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博 士 学 位 论 文

慢病毒基因载体系统的拓展应用性研究

**Studies of lentiviral system for gene transferring and  
its expansile application**

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目 录

目 录	IV
CONTENTS	VIII
摘 要	I
ABSTRACT	III
1 前 言	1
1.1 慢病毒整合研究进展	1
1.1.1 慢病毒整合机制	1
1.1.2 整合酶分子结构	3
1.1.3 整合酶的突变体	5
1.2 CRE/LOXP 系统的研究进展	5
1.2.1 Cre 酶的分子结构和作用机制	5
1.2.2 Cre 酶活动的定位与追踪	7
1.3 线粒体介导的细胞凋亡研究进展	8
1.3.1 线粒体与凋亡	9
1.3.2 p53 蛋白与细胞凋亡	13
1.4 本研究的技术路线	24
2 实验材料与仪器设备	27
2.1 材料与试剂	27
2.2 主要仪器	28
2.3 试剂配方	29
3 实验方法	31
3.1 基因片段的克隆	31
3.1.1 基因片段的高保真扩增	31
3.1.2 基因定点替换突变的高保真扩增	33
3.1.3 目的片段的纯化和回收	34
3.1.4 目的片段的 T/A 连接和转化	34
3.2 慢病毒包装方法和病毒滴定	38

3.2.1 磷酸钙转染法	38
3.2.2 PEI 转染法	39
3.2.3 Sofast 转染法	39
3.2.4 病毒滴定方法	39
<b>3.3 载体构建</b>	<b>40</b>
3.3.1 线性化载体和插入片段的获取	40
3.3.2 酶切片段的纯化和回收	47
3.3.3 载体的连接	47
3.3.4 载体的转化和质粒提取	55
3.3.5 目的载体的鉴定	55
<b>3.4 几种整合酶缺陷慢病毒特性的比较</b>	<b>59</b>
3.4.1 整合酶缺陷慢病毒的包装和病毒滴度的测定	59
3.4.2 整合酶缺陷慢病毒的残留整合酶活性比较	59
3.4.3 整合酶缺陷慢病毒介导外源基因表达的检测	59
3.4.4 检测整合酶缺陷慢病毒感染细胞后的存在形式	60
3.4.5 检测整合酶缺陷慢病毒介导的基因表达的动态变化	61
<b>3.5 Cre/LoxP 系统的优化</b>	<b>61</b>
3.5.1 pPRIME-CMV-Cre-2A-EGFP/RFP 慢病毒的包装和滴度测定	61
3.5.2 pPRIME-CMV-Cre-2A-EGFP/RFP 慢病毒感染细胞后的基因表达检测	61
3.5.3 Cre 酶活性的细胞内检测	62
3.5.4 Cre 酶对含靶序列载体的切割	64
<b>3.6 DNA 损伤修复蛋白 RAD51 和 KU70 与线粒体介导的细胞凋亡关系研究</b>	<b>64</b>
3.6.1 鸡 Ku70 干扰基因效果的 RNA 水平检测	65
3.6.2 鸡 Ku70 干扰基因效果的蛋白质水平检测	65
3.6.3 鸡 Rad51 抗血清制备	66
3.6.4 BiFc 方法研究蛋白质之间的相互作用	70
3.6.5 检测鸡 Rad51 蛋白过量表达对 Bax 蛋白含量的影响	71
3.6.6 PI 法检测多种蛋白对 STS 诱导的细胞凋亡的影响	71
3.6.7 TUNEL 法检测多种蛋白对 STS 诱导的细胞凋亡的影响	72
<b>4 结果与分析</b>	<b>75</b>

