 48, the PD dramatically increased reaching 1.2 PD/day. After the dramatic amplification period, the PD for this cell line fluctuated between 0.2 and 0.9 until passage 64. Though minor fluctuations were found until passage 86, the PDs stabilized around 1.2 until passage 100 (Figure 1A). Unlike CELi-si-p53, CELi-im and CELi-vector displayed unstable cell proliferation from early- to the passage numbers (data not shown). In CELi-im, PDs largely fluctuated between 0.2 and 1.1 until passage 85, and became stable thereafter until passage 100 (Figure 1B). The CELi-vector cells also showed big fluctuation in PDs ranging between less than 0.1 and 1.2 until passage 61. After this unstable stage, PDs continuously increased until passage 78 with cells exhibiting stable cell proliferation until passage 100 (Figure 1C).

### **4.2. The morphologies and growth rates of immortal liver cell lines**

The morphologies and growth rates of the three CELi cell lines at passage 100 were examined for 4 days (Figure 2 and 3). Though morphologies were similar between the three cell lines, CELi-im and CELi-si-p53 were more roundish in shape and fatter compared to the thin and small CELi-vector cells (Figure 2). After growing for 3 days, morphologies were not distinguishable under microscopy since culture dishes were confluent by excessive cell numbers. Growth rates of three CELi cell lines were compared to CEL1° cells and to cells from chemically induced chicken hepatoma cell line, LMH (Figure 3). While CEL1° and LMH cells showed no increase in cell numbers (CEL1°) or exhibited slow growth reaching  $6 \times 10^5$  cells from  $1 \times 10^5$  (LMH), all three CELi cell lines showed much greater growth rates over the 4 day period examined. Growth rates of CELi-im and CELi-vector showed similar levels at all time points, while CELi-si-p53 exhibited slower growth than CELi-im and CELi-vector, with all 3 cultures exhibiting much greater growth rate than CEL1° and LMH cells. The fact that newly established CELi cell lines can proliferate stably and rapidly suggests CELi cell lines can become alternative cellular substrates for virus propagation if cells are permissive to infection with avian viruses.

#### **4.3. Altered expression of cell cycle regulatory genes**

To characterize genetic alterations to induce rapid cell divisions in CELi cell lines, transcriptional changes of cell cycle regulatory genes such as p53, retinoblastoma (Rb), Mdm2, E2F-1, p21<sup>WAF1</sup>, p19<sup>ARF</sup>, p16<sup>INK4a</sup> and c-myc were determined by RT-PCR and qPCR during the progression of immortalization. The expression level of p53 mRNA in all three CELi cell lines was dramatically down-regulated compared to the CEL1° counterpart (Figure 4A, 5A and 6A). Similar results were reported previously regarding immortal CEF cell lines showing that p53 mRNA expression was greatly decreased in immortal CEF cell lines [11-13]. Furthermore, the

down-regulation of p53 is known as a key feature in the immortalization of human cells [29, 30]. Thus, the down-regulation of p53 in CELi cell lines appears to be a typical genetic alteration process during cellular immortalization. As the result of the down-regulation of p53, the expression of p21<sup>WAF1</sup> and Mdm2 mRNAs, which are the transcriptional targets of p53, were found to be down-regulated compared to the CEL1° counterpart when determined by qPCR and end-point RT-PCR (Figure 4C and 4E, Figure 5C and 5E, and Figure 6C and 6E).

However, Rb, another tumor suppressor, was greatly up-regulated in all CELi cell lines (Figure 4B, 5B and 6B) similar to previous reports on other immortal CEF cell lines [11]. The increased Rb expression in immortal chicken cell lines are in contrast to its decreased expression reported in other mammalian cancers [31]. In addition, the mRNA level of E2F-1, which is regulated (suppressed) by binding with Rb, was up-regulated in all CELi cell lines (Figure 4D, 5D and 6D) as shown in immortal breast chicken embryo fibroblast (BCEFi) and heart chicken embryo fibroblast (HCEFi) cell lines [11]. Generally E2Fs, including E2F-1, function in the cellular proliferation [32]. Moreover, E2F-1 mRNA expression is down-regulated in senescent cells of human diploid fibroblast (HDF), mouse embryonic fibroblast (MEF) and CEF [33]. According to the result of end-point PCR, the increased level of E2F-1 mRNA is much greater than those of Rb compared to the CEL1° counterpart, and hence the absolute expression level of E2F-1 may be much higher than Rb. Greater abundancy of E2F-1 compared to Rb suggests the level of free, not bound to Rb, E2F-1 increased in CELi cell lines and, in turn, may stimulate enhanced cell cycle progression in CELi cells.

According to previous reports [11, 33], the expression level of cyclin A, B2, B3, C, D1 and E were up-regulated in immortal CEF cell lines, but the expression levels of p16<sup>INK4a</sup>, one of the cyclin dependent kinase inhibitors, was down-regulated in immortal CEF cell lines. We

confirmed the down-regulation of p16<sup>INK4a</sup> mRNA expression in all three CELi cell lines (Figure 4G, Figure 5G and Figure 6G) in addition to forecasting the up-regulation or activation of cyclin and cyclin dependent kinases. Interestingly, the expression patterns between p16<sup>INK4a</sup> and E2F-1 were shown in reverse directions as same as showing in the previous study [33].

