Cellular and Molecular Biology

Chang et al., Cell Mol Biol 2014, 60:1
DOI: 10.4172/1165-158X.1000104

Research Article Open Access

Luciferase Expression is more Accurate than GFP to Assess Mirnas-Relevant Oncogenesis *in vivo* Live Imaging Study

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Abstract

Background: *In vivo* live imaging technology was able to assess dynamically tumor processes and biology throughout the entire experiment period depending on a bioluminescent signal from tumor cells generated from expression of Green Fluorescent Protein (GFP) or the firefly luciferase gene. Recently, there is scarely reported a comparison of Luc- and GFP-transfected tumors to assess the biological mechanism of miRNAs using identical model systems.

Methods: We constructed the SGC-7901 cells which involving stability-enhanced Luc- and GFP-coexpression identified by real time PCR (qPCR) and fluorescence microscope, the constructed cells were injected into the nude mice, then we compared the transfection efficiency and accuracy of GFP and luciferase for miRNAs-relevant oncogenesis after 40 days.

Results: We constructed successfully SGC-7901 cell lines coexpressing the stability-enhanced luciferase and GFP. The miR-145 expression of Luc/LV-miR-145 SGC-7901 cells was higher than Luc/LV-ctrl's, and luciferase intensity was consistent with tumor volume and negatively related to miR-145 expression in Luc-transfected SGC-7901 cells, but the phenomenon of GFP was opposite. Conclusion: We believed that luciferase offers distinct advantages over GFP as a transfection and gene expression reporter to assist the studies of miRNAs-relevant oncogenesis *in vivo* live imaging study.

Keywords: Green fluorescent protein; Luciferase; miRNA; Tumor; Molecular imaging

Abbreviations: qPCR: Real Time PCR; GFP: Green Fluorescent Protein; Luc: Luciferase; LV: Lentiviral Vector

Introduction

Live animal imaging is becoming an increasingly common technique to assess dynamically tumor processes and biology throughout the entire experiment period. Bioluminescence imaging systems rely on a bioluminescent signal of green fluorescent protein (GFP) and the firefly luciferase gene from tumor cells [1]. The reporter genes (GFP and luciferase) are usually used as the powerful tools to study genes and regulatory sequence function.

MicroRNAs (miRNAs) are endogenously expressed short noncoding RNAs, 20-24 nucleotides in length, that control gene expression post-transcriptionally, either by degradation of target mRNAs or by inhibition of protein translation [2,3]. Accordingly, altered expression of miRNAs could influence numerous cancer-relevant processes, including proliferation, cell cycle control, apoptosis, differentiation, migration and metabolism. miRNA-145 expression level was extremely reduced in many human cancers such as lung adenocarcinoma [4], colon carcinoma [5], bladder cancer [6], these previous studies suggested the transfection of miR-145, as a tumor suppressor, significantly reduced the growth of cancer cells.

In the present study, we infected GFP-miR-145 lentivirus into the stability-enhanced Luc-SGC-7901 cell lines and injected these reconstructed cell lines into nude mice. Furthermore, we compared the tumor volume, and discussed the discrepancy of the transfection efficiency and accuracy of GFP and luciferase for miRNAs-relevant oncogenesis.

Materials and Methods

Cell culture

SGC-7901 cell lines were obtained from Biomedical Reasearch Center, College of Medicine, Xi'an Jiaotong University and grown in RPIM-1640 (PAA) with 10% fetal bovine serum (FBS, Invitrogen) and supplemented with 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen). Cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Stable transfection of pCAG-Luc expression vector

The day before transfection, SGC-7901 cells were seeded in

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Received October 12, 2013; **Accepted** November 12, 2013; **Published** January 25, 2014

Citation: Chang S, Gao L, Li Z, Ni L, Tong D, et al. (2014) Luciferase Expression is more Accurate than GFP to Assess Mirnas-Relevant Oncogenesis *in vivo* Live Imaing Study. Cell Mol Biol 60: 104. doi: 10.4172/1165-158X.1000104

antibiotic-free medium. pCAG-Luc vector was transfected into SGC-7901 cells using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen), cultured by selection with 600 μg/ml G418 (Invitrogen) containing medium for 2 weeks. Single cell clones were picked and cultured in medium (RPIM-1640) containing 300 μg/ml G418 for further study. The expression level of luciferase in transfected SGC-7901 cells was identified by quantitative real-time RT-PCR after further selection and expandation.

