Newcastle disease virus (NDV) is an important avian pathogen and causes substantial economic losses to the poultry industry worldwide (Yusoff & Tan, 2001). It is classified in the genus *Avulavirus* of the family *Paramyxoviridae*, which is an enveloped virus with a negative-strand RNA genome that encodes at least six viral proteins (Alexander, 2000). The envelope of NDV contains two viral surface glycoproteins, the haemagglutinin-neuraminidase (HN) and fusion (F) proteins. The HN protein is involved in attachment to host-cell receptors and release of virions, and the F protein mediates fusion of the viral envelope with the host-cell plasma membrane (Mayo, 2002). In addition, the inner surface of the viral envelope is coated with the soluble matrix (M) protein (Klenk *et al.*, 1977), which is considered as the third NDV envelope protein. Like other paramyxovirus M proteins, the NDV M protein is demonstrated to be a nucleocyttoplasmic shuttling protein (Harrison *et al.*, 2010). In addition to functioning for the assembly and budding of viral particles in the cytoplasm and at the cell membrane later in infection (Pantua *et al.*, 2006), the NDV M protein is observed to localize in the nucleus early in infection and becomes associated with nucleoli and remains in this structure throughout infection (Duan *et al.*, 2013; Peebles *et al.*, 1992). This nuclear-nucleolar localization of the M protein is thought to regulate the balance between viral replication and transcription, which is analogous to the measles virus M protein (Iwasaki *et al.*, 2009), and also inhibit host protein synthesis similar to the vesicular stomatitis virus M protein (Rajani *et al.*, 2012). These studies support the notion that NDV M protein is an essential multifunctional viral protein that plays important roles in the virus life cycle.

The M proteins of most paramyxoviruses, including NDV, are reported to have the highly basic, hydrophobic but not membrane-spanning properties (Bellini *et al.*, 1986; Blumberg *et al.*, 1984; Chambers *et al.*, 1986). This is consistent with the known peripheral attachment of the paramyxovirus M proteins to the viral membrane. The basic residues, however, are not distributed uniformly throughout the NDV M protein sequence. Analysis of the charge distribution in M protein showed that the N-terminal 100 aa are somewhat acidic overall, but the remainder of the polypeptide is strongly basic (Chambers *et al.*, 1986). This recent studies have reported that a majority of cellular or viral proteins possess basic residue (arginine or lysine) -rich peptides to mediate their nuclear or nucleolar localization (Nair *et al.*, 2003; Scott *et al.*, 2010). Mutations...
of one or more basic amino acids abrogate the nuclear or nucleolar localization of these proteins. A good example is mutation of lysine to alanine at position 258 in Nipah virus M protein disrupts its nuclear localization and reduces virus replication (Wang et al., 2010). Therefore, this study was undertaken to examine the role of the basic residues at the N-terminal 100 aa in the subcellular localization of NDV M protein. We have also evaluated the effect of basic residue mutation on the replication and pathogenicity of NDV.

In the present study, the M gene of NDV strain Goose/China/JJ1/2000 (GenBank accession number AF431744; Huang et al., 2004) was amplified and cloned into vector pEGFP-C1 (Clontech) to create pEGFP-M. The GFP-M is validated as a suitable system for studying the subcellular localization of M protein (Duan et al., 2013). As expected, the GFP-M protein in transfected BSR cells was localized mainly in the nucleus and nucleolus, with less fluorescence in the cytoplasm (Fig. 1a, lower panels). In contrast, the GFP alone was found in the whole cells (Fig. 1a, upper panel).
panels). When mutation of the N-terminal basic residues arginine (R) or lysine (K) to alanine (A) in the GFP-M protein was investigated, only GFP-M with the R42A mutation exhibited the cytoplasmic localization (Fig. 1b). Sequence analysis of 187 complete M protein amino acid sequences obtained from GenBank revealed that the residue of some NDV strains at position 42 was K (data not shown), but R to K substitution did not alter M’s nuclear localization (Fig. 1b). Meanwhile, WT M (Mwt) or its mutants fused with a small Flag tag showed identical localizations (Fig. 1c), demonstrating that the localization change of M mutants was not relevant to the fused tag. In
addition, co-immunoprecipitation assays revealed that M carrying either the R42A or the R42K mutation did not affect its interaction with viral HN protein and nucleocapsid (NP) protein (Fig. 1d). We next aligned the M protein sequences of 13 viruses from different genera within the family Paramyxoviridae. Interestingly, there are 11 R and two K residues at this position (Fig. 1c). These results suggested that R42 could potentially compromise functions similar to the residue R42. Therefore, we confirmed that this R residue in most of paramyxovirus M proteins was conserved and might serve important roles in virus replication.