<b>4.1 基因克隆和鉴定</b> .....	<b>75</b>
4.1.1 成功克隆整合酶的基因片段 .....	75
4.1.2 Cre 酶基因的克隆和鉴定 .....	75
4.1.3 EGFP 和 RFP 基因的克隆和鉴定 .....	76
4.1.4 SfiI-EGFP-PaeI 基因的克隆和鉴定 .....	77
4.1.5 BstBI-PGK-SfiI 的克隆和鉴定 .....	77
4.1.6 鸡 Rad51 基因的克隆和鉴定 .....	78
4.1.7 鸡 Ku70 基因的克隆和鉴定 .....	79
4.1.8 ApaI-PGK-NheI 的克隆和鉴定 .....	79
4.1.9 鸡 Rad51 原核表达中的 N 端、C 端和全长克隆和鉴定 .....	80
4.1.10 鸡泛素连接酶 (Mdm2) 基因的克隆和鉴定 .....	80
4.1.11 用于 BiFc 的鸡 P53 基因的克隆和鉴定 .....	81
4.1.12 用于 BiFc 的鸡 Rad51 基因克隆、缺失克隆和鉴定 .....	82
4.1.13 用于 BiFc 的鸡和人 Ku70 基因克隆、缺失克隆和鉴定 .....	83
4.1.14 用于 BiFc 的人 Bax 基因的克隆和鉴定 .....	84
<b>4.2 载体构建</b> .....	<b>85</b>
4.2.1 整合酶基因的的定点替换突变 .....	85
4.2.2 慢病毒包装载体内整合酶基因定点突变相关载体的构建 .....	87
4.2.3 Cre /LoxP 系统相关载体的构建 .....	88
4.2.4 DNA 损伤修复蛋白与凋亡关系研究中相关载体的构建 .....	91
<b>4.3 几种整合酶缺陷慢病毒特性的比较</b> .....	<b>101</b>
4.3.1 整合酶缺陷慢病毒的包装和病毒滴度的测定 .....	101
4.3.2 整合酶缺陷慢病毒的残留整合酶活性比较 .....	101
4.3.3 整合酶缺陷慢病毒介导外源基因表达的分析 .....	102
4.3.4 整合酶缺陷慢病毒感染细胞后的存在形式 .....	103
4.3.5 整合酶缺陷慢病毒介导的基因表达的动态变化 .....	104
<b>4.4 CRE/LOXP 系统的优化</b> .....	<b>105</b>
4.4.1 pPRIME-CMV-Cre-2A-EGFP/RFP 慢病毒的包装和滴度测定 .....	105
4.4.2 pPRIME-CMV-Cre-2A-EGFP/RFP 慢病毒感染细胞后的基因表达检测 .....	106

4.4.3 Cre 酶活性的细胞内检测.....	107
4.4.4 Cre 酶对含靶序列载体的切割.....	109
<b>4.5 DNA 损伤修复蛋白 RAD51 和 KU70 与线粒体介导的细胞凋亡关系研究</b>	<b>110</b>
4.5.1 鸡 Ku70 干扰基因效果的 RNA 水平检测 .....	111
4.5.2 鸡 Ku70 干扰基因效果的蛋白质水平检测 .....	112
4.5.3 鸡 Rad51 抗血清制备.....	113
4.5.4 鸡 Rad51 与鸡 P53、鸡 Ku70 与人 Bax 以及人 Ku70 与人 Bax 之间的相互作用的检测 .....	115
4.5.5 鸡 Rad51 蛋白过量表达对 Bax 蛋白含量的影响 .....	117
4.5.6 PI 法检测多种蛋白对 STS 诱导的细胞凋亡的影响.....	120
4.5.7 Tunel 法检测多种蛋白对 STS 诱导的细胞凋亡的影响 .....	122
<b>5 讨 论.....</b>	<b>123</b>
5.1 慢病毒在基因功能分析和治疗的应用中存在的问题.....	123
5.2 CRE/LOXP 系统优化在基因功能分析中的应用价值.....	124
5.3 KU70 和 RAD51 与线粒体介导的细胞凋亡关系研究 .....	125
5.3.1 Rad51 过量表达与 Bax 蛋白表达水平的关系 .....	125
5.3.2 Ku70 干扰与线粒体凋亡的关系.....	125
5.3.3 BiFc 方法研究 Rad51 蛋白与 p53 蛋白、Ku70 蛋白与 Bax 蛋白的相互关系.....	126
5.3.4 cMDM2 对 p53 蛋白的调节对线粒体介导的细胞凋亡的影响.....	127
<b>6 结 论.....</b>	<b>128</b>
<b>参考文献.....</b>	<b>130</b>
<b>致 谢.....</b>	<b>142</b>

