Inhibition of infectious bursal disease virus infection by artificial microRNAs targeting chicken heat-shock protein 90

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Infectious bursal disease virus (IBDV) causes an important disease in young chickens. Chicken heat-shock protein 90 (cHsp90) has been shown to be a functional component of the cellular receptor complex for IBDV infection. This study demonstrates the inhibitory effect of vector-expressed anti-cHsp90x microRNA (miRNA) on IBDV infection. The reporter vectors pcHsp90x-EGFP and pcHsp90β-EGFP were constructed to facilitate effective miRNA selection. Two anti-cHsp90x and one anti-cHsp90β miRNA-expression vectors were constructed for a stable transfection study. Poly(A)-tailed RT-PCR detected sequence-specific miRNA transcription in transfected cells. Semiquantitative RT-PCR showed inhibition of cHsp90x transcription in transfected cells. A virus-titration assay showed that the anti-cHsp90x miRNA, but not the anti-cHsp90β miRNA, had inhibitory effects on IBDV infection. These results suggest that cHsp90x is a functional component of the cellular receptor complex for IBDV infection, and that anti-cHsp90x miRNA could be used as an anti-IBDV reagent.

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viral structural protein genes (Gao et al., 2008; Sajjanar et al., 2011) and artificial microRNA (miRNA) targeting VP1 or VP2 transcription (Wang et al., 2009, 2010). However, IBDV is antigenically variable and undergoing rapid mutation (Müller et al., 2003), which must be addressed in order to establish a viable RNAi approach.

IBDV enters the host cell by binding to cellular receptors; several different membrane proteins have been shown to interact with IBDV of different virulence (Delgui et al., 2009; Luo et al., 2010). Lin et al. (2007) demonstrated that chicken heat-shock protein 90 (cHsp90) is a functional component of the cellular receptor complex essential for IBDV infection. Among the cellular factors involved in the attachment of IBDV subviral particles to chicken fibroblast DF-1 cells, cHsp90 has been identified to be a dominant factor by mass spectrometry. In addition, both Hsp90 and anti-Hsp90 can inhibit infection of DF-1 cells by IBDV. In this study, we investigated the feasibility of suppressing IBDV infection by using vector-expressed anti-cHsp90 miRNAs. The results show that anti-cHsp90x, but not anti-cHsp90β, miRNA has an inhibitory effect on IBDV infection.

We constructed two reporter vectors to facilitate effective miRNA selection. The cHsp90x and cHsp90β cDNAs were amplified from DF-1 cells using RT-PCR and their identities were confirmed by sequence analysis. Each of the two cDNAs was fused in frame with the EGFP reporter gene in the eukaryotic expression vector pEGFP-N1 (Clontech), resulting in the reporter vectors pCHsp90x-EGFP and pCHsp90β-EGFP. The two reporter vectors were transfected into DF-1...
cells and the expression of the cHsp90–EGFP fusion genes was confirmed by RT-PCR and fluorescence microscopy.

We used a reporter assay to select miRNAs effective against cHsp90 transcription. The nucleotide sequences for cHsp90x (GenBank accession no. X07265) and cHsp90β (X70101) were submitted to siRNA prediction using a web-based siRNA-design tool (http://www.genscript.com). Among ten potential siRNAs presented for each sequence, four anti-cHsp90x and three anti-cHsp90β siRNAs were selected for miRNA30-like hairpin synthesis. For control purposes, miVP2con [targeting the VP2 sequence of IBDV with 3-mer mismatches (Wang et al., 2010)] was included in all experiments. The miRNA30-like hairpins plus chicken miRNA-flanking sequences were generated by PCR (Das et al., 2006) using the siRNA-specific primer pair plus general primer pair (Supplementary Table S1, available in JGV Online). Each PCR product was co-transfected into DF-1 cells with the reporter vector pRFPRNAiC vector (ARK-Genomics), which is tailored for miRNA expression in chickens using a red fluorescent protein (RFP) reporter gene (Das et al., 2006). Each miRNA vector was co-transfected into DF-1 cells with the reporter vector pcHsp90x-EGFP or pcHsp90β-EGFP. The cell cultures were examined for RFP- and EGFP-positive cells by confocal fluorescence microscopy 48 h post-transfection, representing transfection efficiency and cHsp90x–EGFP or cHsp90β–EGFP expression, respectively. Compared with co-transfection with the control miVP2con vector, co-transfection with three out of four anti-cHsp90x and two out of three anti-cHsp90β miRNA vectors resulted in a significant decrease in the number of EGFP-positive cells. According to their higher gene-silencing effects on cHsp90 transcription, two anti-cHsp90x miRNAs (micHsh90x1 and micHsh90x3) and one anti-cHsp90β miRNA (micHsh90β9) were selected for a stable transfection study.