To further analyse whether disruption of M protein nuclear localization affects NDV replication and pathogenicity, a cDNA clone of NDV strain ZJ1 expressing GFP (pNDV/ZJ1GFP) (Hu et al., 2007) was used to introduce individual amino acid substitution in the M protein (M/R42A or M/R45A). The point mutagenesis was performed as previously described (Duan et al., 2014). Results of the mutant viruses rescue showed that the haemagglutinin (HA)-positive allantoic fluid was detected in the rescued virus rZJ1GFP and rZJ1GFP-M/R45A, but two extra egg passages were required for rZJ1GFP-M/R42A to be detected by HA test (data not shown), indicating that the R42A mutant virus grew slowly in chicken eggs. In addition, DF-1 cells (a chicken embryo fibroblast cell line) infected with serial 10-fold dilutions of the viruses were examined. As expected, cells infected with rZJ1GFP, rZJ1GFP-M/R42A or rZJ1GFP-M/R45A exhibited green fluorescence at 12 and 24 h post-infection (p.i.) in comparison with rZJ1 (Fig. 1f, upper panels), demonstrating the successful rescue of the viruses. Moreover, indirect immunofluorescence assay using anti-M polyclonal antibody obtained the same localizations as plasmids expressing GFP-tagged Mwt or its mutants (Fig. 1f, lower panels). To further determine the stability of each M gene mutant, the recovered virus was plaque purified and passed five times in 9-day-old specific pathogen free (SPF) chicken eggs. Sequence analysis of the M gene in the mutant virus after five passages showed that the introduced mutation was unaltered, and no compensatory mutations in other viral genes were observed (data not shown).

We then evaluated the biological characteristics of the parental and mutant viruses. Three internationally accepted pathogenicity tests, the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI), were performed according to the standard procedures (Alexander, 1989). The MDT result showed a significant increase (P<0.01) in the time required by the M/R42A mutant virus (115 h) to kill 9-day-old chicken embryos when compared with that required for rZJ1GFP (52 h) and rZJ1GFP-M/R45A (58 h) (Table 1). The results of ICPI and IVPI values revealed that both of the values of rZJ1GFP-M/R42A were lower than that for rZJ1GFP and rZJ1GFP-M/R45A (Table 1). In addition, the growth ability of the mutant virus in the embryonated eggs and cell cultures measured by 50% egg infectious dose (EID50) and TCID50 test also showed a significant decrease in virus titres (P<0.01) (Table 1). Together, these results demonstrated that disruption of M protein nuclear localization caused by M/R42A mutation significantly attenuated the virus.

To compare the in vitro growth characteristics of the mutant viruses with the parental virus, we performed multicycle growth kinetics in DF-1 cells. The results analysed using the independent samples t-test showed that the virus titres of rZJ1GFP-M/R42A from 12 to 60 h p.i. were significantly lower than that of rZJ1GFP and rZJ1GFP-M/R45A (P<0.05) (Fig. 2a). And the cytopathic effect and expression of GFP in rZJ1GFP-M/R42A-infected cells were much higher than rZJ1GFP and rZJ1GFP-M/R45A at 36 h p.i. (Fig. 2b). Moreover, plaque assays were used to evaluate the pathogenicity of the mutant viruses as previously described (Hu et al., 2011). The plaques produced by the viruses were measured using the GNU image manipulation program version 2.8 (http://www.gimp.org) (Negovetich & Webster, 2010). As shown in Fig. 2c, DF-1 cells infected with rZJ1GFP-M/R42A developed large plaques with a mean size of 2.78±0.40 mm and 2.55±0.30 mm, respectively. While cells infected with rZJ1GFP-M/R45A developed much smaller plaques with a mean size of 0.85±0.33 mm. These data suggested that the R42A mutation reduced the replication and pathogenicity of NDV in avian cells.

We further investigated the in vivo pathogenesis assessment in chickens, 36 4-week-old SPF White Leghorn chickens were randomly allocated in three experimental groups, consisting of rZJ1GFP- (n=12), rZJ1GFP-M/R42A- (n=12) and mock- (PBS, n=12) infected groups. Birds were inoculated intranasally with 10^6.0 TCID50 of each virus in a volume of 0.1 ml. The subsequent experiments were performed as described previously (Susta et al., 2010, 2013). Results showed that birds inoculated with the parental virus rZJ1GFP presented the typical clinical symptoms, such as head twitch, hemiparesis and paralysis at

