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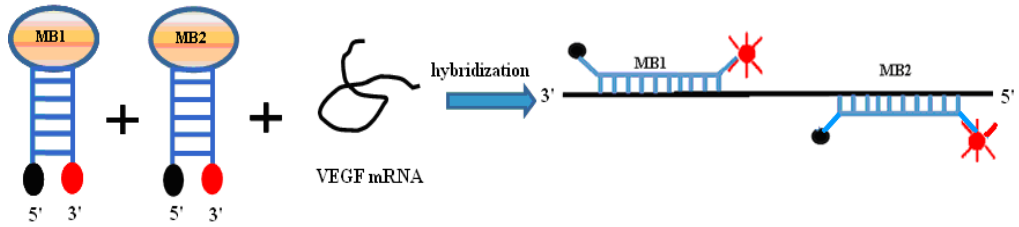
A fluorescence method with wide promising application was developed for VEGF mRNA assay with high accuracy and specificity by applying dual molecular beacons as probes.



A rapid and simple method to assess the changes of human VEGF mRNA based on dual molecular beacons

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Abstract

The vascular endothelial growth factor, or VEGF, is a key signal used by oxygen-hungry cells to promote growth of blood vessels and a key regulator of angiogenesis in a variety of physiologic and pathologic processes. It binds to specialized receptors on the surfaces of endothelial cells and directs them to build new vessels in vascular angiogenesis, and fulfills this role by modulating intracellular signaling and gene expression in response to chemical and physiological stimuli. Thus, assessing change of VEGF mRNA is essential to understand how physiological and pathological processes modulate angiogenesis. Here, dual molecular beacons were applied to detect the changes of VEGF mRNA in hepatocarcinoma cells with different treatments, an optimized hybridization assay for VEGF mRNA took ~30 min to perform and levels of VEGF mRNA were further validated by real-time qRT-PCR. In addition, methods of RNase H digestion and blocking oligonucleotides were used to verify the reliability of the fluorescence change caused by the beacons-mRNA hybridization. Based on these findings, it can be concluded that the new method with advantage of simplicity, rapidity and efficiency has great promise for assessing gene expression analysis of clinic or other biological samples.

INTRODUCTION

Human vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and a key regulator of angiogenesis in a variety of physiologic and pathologic processes [1-3]. This gene, which resides on chromosome 6p21.3, can produce various splice variants (subscripts denote the number of amino acids after signal sequence cleavage) including $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{148}$, $VEGF_{145}$ and $VEGF_{121}$ through alternative splicing of 8 exons [4-6]. Many evidences have indicated that the increased VEGF expression contributes to the VEGF-driven angiogenesis in hypoxic areas of solid tumors [9-10], whereas inhibition of VEGF activity by neutralizing antibodies prevents tumor growth and regression [7-8]. In addition, it

has been reported that this gene also plays an important role in the etiology of retinal disorders and in wound repair [11-12]. Thus, the detection of VEGF mRNA change is necessary for basic scientific study, clinic diagnosis and the development of targeted therapy drugs.