**CONTENTS**

<b>ABSTRACT</b> .....	<b>III</b>
<b>1 INTRODUCTION</b> .....	<b>1</b>
1.1 INTRODUCTION OF INTEGRATION WITH HIV.....	<b>1</b>
1.1.1 The mechanism of integration with HIV .....	1
1.1.2 The molecular structure of integrase.....	3
1.1.3 lentivirus with mutated integrase .....	5
<b>1.2 THE PROGRESS OF CRE/LOXP SYSTEM</b> .....	<b>5</b>
1.2.1 The molecular structure and mechanism of Cre .....	5
1.2.2 The orientation and trace of Cre recombinase.....	7
<b>1.3 THE PROGRESS OF APOPTOSIS MEDIATED BY MITOCHONDRION</b> .....	<b>8</b>
1.3.1 Mitochondrion and apoptosis .....	9
1.3.2 The p53 protein and apoptosis in cell .....	13
<b>1.4 THE PROCEDURE OF OUR EXPERIMENTS</b> .....	<b>24</b>
<b>2 MATERIALS AND EQUIPMENT</b> .....	<b>27</b>
<b>2.1 MATERIALS AND REAGENTS</b> .....	<b>27</b>
<b>2.2 EQUIPMENT</b> .....	<b>28</b>
<b>2.3 REAGENTS FORMULA</b> .....	<b>29</b>
<b>3 METHODS</b> .....	<b>31</b>
<b>3.1 GENE CLONE</b> .....	<b>31</b>
3.1.1 Cloning of gene with high fidelity PCR.....	31
3.1.2 Site-directed mutation with high fidelity PCR .....	33
3.1.3 Purification and extraction of targeted bands.....	34
3.1.4 Ligation of targeted bands with T/A vector and transformation .....	34
<b>3.2 METHODS OF LENTIVIRUS PACKAGING AND TITERING</b> .....	<b>38</b>
3.2.1 Lentivirus packaging with calcium phosphate precipitation .....	38
3.2.2 Lentivirus packaging with PEI reagent .....	39
3.2.3 Lentivirus packaging with Sofast reagent .....	39
3.2.4 Method of Lentivirus titering.....	39
<b>3.3 VECTOR CONSTRUCTION</b> .....	<b>40</b>
3.3.1 Linearization of vectors and acquisition of insertional fragments.....	40
3.3.2 Purification and extraction of targeted bands with enzyme digestion .....	47



3.3.3 Vector ligation .....	47
3.3.4 Transformation of ligated vectors and isolation of plasmids .....	55
3.3.5 Identification of targeted construction .....	55
<b>3.4 COMPARISON OF CHARACTERISTICS IN INTEGRASE-DEFICIENT LENTIVIRUS ..</b>	<b>59</b>
3.4.1 Packaging and titering of integrase-deficient lentivirus .....	59
3.4.2 Comparison of residual activity of integrase with integrase-deficient lentivirus .....	59
3.4.3 Analysis of the exogenous-gene expression mediated by integrase-deficient lentivirus .....	59
3.4.4 Forms of lentivirus in cells infected with integrase-deficient lentivirus .....	60
3.4.5 Dynamic expression of exogenous-gene mediated by integrase-deficient lentivirus .....	61
<b>3.5 OPTIMIZATION OF CRE/LOXP SYSTEM .....</b>	<b>61</b>
3.5.1 Packaging and titering of pPRIME-CMV-Cre-2A-EGFP/RFP lentivirus .....	61
3.5.2 Examination of gene expression in cells infected with pPRIME-CMV-Cre- 2A-EGFP/RFP lentivirus .....	61
3.5.3 Examination of Cre activity in cells .....	62
3.5.4 The capability of Cre to remove the fragments between two Loxps in pPRIME- 4Lox-PGK - EGFP vector .....	64
<b>3.6 CORRELATION OF APOPTOSIS MEDIATED BY MITOCHONDRION WITH RAD51 AND KU70 .....</b>	<b>65</b>
3.6.1 Check the effect of RNA interference against chicken Ku70 on RNA level .....	65
3.6.2 Check the effect of RNA interference against chicken Ku70 on protein level .....	65
3.6.3 Prokaryotic expression of chicken Rad51 protein and generation of its antiserum .....	66
3.6.4 Study protein interaction with BiFc method .....	70
3.6.5 Effect of chicken Rad51 overexpression on the level of chicken Bax .....	71
3.6.6 Examination of the apoptotic effect caused by multiple protein and STS with PI method .....	71
3.6.7 Examination of the apoptotic effect caused by multiple protein and STS with Tunel method .....	72
<b>4 RESULTS AND ANALYSIS .....</b>	<b>75</b>