### Table 1. Biological characteristics of the parental and mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Pathogenicity</th>
<th>Virus titre</th>
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<tr>
<td></td>
<td>MDT (h)</td>
<td>ICPI</td>
</tr>
<tr>
<td>rZJ1GFP</td>
<td>52</td>
<td>1.91</td>
</tr>
<tr>
<td>rZJ1GFP-M/R42A</td>
<td>115</td>
<td>1.64</td>
</tr>
<tr>
<td>rZJ1GFP-M/R45A</td>
<td>58</td>
<td>1.89</td>
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</table>
Characterization of NDV M mutation R42A

(a) Virus titre (log_{10} TCID_{50} ml^{-1})

(b) GFP Merge

(c) rZJ1GFP rZJ1GFP-M/R42A rZJ1GFP-M/R45A

(d) Virus titre (log_{10} TCID_{50} g tissue^{-1})

(e) Spleen HE Spleen IHC Thymus HE Thymus IHC Bursa HE Bursa IHC
5 days p.i., and were humanely euthanized. Whereas birds inoculated with the mutant virus rZJ1GFP-M/R42A presented delayed and mild clinical signs, which were characterized by the observation that three birds had neurological signs at 9 days p.i. and two of them died at 10 days p.i., and there were no deaths in the subsequent days (Table S1, available in the online Supplementary Material). To evaluate the replication of rZJ1GFP-M/R42A in chicken tissues at 5 days p.i., viral load in visceral organs was assessed by titration [log_{10} TCID\textsubscript{50} (g tissue)^{-1}] in DF-1 cells using the method of Reed and Muench (Reed & Muench, 1938). Results showed that the mutant virus rZJ1GFP-M/R42A only replicated in spleen, lung, thymus and bursa of Fabricius, and only to a small extent; by contrast, the parental virus rZJ1GFP replicated in multiple tissues and had relatively higher virus titres in the lymphoid organs (spleen, thymus and bursa of Fabricius) (Fig. 2d). Moreover, the results of histopathology and immunohistochemistry detection of the lymphoid organs showed that birds inoculated with rZJ1GFP-M/R42A displayed much milder histological changes and much weaker immunostaining intensity than that for rZJ1GFP, while mock-infected birds did not present any microscopic lesions and immunoreactivity (Fig. 2e, Table S1). Furthermore, birds inoculated with rZJ1GFP-M/R42A shed the highest amount of virus in oral secretions at 2 days p.i. (mean virus titre 10^{3.35} TCID\textsubscript{50} 0.1 ml^{-1}). But rZJ1GFP-M/R42A shed the highest amount of virus (10^{1.75} TCID\textsubscript{50} 0.1 ml^{-1}) in oral secretions at 7 days p.i., indicating more delayed and much lower virus replication in the shedding of the mutant virus.

In summary, we investigated the role of R42 in the subcellular localization of M protein and in the replication and pathogenicity of NDV. Previous study showed that NDV M protein is localized in the nucleus via a bipartite nuclear localization signal (NLS) independent of other viral proteins (Coleman & Peeples, 1993; Peeples et al., 1992). Mutations of the key basic residues in NLS disrupt the nuclear localization of M protein (Coleman & Peeples, 1993), but the effect of disruption of M protein nuclear localization on NDV replication is not known. Several studies have indicated that the nuclear localization of NDV M protein is to regulate the replication and transcription of the viral genome and inhibit host cell functions, which is similar to the functions of some paramyxovirus M proteins (Iwasaki et al., 2009; Rajani et al., 2012). A recent report only showed that NDV replication is inhibited by RNA interference targeting the M gene in chicken embryo fibroblasts (Yin et al., 2010). However, little is known about the importance of the M protein’s nuclear localization in the replication and pathogenicity of NDV. In this study, we found that the basic residue R42A mutation in the N terminus of M protein could also abrogate its nuclear localization. And a recombinant virus with R42A mutation greatly reduced viral replication in DF-1 cells and attenuated the virulence and pathogenicity of the virus in chickens. It is demonstrated that the virulence of NDV is mainly determined by the cleavage of the F protein and by both the stem region and the globular head of the HN protein (Nagai et al., 1976; Panda et al., 2004; Peeters et al., 1999). In recent years, an increasing number of researchers have focused on the role of F and HN proteins in NDV pathogenesis (Cornax et al., 2013; Dortmans et al., 2009; Estevez et al., 2011; Samal et al., 2011, 2013). In the present study, R42A mutation in the M protein similarly attenuated NDV replication and pathogenicity, indicating that the M protein may also influence the virulence of NDV. Taken together, this is the first report to show that a basic amino acid mutation in the NDV M protein abrogates its nuclear localization and attenuates viral replication and pathogenicity, providing us with new insights into further investigating the role of M protein in the pathogenicity and pathogenesis of NDV.

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References

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