The expression level of p19<sup>ARF</sup> mRNA was up-regulation in all CELi cell lines (Figure 4F, 5F and 6F). Though the major function of p19<sup>ARF</sup> is tumor suppression and induction of apoptosis through binding to Mdm2 stabilizes p53 [34] and prevents cell proliferation [35], recent finding of the p53-independent function of p19<sup>ARF</sup> showed ribosome biogenesis and cell growth stimulation by binding to nucleophosmin/B23 (NPM) [35, 36]. Thus, the up-regulated p19<sup>ARF</sup> mRNAs in CELi cells may play a role in cell growth by increasing ribosome biogenesis and protein synthesis by binding to NPM [36].

The expression levels of c-myc mRNA were varied in the three CELi cell lines. Compared to CEL1° counterpart, the expression levels of c-myc mRNA fluctuated during the whole passage periods in all three CELi cell lines (Figure 4H, Figure 5H and Figure 6H). Though c-myc is a strong proto-oncogene and shows critical functions on cellular proliferation in mammal [26, 27], variable expression patterns found in CELi cells indicate that c-myc in avian cells may not significantly influence cellular proliferation and immortalization.

## **5. Conclusion**

Three newly established CELi cell lines were found to be infection with permissive to avian infectious viruses including ILTV, MDV, and AMPV (data not shown), but those cell lines produced low virus titers except for AMPV propagation (data not shown). Nevertheless, CELi cell lines are still valuable to study further virus propagation due to the rapid proliferation potential and its morphological stability. Therefore, further research is needed to investigate CELis' potential as cellular substrates for the propagation and vaccine production of avian infectious viruses.

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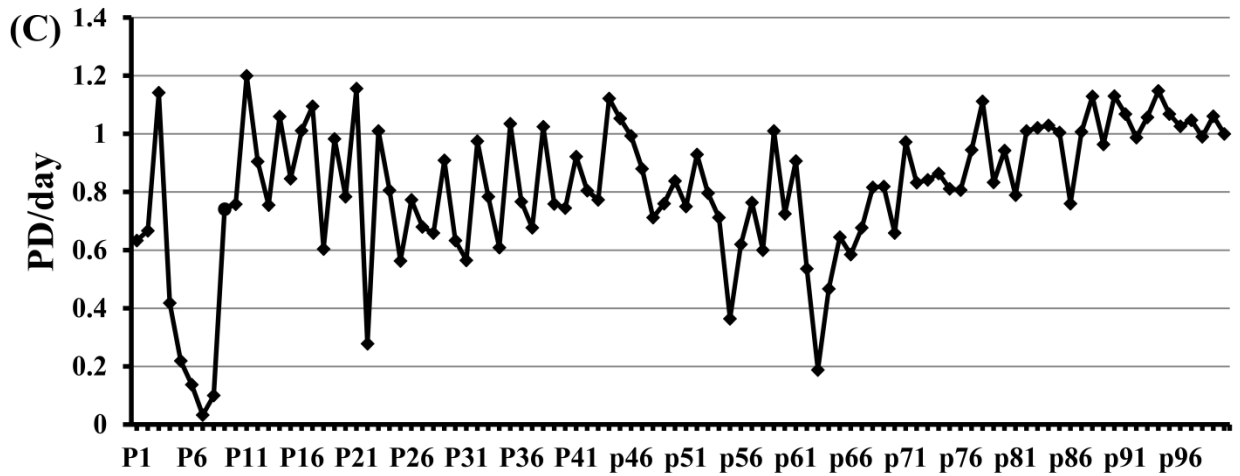
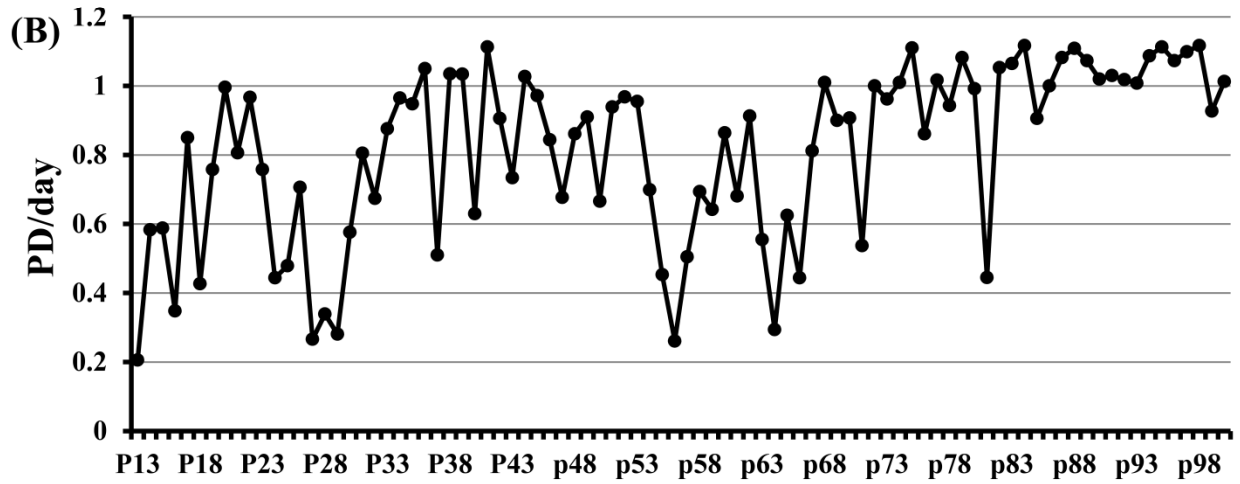
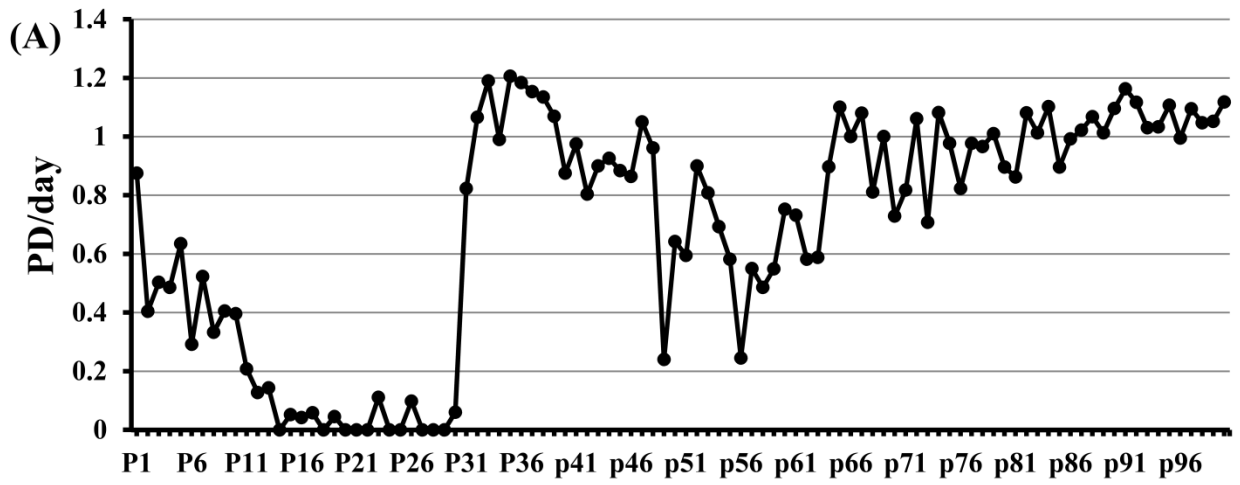