Commonly, the effects of stimulus on VEGF mRNA expression are measured by real-time quantitative RT-PCR (qRT-PCR) of spliced transcripts [13-14]. Although the method used to determine the expression of VEGF has strongly impacted our knowledge of the biological roles that VEGF plays under physiological and pathological conditions, it requires multiple steps, including a reverse transcriptase reaction to make cDNA, and PCR reaction that is tracked by dyes or fluorophores. In addition, it requires some effort to obtain accurate results, and the necessary reagents are expensive. Given the most common method's limitations, we sought to develop a molecular beacons-based method to quickly and efficiently detect VEGF mRNA changes *in vitro*. Molecular beacons are oligonucleotide hairpin probes with an internally quenched fluorophore, whose fluorescence are restored upon excitation when they hybridize with complementary nucleotide sequences [15]. The unique target recognition and strong signal transduction capabilities have led them to be widely applied in the assays of quantitative PCR, protein-DNA interactions, multiplex genetic analysis and mRNA detection *in vivo* and *in vitro*[16]. Until now, new methods based on molecular beacons have been developed to assess mRNA levels of vav, eNOS, ING1 and p21 et al [17-20] and these single beacon based methods without amplification of signal change showed their major advantages of simplicity, efficiency and accuracy on the expression analysis of mRNA. In these detection systems, fluorescent signal intensities, which directly represent the level of targeted mRNA, depends on the concentration and the accessible site number of the target. Thus, the strategy of using multiple beacons will produce stronger fluorescence signal and accordingly increase the detection sensitivity of molecular beacons, comparing with single beacon method. This kind of improvement is very important for those rare samples. Furthermore, the adjacently positioned oligonucleotide probes annealing on the same target RNA can interact cooperatively, increasing the hybridization efficiency through the suppression of secondary structure and/or a base stacking effect [21,22]. These studies enlightened us to design dual molecular beacons that recognize common regions of all VEGF mRNA variants and subsequently develop a homogenous assay to detect changes in VEGF mRNA level *in vitro*.

2 METHODS

2.1 Molecular beacons design and synthesis.

In order to design molecular beacons for specifically hybridizing with VEGF mRNA, we applied MFOLD and RNAdraw programs to analyze secondary structure of human VEGF mRNA (gi: 284172447) for open,

single-stranded regions, then, those regions, which are preserved in all VEGF variants, were subjected to BLAST analysis to rule out any significant homology with other genomic sequence. Finally, the loop of dual molecular beacons based on the sequences of 1316-1337 and 1341-1361 regions of VEGF mRNA (gi: 284172447) were designed, respectively. A 6-nt stem sequence was added to each end of the loop to form the stem. The fluorophore 5(6)-Carboxytetra methylrhodamine (6-TMARA; λex/em=λ521/578 nm) and quencher 4-(4- dimethylaminophenylazo) benzoyl (DABCYL) were added to have the following final molecular beacons design: M1: 5'-CGC TGC GACTCCTCAGTGGGCACACACGCAGCG-3'; MB2: 5' –CGCTGCCG CATAATCTGCATGGTGATGGCAGCG-3', MB3(control): 5'-CGCTCGTGTAGTTCCCGTCATC TTTGT C GAGCG -3', underlined bases indicate the stem sequences. The sequence of blocking oligonucleotide is 5'-GTGTGTGCCCACTGAGGAGTCACATCACCATGCAGATTATGCG-3'. It was used in threefold molar excess to that of beacons to assess the specificity of the assay. Common region of all VEGF mRNA variants was selected to create oligonucleotide primers using Primer 5.0 and RT-PCR was performed to verify the specificity of the primers for their target using total RNA from cultured cells; these primers were judged to be specific for VEGF if RT-PCR produced a single band matching the size of the positive control on agarose gel electrophoresis. VEGF molecular beacons and oligonucleotides were synthesized by TaKaRa Biotechnology (Dalian, China) Co. Ltd.

2.2 Beacon hybridization assay.

A schematic depiction of the assay with dual molecular beacons is shown in Fig. 1. Buffer containing 100 mM Tris-Cl (pH 8.0) and 20 mM $MgCl_2$ was used for hybridization experiments unless otherwise indicated. Mixtures containing 3 μg of total RNA and 100 nM dual molecular beacons were incubated at 45°C for 30 min. Hybridizing conditions were determined from separate experiments with different incubation time and melting curves of molecular beacons. Negative control experiments were performed with HCV RNA or a nonspecific beacon MB3. Background signal was assessed by performing the assay with beacons alone; specific signal was calculated by subtracting background from total fluorescence signal (beacons plus cell total RNA). Each hybridization assay was performed in triplicate. A range of total RNA concentrations from 0 to 5μg were used for determining the sensitivity of dual molecular beacons or single beacon-based method for VEGF mRNA assay. The background signal of fluorescence from dual molecular beacons or single beacon was subtracted from the fluorescence signal from the duplexes of beacons or single beacon with VEGF mRNA. The positive fluorescence difference value indicates the lowest concentration of VEGF mRNA detectable by the two methods.