We generated cell lines for stable miRNA expression. Each miRNA cassette, together with the RFP reporter gene, was excised from the pRFPRNAiC vector and subcloned into the neomycin-selectable marker-containing vector pTarget (Promega). The resultant vectors were transfected individually into DF-1 cells and the cell cultures were submitted to G418 (350 µg ml⁻¹; Sigma) selection. After selection for 2 weeks, three G418-resistant cell clones from each transfection were picked for further study. Sequence-specific miRNA expression in the stably transfected cells was detected by poly(A)-tailed RT-PCR (Wang et al., 2010). An expected 120 bp miRNA was detected in cell cultures transfected with micHsh90x1, micHsh90x3, micHsh90β9 or miVP2con vector, but not in empty vector-transfected cells (Fig. 1).

The gene-silencing effect of the vector-expressed miRNAs on cHsp90 transcription was detected by semiquantitative RT-PCR using the chicken β-actin transcript as the internal reference. Compared with that in miVP2con-expressing cells, cHsp90x transcription was decreased by 83 or 82% in micHsh90x1- or micHsh90x3-expressing cells, respectively, whereas cHsp90β transcription was decreased by 80% in micHsh90β9-expressing cells (Fig. 2). The identities of the RT-PCR amplicons were confirmed by sequence analysis.

We detected the inhibitory effect of the anti-cHsp90 miRNAs on IBDV infection by a virus-titration assay. Stably transfected cells were infected with IBDV strain Lukert (m.o.i. of 1) and the cell supernatants were collected for TCID₅₀ titration at 24, 48, 72 and 96 h post-infection (Wang et al., 2010). Compared with 7.9 log₁₀(TCID₅₀) in the miVP2con-expressing cells, the virus titre was decreased by 4.7 or 5.3 log₁₀ at 24 h post-infection in micHsh90x1- or micHsh90x3-expressing cells, whereas only a marginal decrease (0.9 or 0.2 log₁₀) in virus titre was detected in micHsh90β9-expressing cells (Fig. 3). A similar inhibitory effect of the anti-cHsp90x miRNAs on IBDV infection was detected at 48 h post-infection, which started to diminish from 72 h post-infection (Fig. 3). To confirm the blocking effect of the anti-cHsp90 miRNAs on IBDV infection, total RNA was extracted from stably transfected cells at 48 h post-infection and treated with DNase I to remove the contaminated plasmid DNA. The VP2 sequence was amplified using semiquantitative RT-PCR. Compared with
that in the miVP2con-expressing cells, the VP2 amplicon was decreased by 71 or 79% in micHsh90a1- or micHsh90a3-expressing cells, but not in micHsh90b1-expressing cells.