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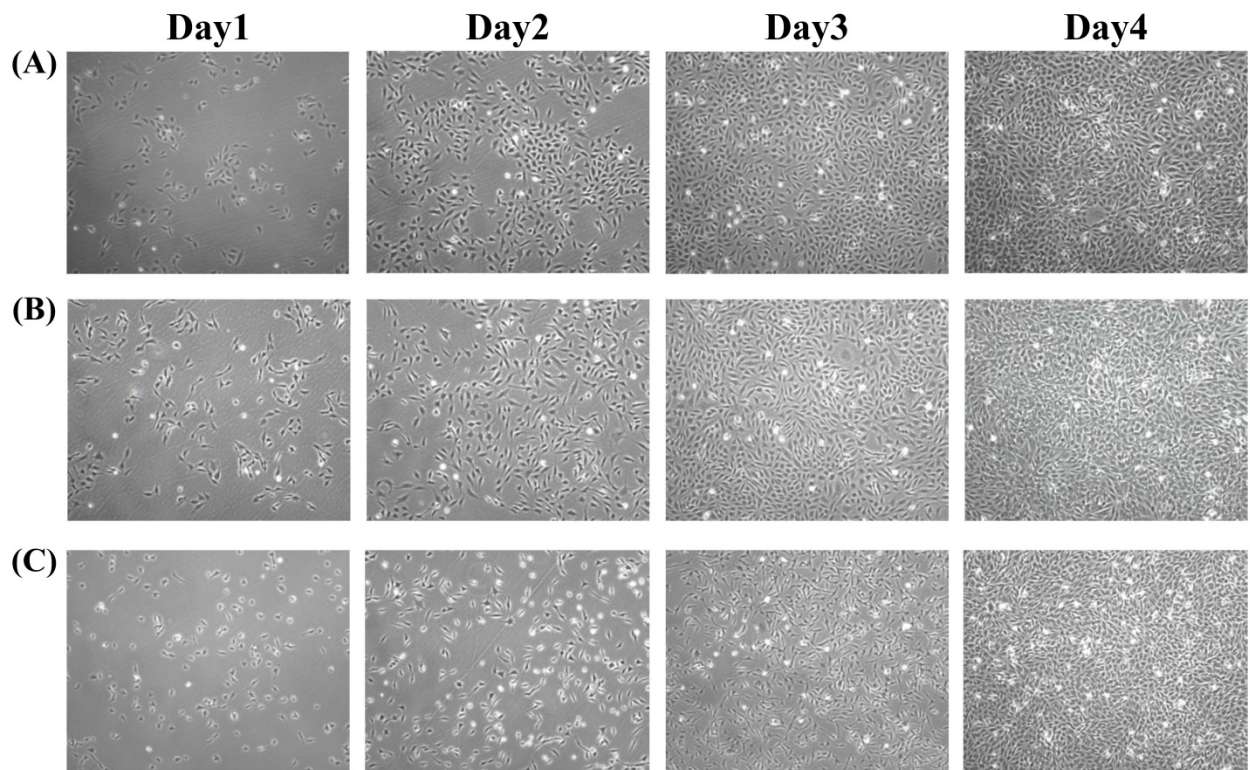
**Table 1.** The primer sets for qPCR and end-point RT-PCR.