Fig.1

2.3 Cell culture and treatment with $CoCl_2$ and H_2O_2

$CoCl_2$ and H_2O_2 were purchased from Sigma. Different concentrations of $CoCl_2$ or H_2O_2 were mixed with DMEM medium (Invitrogen) and added to Huh7 and Huh7.5 cells for different time. Total cellular RNA was isolated from culture cells using TRIzol reagent (Invitrogen) and concentrations were measured by ultraviolet absorbance at 260 and 280 nm and subsequently stored in aliquots at −80°C.

2.4 HCV infection

JFH1 (a unique hepatitis C virus strain) particles were produced in Huh7.5 cells as previously described [23]. All infections were performed by incubation of virus inoculum JFH1 with Huh7.5 cells for 6~9 h before cells were washed and changed into the medium appropriate for the specific cell type and differentiation stage. For the time course of permissiveness, infection at each time point was allowed to proceed for exactly 48 h before cell harvesting. Viral titers of HCV produced were performed with Huh-7.5-based cells and measured in focus-forming units (FFU) per milliliter.

2.5 q RT-PCR.

Real-time PCR was performed in the LightCycler Instrument (Roche Applied Science) in a total volume of 20μL per PCR tube. PCR primers for VEGF were 5'-GGCTGACTCTAGAATTTCTGGAATCT-3' and 5'-GTGGTACAATCATTCCTTGTGCTT-3'. GAPDH primer sequences were 5'-ATGGCACCGTCAAGG CT GAGAAC-3' and 5'-GTTGCTGTAGCCAAATTCGTTGTC-3'. For each reaction, 1 μL of cDNA was placed in a 4 μL 5×Platinum SYBRgreen qPCR Supermix (Takara) containing 0.1 μL of a temperature-released *Taq* DNA polymerase (5 U/μL; Platinum DNA Polymerase; Takara), 0.5 μL of the primers, and 14.5μL DEPC-treated water. The cycling protocol was identical for total VEGF and consisted of an initial 5-min denaturation step at 95 °C for activation of the DNA polymerase, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 63 °C for 15 s, and extension at 72 °C for 20 s.

3 RESULTS AND DISCUSSION

3.1 Optimum of hybridization reaction

The signal to background ratio of dual molecular beacons in target mRNA detection is not only dependent on the design but also assay conditions employed [17]. In order to achieve optimal performance of the assay system, signal changes of molecular beacons caused by VEGF cDNA (an alternative of VEGF mRNA with same sequence) under different Mg^{2+} concentrations (0-50mM) were obtained and compared. It was found that

the variation of Mg^{2+} concentration obviously affected the signal change of hybridization and 20 mM Mg^{2+} was among the most optimal concentration for VEGF cDNA detection (Fig.2). These results suggest that Mg^{2+} plays an important role in the processes of maintaining the stem-loop conformation by neutralizing backbone electrostatic repulsions as well as the hybridization by affecting the free energy change of duplexes [24-25]. Next, we studied the effect of temperature on the hybridization of VEGF mRNA with beacons. Denaturing profiles were obtained by recording the fluorescence intensities of molecular beacons in the presence and absence of target at different temperature. Consistent with previously recommended conditions for DNA/RNA probes, our results showed that background siganl didn't clearly improve upon increasing temperature from 25 to 60°C, while there was a large difference between the sensitivity of 45°C with that of others. These results led us to choose the preferred buffer containing 20mM Mg^{2+} for further evaluation of VEGF mRNA under 45°C due to their overall performance. In addition, we compared the fluorescence change caused by the hybridization of single or dual beacons with VEGF cDNA and found that the simultaneous hybridization of dual beacons with VEGF cDNA can produce an accumulative and weak cooperative effect (Fig.2C).