Lentivirus infection

Lentivirus miR-145 combined with GFP reporter (GFP-miR-145) was synthesized by Shanghai GeneChem Co., Ltd. The empty lentiviral vector (GFP-Ctrl) was used as a control. Stability-enhanced Luc-SGC-7901 cells were infected by lentiviruses according to the manufacturers' protocol.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted from the cells using Trizol reagent (Invitrogen). To quantitate miR-145 expression, 5 ng of total RNA was reversely transcribed using the PrimeScript® RT reagent Kit (Takara Co., Ltd, Dalian, China), The mature miRNA was quantified by quantitative PCR (qRT-PCR) using the SYBR Premix Ex TaqTMII (Takara Co., Ltd, Dalian, China) on an FTC-3000TM System (Funglyn Biotech Inc., Toronto, Canada) using the specific primers for miR-145 and normalized by U6. To measure the mRNA levels of luciferase, total RNA was reversely transcribed using universal primers. qRT-PCR was performed using specific primers for luciferase, and β-action served as an endogenous control. All the primers were listed in Table 1. Relative quantification of miRNAs and mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method, in which $\Delta\Delta Ct = (Ct_{miR\text{-}145}\text{-}Ct_{U6})oscc\text{-}(Ct_{miR\text{-}145}\text{-}Ct_{U6})_{control}$ [7]. All assays were performed in triplicate.

Tumor xenograft live imaging

All experimental procedures were approved by the Animal Experimentation Ethics Committee of Xi'an Jiaotong University. SGC-7901 cells (1×10^6, suspended in 100 μl sterile PBS) xenografts stably co-expressing Luc-GFP combined with miR-145 or the corresponding control vector (ctrl) were injected subcutaneously into the ventral thigh of nude mice. The bioluminescent imaging was measured using Xenogen IVIS Spectrum (USA). After 40 days, following general anesthesia, images were taken and analyzed using Spectrum Living Image 4.0 software (Caliper Life Sciences).

Statistical analysis

SPSS 13.0 software was used for statistical analysis, and diagrams were performed by GraphPad-Prism 5. Two-tailed Student's t test was used for comparisons between groups, and $P<0.05$ was considered statistically significant.

miRNAs	Primers
miR-145	RT primer: GTCGTATCCAGTGCGTGTCGTGGAGTCG-GCAATTGCACTGGATACGACagggatt
	Forward primer: CAGTGCGTGTCGTGGAGT
	Reverse primer: AGGTCCAGTTTTCCCAGG
U6	RT primer: CGCTTCACGAATTTGCGTGTCAT
	Forward primer: GCTTCGGCAGCACATATACTAAAAT
	Reverse primer: CGCTTCACGAATTTGCGTGTCAT
luciferase	Forward primer: TGAGTACTTCGAAATGTCCGTTC
	Reverse primer: GTATTCAGCCCATATCGTTTCAT
β-actin	Forward primer: CCAACCGCGAGAAGATGA
	Reverse primer: CCAGAGGCGTACAGGGATAG

Table 1: Primers for PCR.

Results

Stability-enhanced Luc- and GFP-coexpression in SGC-7901 cell lines

To validate the viability of SGC-7901 cells expressing luciferase after 2 weeks, we found that the expression of luciferase in SGC-7901 cells transfected with pCAG-Luc vector was higher than control group after 40 days (P=0.037) (Figure 1). Furthermore, SGC-7901 cells stably transfected with pCAG-Luc were infected by GFP-miR-145 and GFP-ctrl, respectively. Figures 2A-2D showed that the transfection efficiency of GFP was significantly high in SGC-7901 cells infected lentiviruses using fluorescent microscope, meanwhile, the miR-145 expression of GFP-miR-145 SGC-7901 cells was higher than GFP-ctrls' using qRT-PCR (P=0.037) (Figure 2E).