<b>Gene Name</b>	<b>Forward</b>	<b>Reverse</b>
p53	CCATCCACGGAGGATTATGG	TTCAGCACCGGGGAGTAAGT
RB	TGTGCTGAGATTGGCTCACA	CTGAGAGGCGCTCTTCTTCC
Mdm2	GCCAAATTTTCGGCTTGAAAA	TGTTGTTGGCTGGGAAGTTG
E2F-1	AGCGGAAGCTGAACTTGGAG	CAGGAGACTTTGCCCTCTG
p21 <sup>WAF1</sup>	AAGCGTGCAGGAACCTCTTC	CAGGACCCTCTCCCCTTGA
p19 <sup>ARF</sup>	GGAAGACCTGGGAATGGATG	TGATGGGTGCACCACTGAAT
p16 <sup>INK4a</sup>	GCGGGATGAACTAGCCAACG	GTCCGACCGAAGGAGTTGAC
c-myc	TGTCACGTCAACATCCACCA	ACCCTGCCACTGTCCAATT

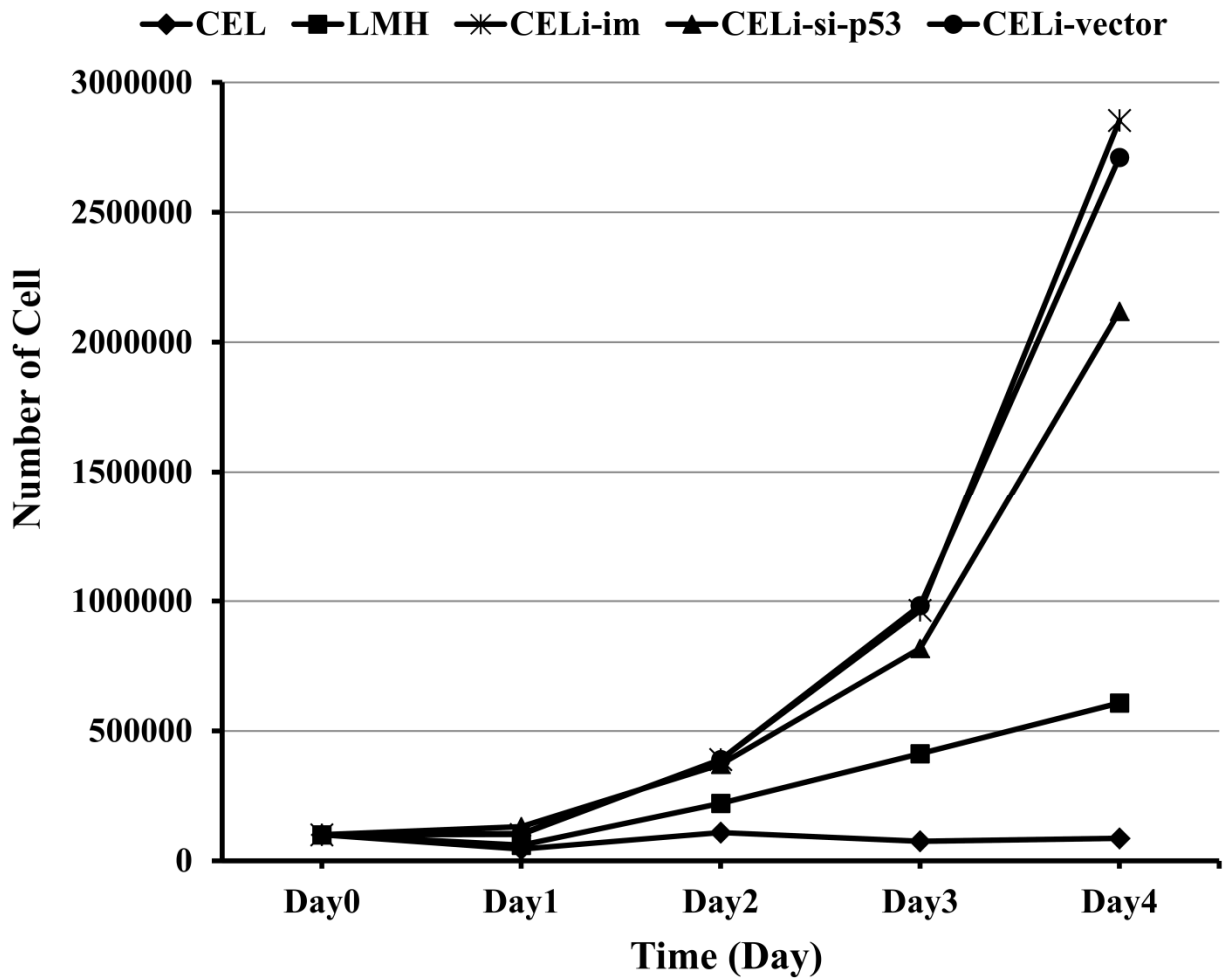


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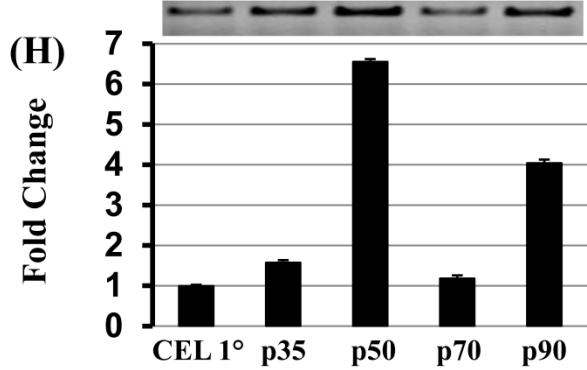