<b>4.1 CLONING AND IDENTIFICATION OF GENE .....</b>	<b>75</b>
4.1.1 Cloning and identification of the fragment comprising integrase .....	75
4.1.2 Cloning and identification of Cre gene .....	75
4.1.3 Cloning and identification of EGFP and RFP .....	76
4.1.4 Cloning and identification of SfiI-EGFP-PaeI .....	77
4.1.5 Cloning and identification of BstBI-PGK-SfiI .....	77
4.1.6 Cloning and identification of chicken Rad51 .....	78
4.1.7 Cloning and identification of chicken Ku70 .....	79
4.1.8 Cloning and identification of ApaI-PGK-NheI .....	79
4.1.9 Cloning and identification of chicken Rad51 fragments involved in Prokaryotic expression .....	80
4.1.10 Cloning and identification of chicken Mdm2 gene .....	80
4.1.11 Cloning and identification of chicken p53 gene involved in BiFc .....	81
4.1.12 Cloning and identification of chicken Rad51 gene、 deleted mutants involved in BiFc .....	82
4.1.13 Cloning and identification of chicken and human Ku70 or deleted mutants involved in BiFc .....	83
4.3.14 Cloning and identification of human Bax gene involved in BiFc .....	84
<b>4.2 VECTOR CONSTRUCTION .....</b>	<b>85</b>
4.2.1 Site-directed mutation in integrase gene .....	85
4.2.2 Site-directed mutation in the integrase gene of packaging vectors .....	87
4.2.3 Vectors construction involved in optimization of Cre/LoxP system .....	88
4.2.4 Vectors construction involved in studying the relations between DNA repair proteins and apoptosis .....	91
<b>4.3 COMPARISON OF CHARACTERISTICS IN INTEGRASE-DEFICIENT LENTIVIRUS</b>	<b>101</b>
4.3.1 Packaging and titering of integrase-deficient lentivirus .....	101
4.3.2 Comparison of residual activity of integrase with integrase-deficient lentivirus .....	101
4.3.3 Analysis of expression from exogenous-gene mediated by integrase-deficient lentivirus .....	102
4.3.4 Forms of lentivirus in cells infected with integrase-deficient lentivirus .....	103
4.3.5 Dynamic exogenous-gene expression mediated by integrase-deficient lentivirus .....	104
<b>4.4 OPTIMIZATION OF CRE/LOXP SYSTEM .....</b>	<b>105</b>

4.4.1 Packaging and titering of pPRIME-CMV-Cre-2A-EGFP/RFP lentivirus	105
4.4.2 Examination of gene expression in cells infected with pPRIME-CMV-Cre-2A-EGFP/RFP lentivirus	106
4.4.3 Examination of Cre activity in cells	107
4.4.4 Capability of Cre to remove the fragment between two Loxps in pPRIME-4Lox-PGK - EGFP vector	109
<b>4.5 CORRELATION OF APOPTOSIS MEDIATED BY MITOCHONDRION WITH RAD51 AND KU70</b>	<b>110</b>
4.5.1 Check the effect of RNA interference against chicken Ku70 on RNA level	111
4.5.2 Check the effect of RNA interference against chicken Ku70 on protein level	112
4.5.3 Generation of antiserum against chicken Rad51 protein	113
4.5.4 Check the interaction between chicken Rad51 and chicken p53, chicken Ku70 and human Bax as well as human Ku70 and human Bax with BiFc method	115
4.5.5 Effect of chicken Rad51 overexpression on the level of chicken Bax protein	117
4.5.6 Examination of the apoptotic effect caused by multiple protein and STS with PI method	120
4.5.7 Examination of the apoptotic effect caused by multiple protein and STS with TUNEL method	122
<b>5 DISCUSSION</b>	<b>123</b>
5.1 PROBLEM IN DISSECTION OF GENE FUNCTIONS AND GENE THERAPY WITH LENTIVIRUS	123
5.2 ADVANTAGES OF OPTIMIZING CRE/LOXP SYSTEM ON THE ANALYSIS OF GENE FUNCTION	124
5.3 RELATIONS BETWEEN APOPTOSIS MEDIATED BY MITOCHONDRION AND KU70 OR RAD51	125
5.3.1 Relation between the Rad51 overexpression and the level of Bax protein	125
5.3.2 Relation between apoptosis mediated by mitochondrion and Ku70 RNA interference	125
5.3.3 Investigation of protein interaction between Rad51 and p53 or Ku70 and	

Bax with BiFc method.....	126
5.3.4 Contribution of cP53 regulated by cMdm2 to the apoptotic effects mediated by mitochondrion .....	127
<b>6 CONCLUSIONS .....</b>	<b>128</b>
<b>REFERENCES.....</b>	<b>130</b>
<b>ACKNOWLEDGE.....</b>	<b>142</b>