Luc-transfected tumor cells were more accurate than GFP in live imaging studies

Firstly, we identified whether miR-145 was involved in tumorigenesis of tumor cells *in vivo*. As shown in the Figure 3A at 40 days after injection, intratumoral delivery of synthetic miR-145 induced a specific inhibitory response and significantly inhibited tumor growth compared with control group, as measured by caliper. To correlate the suppressive tumorigenesis with delivery of miR-145, RNA was extracted from miR-145 and control tumors, and miR-145 expression was assessed by qRT-

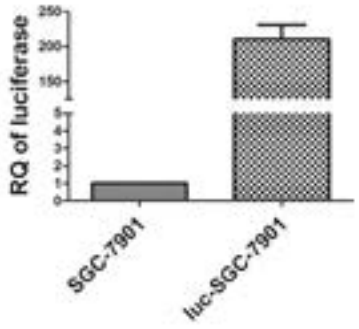

Figure 1: Expression levels of luciferase in SGC-7901 cells transfected with pCAG-Luc vector.

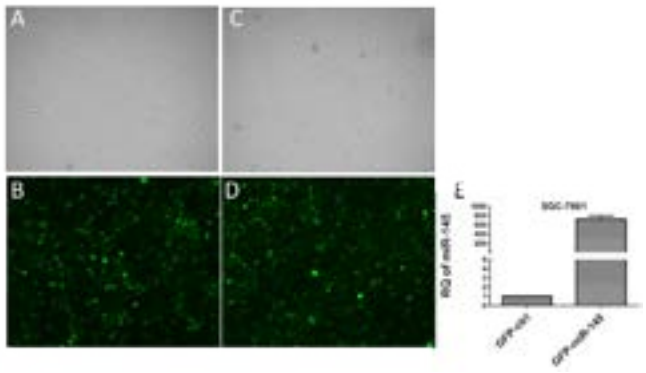

Figure 2: Transfection efficiency of GFP-expressing lentiviruses. (A) Light field of GFP-miR-145. (B) Fluorescent expression of GFP-miR-145. (C) Light field of GFP-ctrl. (D) Fluorescent expression of GFP-ctrl. (E) miR-145 expression of GFP-miR-145 and GFP-ctrl.

PCR. Tumor's volume injected by miR-145 was significantly smaller than control tumor. Moreover, the miR-145 expression of Luc-miR-145 SGC-7901 cells was higher than Luc-ctrl's (P=0.002) (Figure 3B), and luciferase intensity was consistent with tumor volume (Figure 4A) and negatively related to miR-145 expression in Luc-SGC-7901 cells, but the phenomenon of GFP was opposite (Figure 4B).

Discussion

Live imaging technology is rapid, cost-effective, convenient and applied to studying pathological processes and biology *in vivo*. The dysregulated expression of miRNA may impact a multitude of transcripts and profoundly influence cancer-related signalling pathways by targeting up to several hundred mRNAs [8]. Previous studies usually introduced natural or altered miRNAs into cultured tumor cells by transfecting the GFP-miRNA [9] or Luc-miRNA [10], respectively. However, there is scarely reported comparison of Luc- and GFP-cotransfected tumors to assess the biological mechanism of miRNAs using identical model systems. For our study, we made a recombination of GFP- and Luc-miRNA coexpressing mouse tumor model to compare and contrast the transfection efficiency and accuracy of GFP and luciferase.

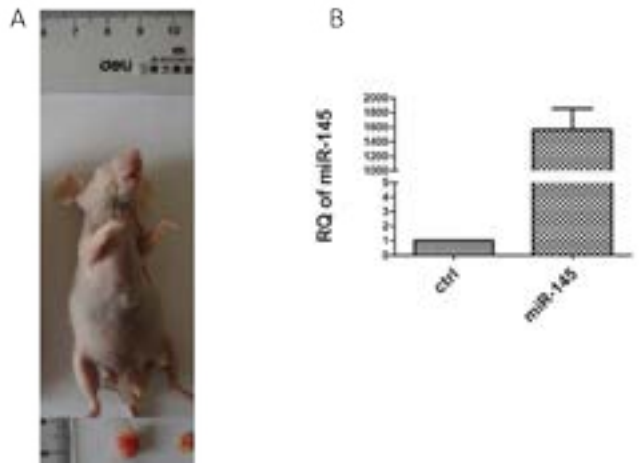

Figure 3: Expression of miR-145 inhibitors tumor growth. (A) Gross morphology of tumors composed of either SGC-7901-ctrl cells or SGC-7901-miR-145 cells at 40 days after injection. (B) Expression level of miR-145 in tumor xenografts was quantified by qRT–PCR.