Fig.2

3.2 Specificity of molecular beacon signal.

Next, the competitive inhibition and RNase H digestion experiments were used to examine the specificity of the system for VEGF mRNA assay. When a blocking strand composed of same sequences as loop sections of the beacons but lacking the fluorophore, stem and quencher, was added in threefold molar excess to the beacons-VEGF mRNA mixture, it caused a dramatic attenuation of fluorescence intensity. To further assess the specificity of the assay, we applied RNase H to digest duplexes of VEGF MBs/mRNA and found that the fluorescence signal decreased when this enzyme was added into the solution. The result demonstrates that digestion of VEGF mRNA in duplexes by RNase H can help molecular beacons restoring the stem-loop conformation and cause fluorescence quenching again (Fig. 3).

In addition, we investigated the influence of genomic DNA on the reliability of this method. By comparing the fluorescence change of molecular beacons caused by mRNA in the presence of genomic DNA or not, we found that the fluorescence change is only associated with the amount of mRNA, but not with genomic DNA (data not shown). These results clearly demonstrate that the method can be applied for target mRNA detection in crude total RNA sample without further purification.

Fig.3

3.3 Differential expression of VEGF mRNA in different cell lines.

Many evidences have showed that the level of VEGF mRNA varied significantly in different cell lines [23]. We firstly investigated whether dual beacons can accurately discriminate the difference of VEGF mRNA in hepatocarcinoma cell lines of Huh7, Huh7.5 and 20986. Total RNA samples extracted from these cell lines were subjected to the dual beacons assay and the quality of these RNA samples was detected by agarose electrophoresis. As seen in supplementary Fig. S1, four different RNA extractions from tumor cells resulted in 28S ribosomal RNA bands that are approximately twice as intense as the 18S ribosomal RNA band [26]. Then, we incubated dual molecular beacons with total RNA of Huh7 cells to investigate whether the molecular beacons are specific for VEGF mRNA detection in a heterogeneous RNA sample. The result shows the fluorescence signals from hybridization assays using the dual molecular beacons. As the concentration of total RNA used in the assay increased from 0.2 to 5 μg, there was an increase in fluorescence signal from dual molecular beacons. However, the sensitivity of single beacon can only attain 0.5 μg total RNA (Fig.4A). These results indicate that dual molecular beacons have the sensitivity and specificity required to VEGF mRNA detection in tumor cells. Then, dual molecular beacons were applied to discriminate the difference of VEGF mRNA in these three kinds of tumor cell line. From results (Fig.4B), it was found that VEGF mRNA level is tightly associated with cell type and the level of VEGF mRNA in Huh7. cells is two folds higher than that of Huh7 and 20986 cells, while there is no difference between Huh7 and JFH1 cells. Then, the efficacy of the beacons-based approach for measuring VEGF mRNA was verified by qRT-PCR and the results were indicated in Fig.4C. From the results, it was found that the difference of VEGF mRNA observed by the two methods was similar. In order to further confirm the reliability of qRT-PCR, we detected RT-PCR products of VEGF mRNA by using agarose gel electrophoresis and the result showed a VEGF band with expected size (259 bp) (Fig.S2). From these results, we can conclude that the dual beacons can be reliably applied for VEGF mRNA detection.

Fig.4

3.3 Changes in VEGF expression in response to $CoCl_2$ treatment.

$CoCl_2$ has been shown to be a regulator of VEGF mRNA expression both in vitro [27-28] and in vivo [29]. We wanted to determine whether molecular beacons could accurately quantify the effects of $CoCl_2$ on VEGF mRNA expression. Beacons were incubated with RNA from Huh7 that had been incubated with regular media or media supplemented with different concentrations of $CoCl_2$ for 24 h. Figure 5A shows a 2.3 to 6 folds increase in beacon signal that was detected in RNA from $CoCl_2$ treated Huh 7 cells compared with the control. The manner of VEGF mRNA increase is $CoCl_2$ concentration-dependent in some extent. However, the level of VEGF mRNA changed weakly in 20986 cells treated with $CoCl_2$ compared with control cells (Fig.5C). More

interestingly, there was no effect of $CoCl_2$ on VEGF mRNA in Huh7.5 cells (Fig.5E). These results also demonstrate the different responsibility to $CoCl_2$ treatment caused by the difference of cells origin. The efficacy of the beacon-based approach for measuring VEGF mRNA was verified by qRT-PCR (Fig.5B, D, F) and the induced increase in VEGF mRNA observed by two methods was similar with each other.