厦门大学博硕士论文摘要库

## 摘要

慢病毒载体因为具有能感染分裂和未分裂的宿主细胞，并且带给宿主细胞轻微的免疫原性的优点，而在转基因改造、基因功能分析和基因治疗中得到了广泛的应用。但是，慢病毒载体的整合特性，尤其是它整合到基因组的转录活性区域或邻近活性区域的特性，使基因功能分析的结果产生未知干扰，并给实际应用带来风险。慢病毒的整合可能会引起突变，或者改变非目的基因的表达从而对目的基因的功能研究产生影响，而且病毒序列整合到宿主细胞基因组中可能会在转基因操作中带来长期和有害的影响。我们的研究旨在利用慢病毒的优良特性的同时，通过其他的改进措施来解决慢病毒整合带来的消极影响，拓展慢病毒的应用性。

为了避免插入突变的风险，我们对慢病毒包装系统的 pMDLg/pRRE 载体上的整合酶中的 5 个氨基酸位点进行定点错义突变，并对慢病毒载体相关部件和包装技术进行优化。获得了 IDLV-D64A、IDLV-D116A、IDLV-R262A-R263A-K264H、IDLV-D64A-D116A、IDLV-D64A-D116A-R262A-R263A-K264H 五种整合酶缺陷的慢病毒，通过对这 5 种慢病毒的包装效率、整合酶缺陷慢病毒所介导的外源基因表达能力、残留整合酶活性、整合缺陷形成的 2LTR 与慢病毒基因组的比率以及介导外源基因表达能力的持续性的比较，综合分析其应用的可行性和安全性。我们发现整合酶上这些位点的突变并没有协同效应或相加效应，而 IDLV-D64A 病毒可有效用于基因功能分析和基因治疗。

目前，对于小鼠等少数胚胎干细胞技术成熟的模式动物，可以通过同源重组技术获得稳定的基因改造；对于多数的其他动物，还需要依赖慢病毒整合来获得稳定的转基因细胞和转基因动物，需要利用 Cre 酶切除整入基因组的病毒序列残存来消除转基因的安全隐患。Cre 酶定位切除还在小鼠的条件性基因敲除中广泛应用。为了对 Cre 酶的表达和发挥功能的情况进行定位和监视，我们构建了由 CMV 驱动的 Cre 重组酶和荧光蛋白联合表达的慢病毒载体。该载体中 Cre 重组酶与绿色/红色荧光蛋白通过口蹄疫病毒的 2A 序列藕联，我们发现这种改建不影响病毒的包装和感染以及 Cre 的功能活性，而荧光报告却精准有效。在载体病毒

中我们还引入了突变型 LoxP 中重组活性很高的位点 Lox5171, 将两组 LoxP 位点同向置于一个慢病毒载体中, 借助 Cre 酶的剪切能将残留病毒片段最小化。这些改建提供了利用慢病毒整合获得安全转基因的改进方案。

有报道指出细胞内 Rad51 和 Ku70 蛋白含量的改变能大大提高细胞的基因打靶效率, 但也有资料指出 Rad51 和 Ku70 蛋白与细胞凋亡密切相关。对于在提高基因打靶效率中, 改变这两种蛋白含量对细胞凋亡所产生的影响尚不清楚。我们首先通过双分子荧光互补技术 (BiFc) 对细胞的 Rad51 与 p53、Ku70 与 Bax 两组蛋白之间的相互作用进行了研究, 研究结果表明 Rad51 与 p53、Ku70 与 Bax 之间都有相互作用。在鸡 DF-1 细胞中对 Rad51 蛋白过量表达或 Ku70 基因的表达进行有效干扰后, 用流式细胞技术和 Tunel 对 DF-1 细胞凋亡情况进行检测, 我们发现: 在没有凋亡胁迫时, 这两种蛋白的含量变化对 DF-1 细胞凋亡的影响很小; 但 Rad51 和 Ku70 的过量表达能阻抑 DF-1 细胞在 STS 诱导下的细胞凋亡, 而 Ku70 的干扰则促进细胞在 STS 诱导下的 DF-1 细胞凋亡, Rad51 能在一定程度上减弱 Ku70 干扰引起的 DF-1 细胞凋亡作用。与 Rad51 相似, 过表达能促进 p53 蛋白进行泛素化降解的 Mdm2 蛋白也能一定程度上抑制 STS 对 DF-1 细胞的凋亡诱导作用。已有的资料表明 P53 的蛋白修饰对于 Bax 的转录是必需的, 但我们的 Western 结果显示: 尽管 Rad5 的过表达能增加其与 P53 蛋白结合, 但 Bax 含量却没出现预期的变化, 表明 Rad51 蛋白并没有参与 Bax 基因表达的调节, 它对 DF-1 细胞凋亡的阻抑可能主要通过它的 DNA 修复功能而起作用。这些结果表明, 这些基因参与的细胞凋亡中的作用在哺乳动物和其它脊椎动物中具有普遍性, Rad51 的过表达和 Ku70 的有效干扰可用于提高细胞的基因打靶效率, 且并不会因为它们干扰细胞凋亡而造成明显的不利影响。