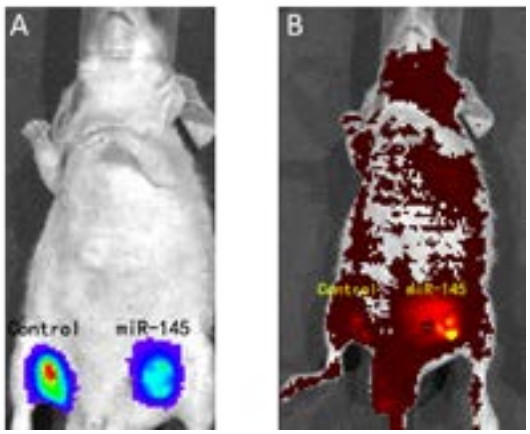

Figure 4: Imaging results for mice inoculated with SGC-7901 cells. (A) Luciferase intensity of SGC-7901 cells. (B) GFP intensity of SGC-7901 cells.

Here, we clearly found that Luc-ctrl intensity correlated significantly and linearly with tumor volume, and negatively related to miR-145 expression, interestingly, the trends of GFP was opposite. Based on comprehensive consideration of our results, we believed that Luc-tumor cells are more persuasive than GFP-tumor cells for studies of miRNA-relevant oncogenesis. What caused the differences between Luc- and GFP-tumor cell lines?

It is vital to determine transfection efficiency, including percent transfected cells and average reporter expression in a cell population. Both approaches usually gave similar trends, however, Vidugiriene et al. [11] believed that the differences in reporter activity were much greater than differences in percent transfection [11]. In our study, average reporter expression was used to identify the transfecion efficiency of luciferase using the qRT-PCR, and for GFP, determined by percent transfected cells, together with average reporter expression using fluorescence microscope, the later shown the consistency between two approaches.

Endogenous activity is distinguishing characteristic of GFP and luciferase reporter protein. Luciferase typically no endogenous activity in host cells to interfere with quantitation [12], and on the other hand, Hoffman [13] had studied the use of low light imaging technologies with GFP-transfected tumor cells for tumor growth, metastasis, and angiogenesis in mouse models. Although GFP is not originated from mammalian cells, some primary cells, such as monocytes and macrophages, are autofluorescent, contributing background and limit fluorescent reporter detection [11]. Similarly, other investigators also detected high backgrounds with fluorescent imaging of mice due to endogenous macromolecules in blood and other tissues, which could appear as false-positive lesions when concentrated in the intestines [14,15]. As mentioned above, in the present study, we could also find some false-positive lesions of GFP in other places besides tumor through live imaging study, however, luciferase intensity was consistent with tumor volume and miRNA expression.

Furthermore, it is necessary to clarify the difference of GFP expression, Caceres et al. [16] studied live imaging using the same human breast tumor cell lines (MCF-7) transfected with GFP or luciferase, their findings suggested that it was possible to image superficial MCF-7 tumors transfected with either Luc or GFP, but tumors at distant sites and in deep tissue could only be detected using Luc-transfected cells. So we supposed that injection depth might exceed the subcutaneous tissue, reducing the sensitivity of GFP when operating, it indicated that we should operate the strict control of injection depth through continual practice to reduce the error of GFP.

In conclusion, luciferase had distinct advantages over GFP as a gene expression reporter to benefit the studies of miRNAs-relevant oncogenesis. It was critical not only to choose the proper reporter and the transfection efficiency approach on the basis of different injection locations (intravenous, intraperitoneal or subcutaneous), but also to monitor cell health. As Jessamy et al. [17] had identified that luciferase expression did not impact tumor cell growth *in vitro* or *in vivo*.

Acknowledgements

This study was supported in part by the Key Science and Technology Program of Shaanxi Province (No.2010ZD KG-50), the Project of Technological Innovation Planing in Shannxi Province (2012KTCL03-17) and Natural Science Foundation of China (No. 81272957 and No.81171398).

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