Fig.5

3.5 VEGF expression in response to H_2O_2 treatment.

H_2O_2 has been shown to influence VEGF expression in cultured cells [30-32]. To determine whether the new assay could be used to accurately measure changes in VEGF expression in response to H_2O_2 treatment, beacons were incubated with total RNA from Huh7 and Huh7.5 cells that had been incubated with regular media or media supplemented with H_2O_2 for 24 h. We found 4-fold increase in beacons signal due to hybridization to VEGF mRNA from H_2O_2-treated cells compared with that of control cells (Fig. 6). However, H_2O_2 treatment cannot significantly change VEGF level in 20986 cells. In the following experiment, we applied qRT-PCR method to detect VEGF levels of these samples. Results indicated that the fold of change obtained by the fluorescence method was similar to that of VEGF mRNA detected by qRT-PCR (Fig. 6).

Fig.6

3.6 VEGF expression in response to HCV infection.

HCV infection is one of the major causes of chronic hepatitis, liver cirrhosis, which subsequently leads to hepatocellular carcinoma, through the stabilization of hypoxia-inducible factor 1α (HIF-1α) [33-35]. HIF-1α induction in turn leads to the stimulation of VEGF [33-35]. To fully validate the potential value of the new method and propose a relevant application in the field of virus research, we examined the effect of HCV infection on VEGF mRNA level. Dual beacons were incubated with total RNA samples from Huh7.5 and Huh7 cells that had been incubated with media supplemented with HCV JFH1(MOI=0.1) for 24 h. We found a 1.6 folds increase of beacons signal from JFH1-treated Huh7.5 cells comparing with that of control. The fold of change obtained by this method was similar to that obtained by qRT-PCR. As expected that there was no difference of beacons signal between Huh7 cells infected with JFH1 or not. because Huh 7 cell line is difficultly infected by JFH1 (Fig.7). These results demonstrate that the new method can be applied to evaluate the effect of JFH1 on VEGF expression as well as monitor process of HCV transfection.

Fig. 7

4 DISCUSSION

Molecular beacons have become a very useful tool for homogeneous single-stranded mRNA detection due to their ability to differentiate between bound and unbound states and their improved specificity over linear probes [16]. In the present study, we have described a dual beacons-based method for the expression analysis of VEGF and presented data demonstrating that VEGF mRNA changes can be assayed without any amplification of the detected signal. As a key signal to promote growth of blood vessels and a key regulator of angiogenesis [1-3], VEGF mRNA is known to be modulated by several physiological, pathophysiological stimuli[6-8], or HCV transfection[33-34]. From experimental results, we find that dual beacons can reliably quantify changes of VEGF mRNA caused by different treatment, no matter whether the treatment produces a large change or a more modest change, even no change of this gene. Moreover, the new method was applied for evaluating the change of VEGF mRNA caused by NS398, an inhibitor of VEGF [36-37] and the result indicated that the level of VEGF mRNA in tumor cells reduced about 45% after treating with this drug for 24 h (supplementary Fig.S3). The data showed that the new method, by combing with multiwell-plate reader technology, can be used for fast and high throughput screening of target drugs of VEGF *in viro*. In addition, it is also hopeful for the assessment of other genes in heterogeneous RNA samples derived from either cell cultures or whole tissues by replacing the loop sequences of molecular beacons.

Although real-time RT-PCR (qRT-PCR) is currently the most common method for quantifying a specific RNA species from cellular and tissue sources [14], it can not avoid the inherent drawbacks of PCR such as long time consuming and high variability of results. While the dual molecular beacons-based method described in this report is fast, relatively simple, more specific and cost-effective. Except for the advantages of molecular beacons method, it was worth mentioning that although the dual molecular beacons increased the sensitivity of target mRNA detection in some extent comparing with that of single molecular beacon, it is difficult to be applied for the target mRNA assay with low abundance, which is the main advantage of the role of real-time RT-PCR. Nevertheless, the method of dual molecular beacons developed in this study provides an option for mRNA detection, discriminating alternatively spliced transcripts and analysis of large mRNA segments *in vivo*. However, the parameters of high nuclease stability and high S/N ratio of MB probes should be optimized for these tasks, which are necessary for ensuring high reliability of the results.