**关键词:** 转基因技术 慢病毒载体系统 整合酶缺陷慢病毒 Cre/LoxP系统 细胞凋亡

**ABSTRACT**

Lentiviral vectors have been extensively implicated in transgenic modification, gene function analysis and gene therapy because they are able to infect a wide range of dividing and non-dividing host cells with weak immunogenicity. However, their property of integration, preferentially to integrate into or close to transcriptionally active genome regions, gives rise to big puzzle for gene function analysis or serious potential risk in their applications. Lentiviral integration possibly causes mutation or altered expression of non-targeting genes that would affect the function study of aimed genes, and the integration of viral sequence into the cell genome would leave a risk long-term harm in transgenic manipulations. This study concerns the technologies and the related problems in overcoming the integrative risks while making use of the superiority of the lentiviral vectors, with an aim to expand their application.

To conquer the risk of insertional mutagenesis, we performed missense mutation of five amino acids in integrase of packaged lentiviral vectors derived from pMDLg/pRRE and optimizations of lentiviral components and packaging protocols. We have obtained five different integrase-deficient lentivirus (IDLVs), named IDLV-D64A、 IDLV-D116A、 IDLV-R262A -R263A-K264H、 IDLV- D64A-D116A and IDLV-D64A-D116A-R262A-R263A-K264H. By comparative studies of important characteristics of IDLVs including efficiency of virus packaing, residual integrase activity, capability of exogenous-gene expression and ratio of 2LTR to total HIV DNA, we found that the mutations on these sites of the integrase did not have synergistic or additive effect and the IDLV-D64A mutant is safe and effective vehicle for applications such as gene repair, knock in and knock out, where transient expression is sufficient.

At present, stable gene modification could be achieved by homologous recombination only in a few animals such as mice where technology of embryonic stem cells has been well established; in most other animals, stable gene transfer needs

to make use of the lentiviral integration and the Cre recombinase is usually used to remove the viral sequence that has integrated into the host genome. The Cre/LoxP system is also intensively applied in conditional KO in mice. To trace precisely the presence of Cre expression and to estimate better the effectiveness of the Cre activity, we generated a lentiviral vector to allow the coordinate expression of the Cre recombinase and the RFP or EGFP intermediated with FMDV 2A region driven by a single CMV promoter, and this modification of construct also can package and infect cells effectively. We demonstrated that the coordinate expression with RFP/EGFP with Cre mediated by FMDV 2A region did not influence the Cre activity. To minimize the residual fragment of lentivirus on genomic DNA of host cells, we introduced Lox5171, which has been showed to have strong activity of recombination, into a lentiviral vector. These modifications provided improved solutions to the safety problems in transgenic trials on many animals.

Recent studies demonstrated that the efficiency of gene targeting could be improved by overexpression of Rad51 protein or inhibition of Ku70 protein, and the Rad51 and Ku70 proteins has been showed involved in apoptosis. However, it is not clear whether altered concentration of these proteins in achieving an increased gene-targeting efficiency would give rise to apoptotic problems. We first studied the protein interactions between Rad51 and p53 or Ku70 and Bax by using BiFc technology, and found that these protein interactions did exist. In chicken DF-1 cells, Rad51 overexpression or Ku70 inhibition has little effect on the apoptosis of cells under no apoptotic stress as examined with the FACS or TUNEL assay; nevertheless, overexpression of Rad51 or Ku70 could inhibit STS-induced apoptosis, effective Ku70 knockdown could enhance STS-induced apoptosis, and Rad51 can reduce to some extent the apoptosis with Ku70 knockdown under the apoptotic stress condition. Similar to Rad51, overexpression of chicken Mdm2, a protein that can increase P53 degradation via ubiquitination, could also have an effect on protecting cells from apoptotic stress. It has been shown that the modification of P53 protein is necessary for Bax expression, whereas western analysis from our experiment did not detect decreasing expression of Bax with Rad51 overexpression as we expected, although



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