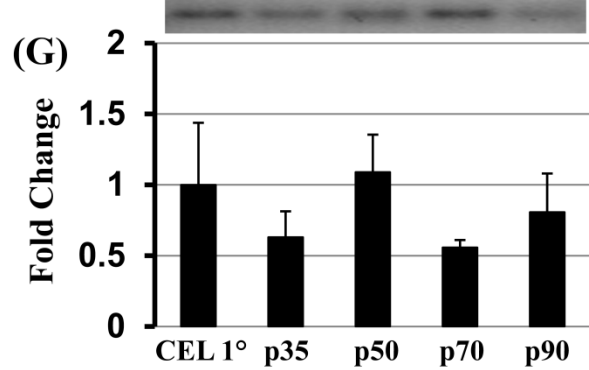
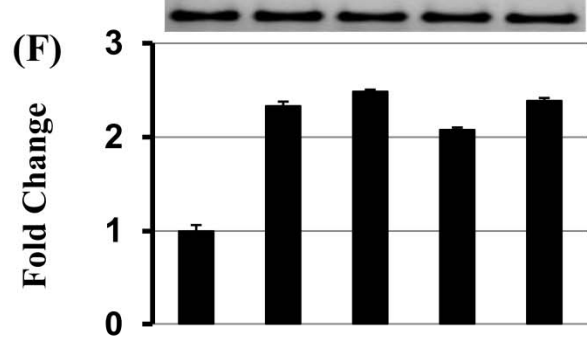
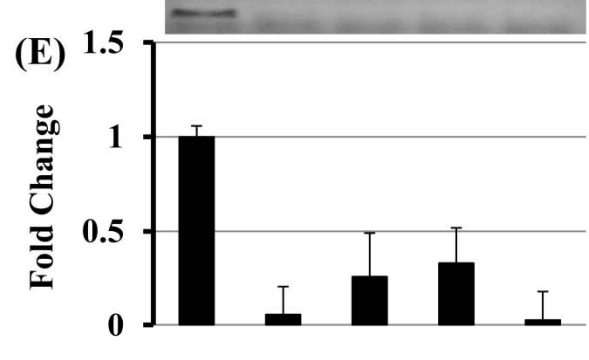
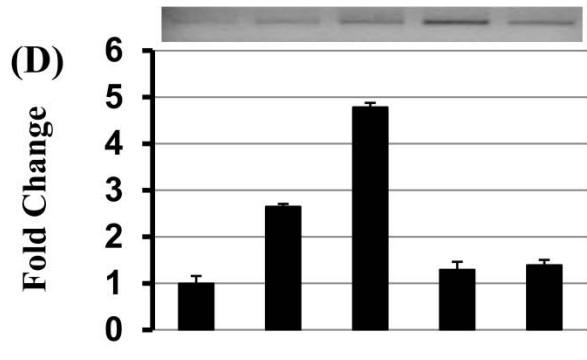
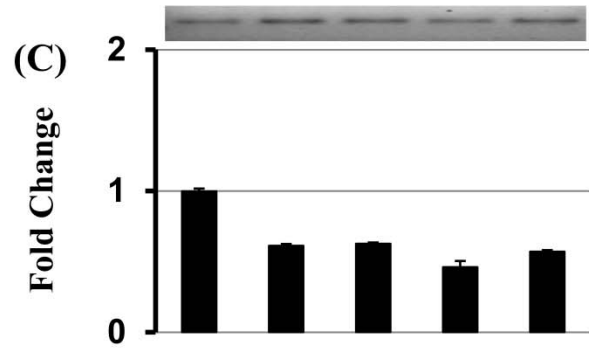
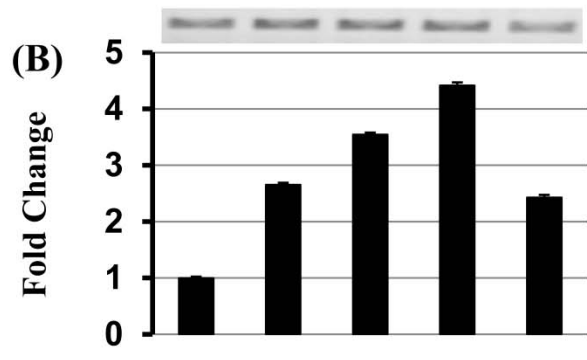
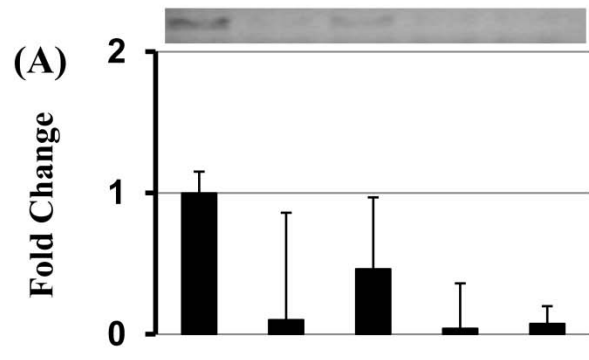
**Figure 1.** Growth curve during immortalization for chicken liver cell lines. Population doublings (PD) per day for each cell lines were determined by passaging every 4-5 days. The X-axis presents passage numbers, and the Y-axis indicates PD/day. (A) CELi-si-p53; (B) CELi-im; (C) CELi-vector.