5 Conclusion

We have developed a simple, direct and highly specific method for assessing changes in VEGF mRNA in complicated samples. The analytical performance of the method was validated through experiments based on qRT-PCR, which were specifically designed, synthesized, and quantified in common. We believe that the

method with rapid detection capability can be widely used as a promising tool for the elucidation of its role in promoting physiologic and pathologic angiogenesis as well as a model of mRNA detection for other spliced variants of VEGF.

Acknowledgements

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Fig.1 The Schematic diagram of VEGF mRNA detection based on dual molecular beacons.

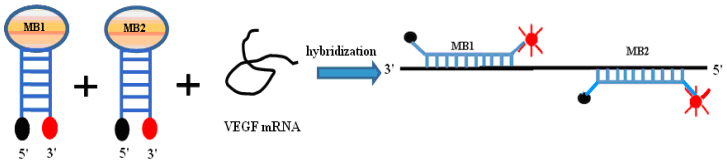


Fig.2 Effects of Mg^{2+} (A) and temperature (B) on the hybridization process of dual molecular beacons (DMB) with VEGF cDNA. [DMB]=100 nM, cDNA=250 nM.

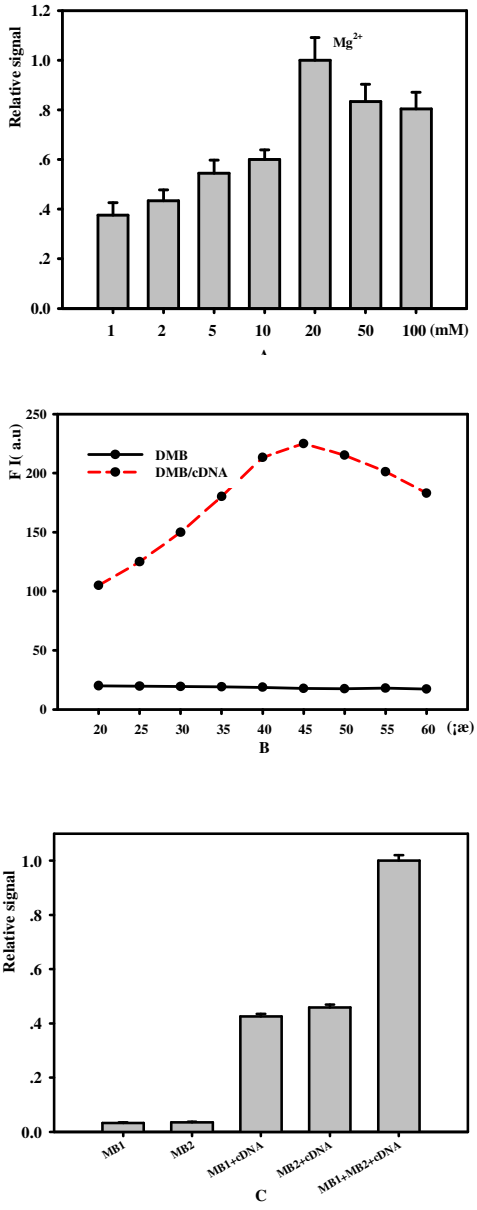


Fig.3. A: specificity analysis of VEGF mRNA detection based on the dual molecular beacons. [DMB]=100 nM, total RNA=3μg. [RNase H]=200U/mL.