Currently, two main RNAi strategies have been explored to suppress virus infection and/or replication by targeting either viral or host gene(s) essential for virus replication (Martineau & Pyrah, 2007). For the first strategy, the viral gene(s) essential for fundamental process (e.g. polymerases and master regulators) and/or acting early in the virus life cycle are normally selected as the RNAi target(s). However, many RNA viruses such as IBDV can escape the silencing effect due to the high sequence specificity of the RNAi mechanism and rapid mutation rates of viral genes (Fechner et al., 2007). On the other hand, the cellular receptors for virus binding and/or entry are highly conserved proteins and thus can avoid the escape of gene-silencing effects by different viral strains (Haywood, 1994; Fechner et al., 2007).

cHsp90 has been shown to be a functional component of the cellular receptor complex essential for IBDV binding (Lin et al., 2007), which warranted us to investigate the feasibility of inhibiting IBDV infection using an anti-cHsp90 RNAi strategy. To facilitate effective miRNA selection, we constructed reporter vectors for co-transfection with the miRNA vectors, by which effective miRNAs could be screened easily according to reduction of EGFP-transcription, by which effective miRNAs could be screened easily according to reduction of EGFP-positivity in the co-transfected cell cultures. Among seven candidate miRNAs tested using the reporter assay, five of them were shown to have significant inhibitory effects on cHsp90α or cHsp90β transcription, confirming the high reliability of the web-based siRNA-design tool and our miRNA-expression system.

The claim that cHsp90 is a functional component of the cellular receptor complex for IBDV binding is drawn from indirect evidence using an anti-human Hsp90α mAb (Lin et al., 2007); this cannot rule out the possibility of cross-reactivity between cHsp90α and cHsp90β, as the two proteins are 85% identical (Brown et al., 2007). Our stable transfection experiments showed that the anti-cHsp90α miRNA, but not the anti-cHsp90β miRNA, had an inhibitory effect on IBDV infection, providing direct evidence that cHsp90α, but not cHsp90β, is a functional component of the cellular receptor complex for IBDV infection. The reason(s) for incomplete suppression of IBDV infection could be due to partial suppression of cHsp90α expression by the anti-cHsp90α miRNAs (Fig. 1).

The available experimental data suggest that multiple cellular molecules are involved in IBDV binding. For example, Nieper & Müller (1996) found that two proteins (40 and 46 kDa) were the common receptors on chicken embryo fibroblasts for the two serotypes of IBDV, as well as specific ones for each serotype. Setiyono et al. (2001) showed that three proteins (70, 82 and 110 kDa) are involved in virulent IBDV binding to lymphoblastoid cells. Lin et al. (2007) demonstrated that cHsp90 is a functional component of the cellular receptor complex for IBDV binding. More recently, surface IgM and/or α4β1 integrin were found to be one of the putative membrane sites for IBDV binding (Delgui et al., 2009; Luo et al., 2010). These proteins may act as the co-receptors and/or receptor complex for IBDV recognition and/or binding. In the case of cHsp90α, it remains to be defined whether the protein is also a key component of the cellular receptor complex for virulent IBDV infection of chicken B-lymphocytes in the BF. This issue can be addressed by using a similar RNAi strategy in chicken B-lymphoid cell lines. As an in vivo anti-IBDV RNAi approach, however, care must be taken to avoid a negative impact on cell functions, as Hsp90 facilitates the maturation and/or activation of over 100 ‘client proteins’ involved in signal transduction and transcriptional regulation (Brown et al., 2007). Nevertheless, there are two closely related Hsp90 isoforms, Hsp90α and Hsp90β, which share similar functions in eukaryotes (Goetz et al., 2003). In humans, Hsp90 has been used as an attractive target for anti-cancer drug development and Hsp90 inhibitors have been shown to have significant anti-tumour activity against a broad variety of cancers in clinical trials (Banerji, 2009). These data imply that cHsp90α is a usable RNAi target for establishing an anti-IBDV approach.
In summary, our experimental data suggest that vector-expressed anti-cHsp90α miRNA has a significant inhibitory effect on IBDV infection, and that cHsp90α, but not cHsp90β, is a functional component of the cellular receptor complex for IBDV infection. Further studies are needed to define whether cHsp90α is a common component of the cellular receptor for both avirulent and virulent IBDV infection prior to establishing a viable anti-IBDV RNAi approach.

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References