**Figure 2.** The morphologies of CELi cell lines. Cells were visualized for four days using a phase contrast microscope at 100X magnification. (A) CEL-si-p53; (B) CEL-im; (C) CELi-vector.



**Figure 3.** Growth rate of CELi cell lines. Each cell line was plated at a density of  $1 \times 10^5$  cells/6 cm dish and the number of cells was calculated every day for 4 days. X-axis indicates day, and Y-axis depicts the number of cells. The asterisk, the closed circle, the closed triangle, the closed square and the closed diamond indicate CELi-im, CELi-si-p53, CELi-vectors, LMH cell lines and CEL cell line, respectively.

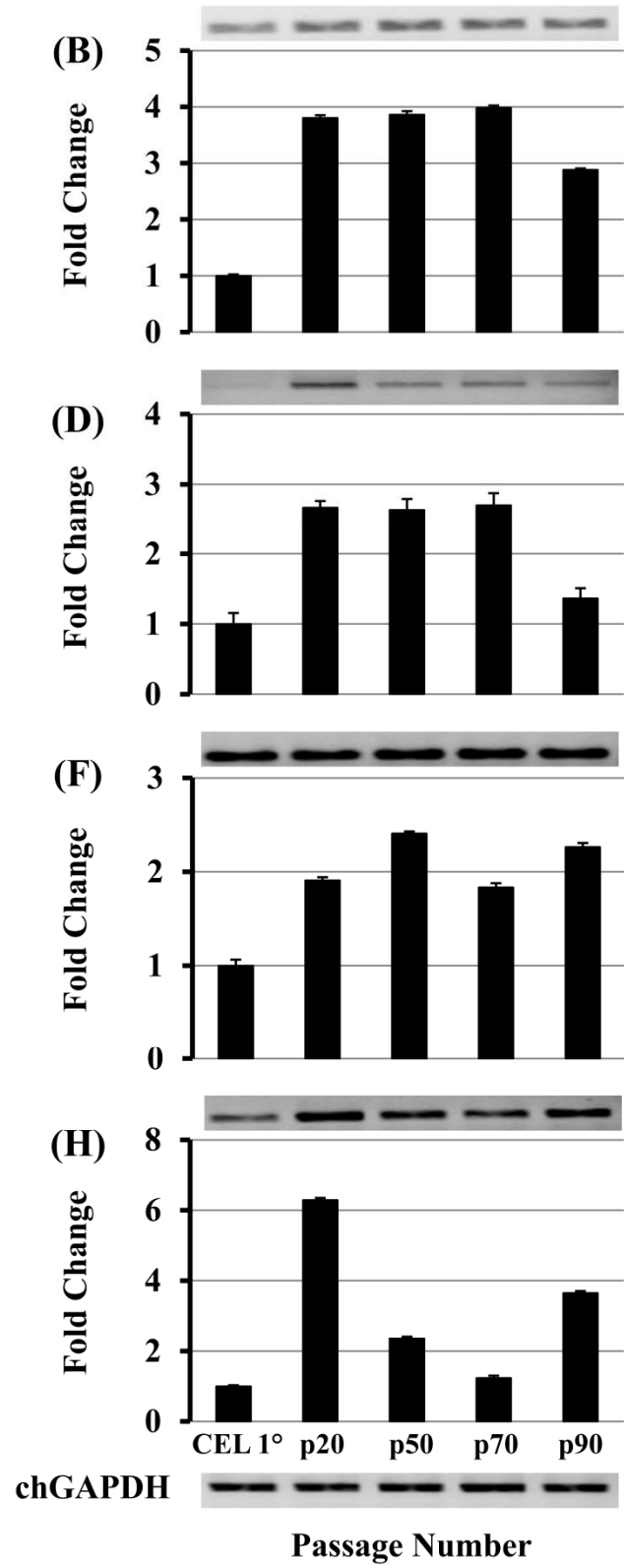
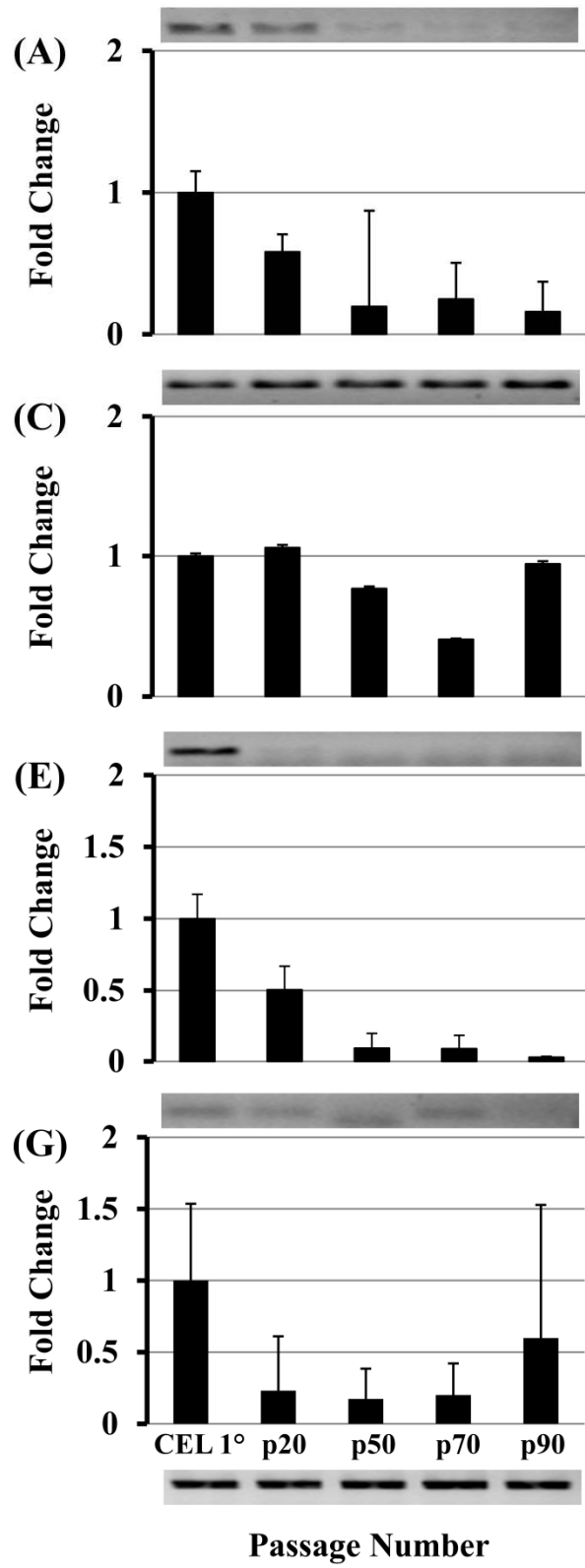