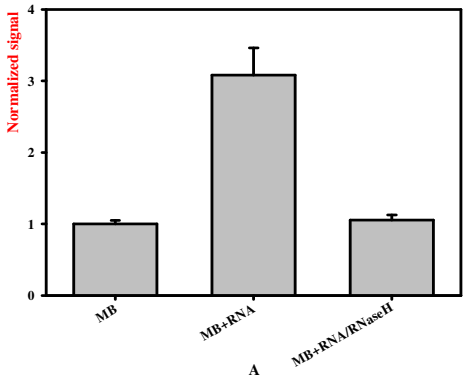
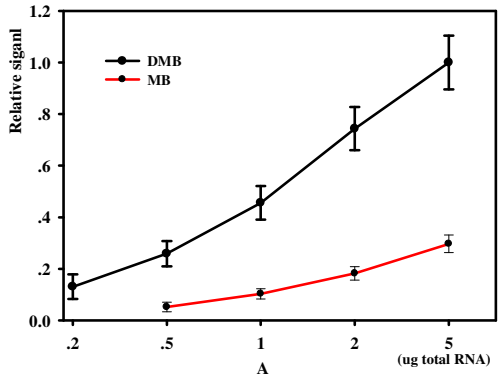
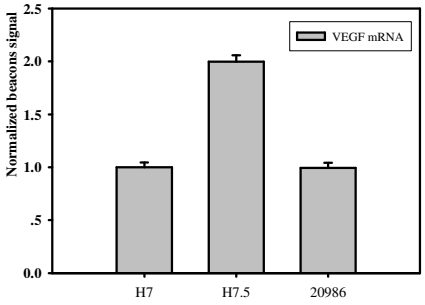


Fig.4. (A) Sensitivity comparison between single and dual beacons for VEGF mRNA assay. Total RNA was extracted from Huh 7 cells. [MB]=100nM. Detection of VEGF mRNA changes in different human hepatocarcinoma cells based on dual beacons (B) and qRT-PCR methods (C).



A



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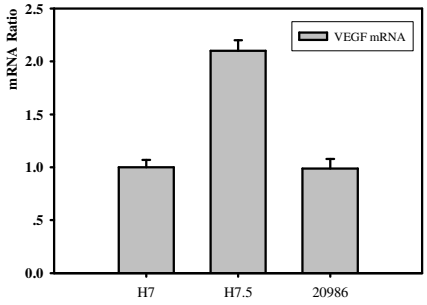


C

Fig.5. Detection of VEGF mRNA expression in human hepatocarcinoma cells Huh7, 20986 and Huh7.5 exposed to $CoCl_2$ or not based on beacons and qRT-PCR methods. [$CoCl_2$]=50,100 and 200 μM, respectively.