Passage Number

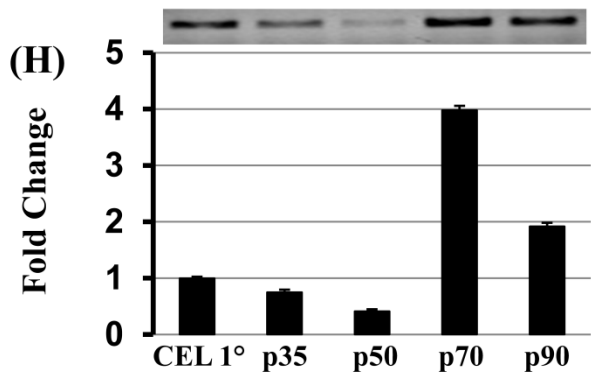
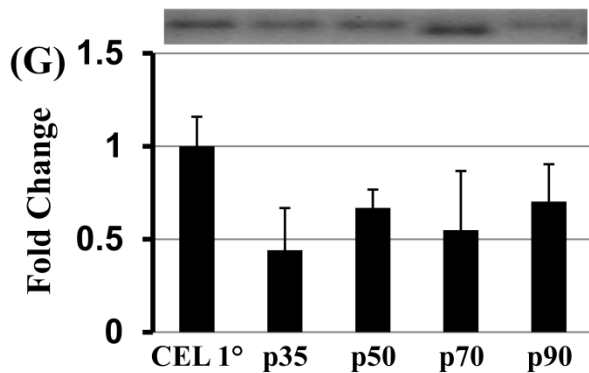
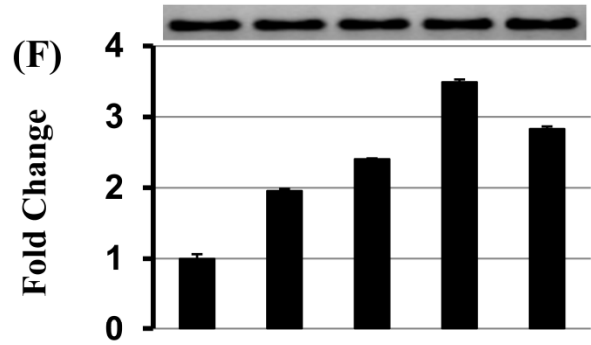
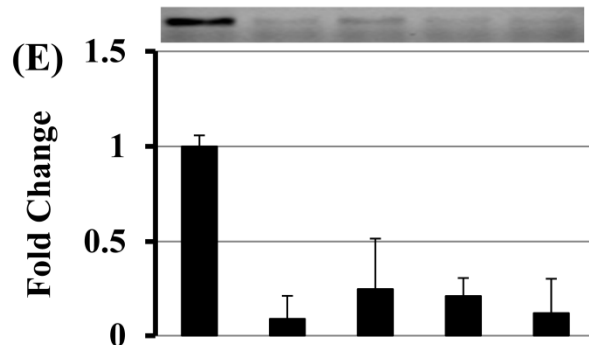
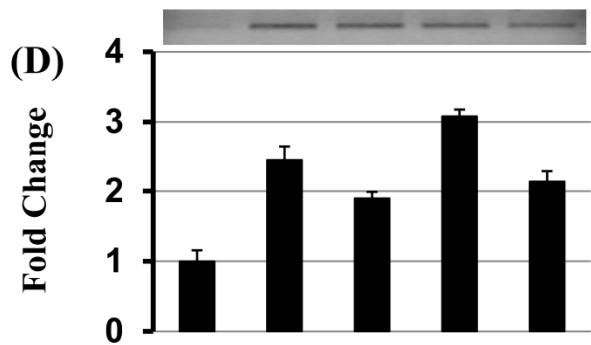
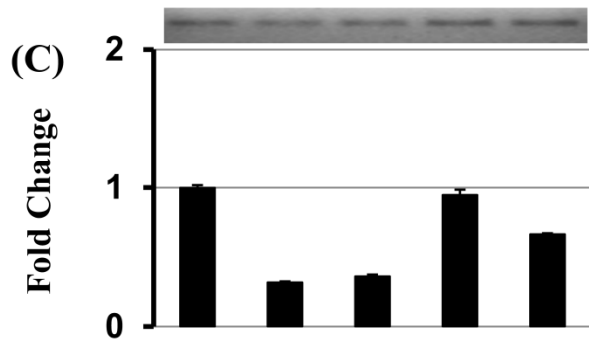
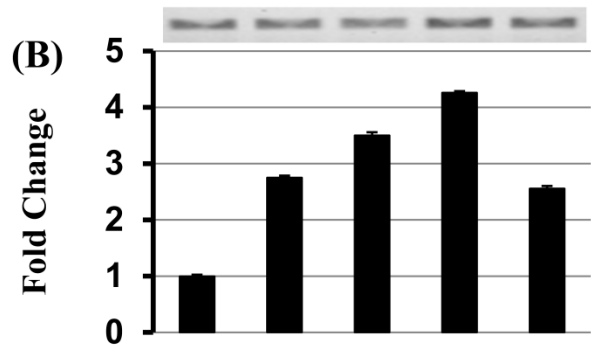
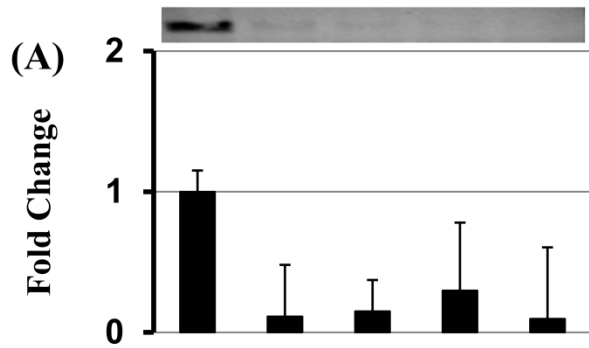
Passage Number



**Figure 4.** Expression of mRNA for cell cycle regulatory genes in CELi-si-p53. The end-point RT-PCR and qPCR results were displayed for each gene examined. X-axis indicates the passage numbers of designated cell lines, and the Y-axis presents fold change values of relative expression (REL). (A) p53; (B) Rb1; (C) Mdm2; (D) E2F1; (E) p21; (F) p19; (G) p16; (H) c-myc.



**Figure 5.** Expression of mRNA for cell cycle regulatory genes in CELi-im. The end-point RT-PCR and qPCR results were displayed for each gene examined. X-axis indicates the passage numbers of designated cell lines, and the Y-axis presents fold change values of relative expression (REL). (A) p53; (B) Rb1; (C) Mdm2; (D) E2F1; (E) p21; (F) p19; (G) p16; (H) c-myc.



chGAPDH



Passage Number

Passage Number

**Figure 6.** Expression of mRNA for cell cycle regulatory genes in CELi-vector. The end-point RT-PCR and qPCR results were displayed for each gene examined. X-axis indicates the passage numbers of designated cell lines, and the Y-axis presents fold change values of relative expression (REL). (A) p53; (B) Rb1; (C) Mdm2; (D) E2F1; (E) p21; (F) p19; (G) p16; (H) c-myc.