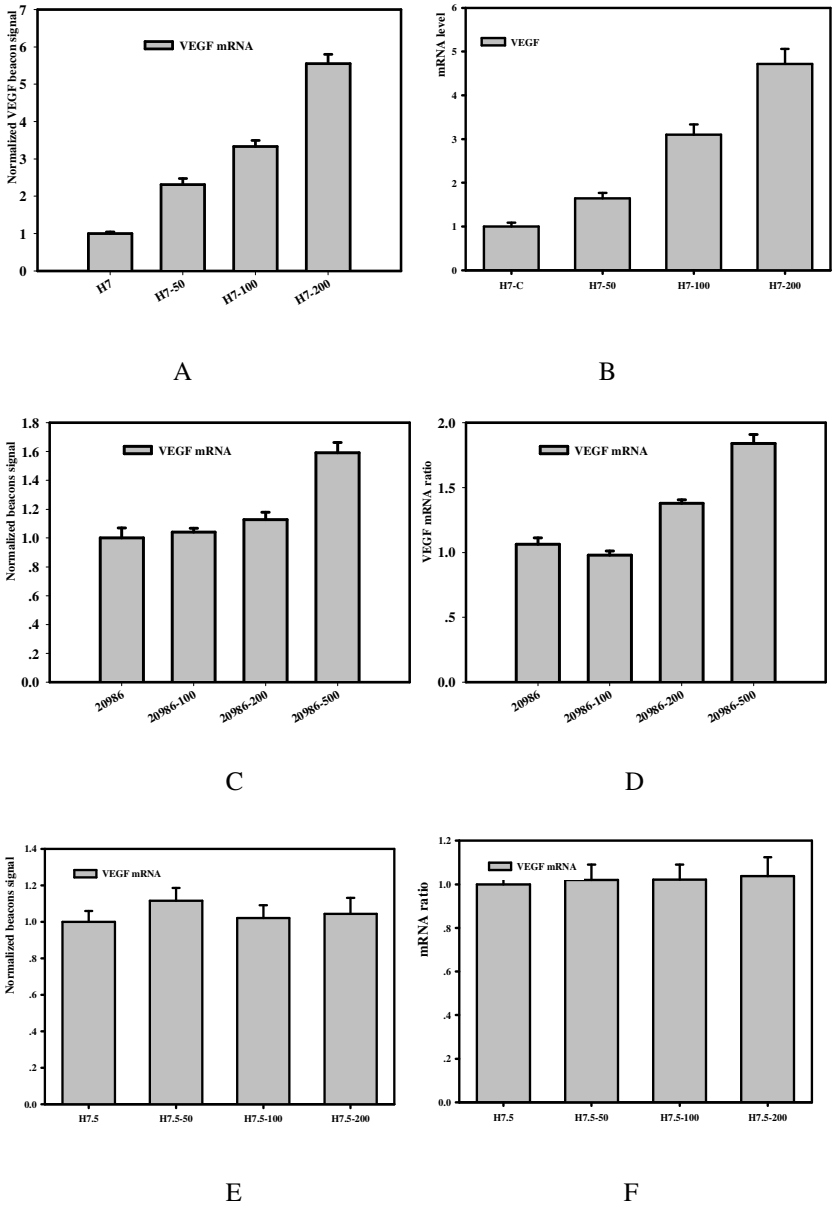


Fig.6 Detection of VEGF mRNA in different cells with H_2O_2 treatment or not based on dual beacons and qRT-PCR methods. Fluorescence signal expressed as a ratio to beacons signal from control cells. [H_2O_2]=500μM

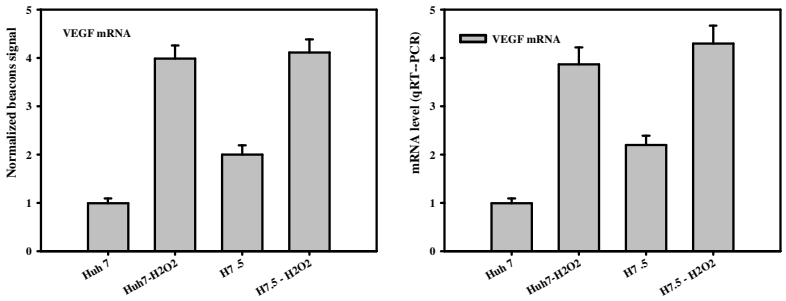
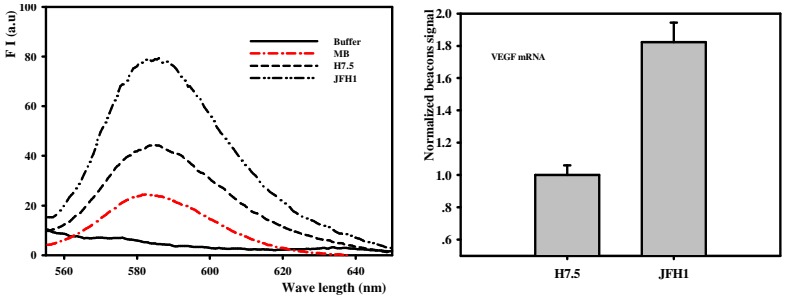


Fig.7 Changes in VEGF mRNA expression in human H7.5 cells exposed to HCV JFH1 infection. A: fluorescence signal from beacons hybridized to VEGF mRNA extracted from Huh7.5 cells with JFH1 infection (MOI=0.01, 24h) or not (control), expressed as ratio to control beacon signal (n =3). A: fluorescence wavelength scan curves from beacons hybridized to mRNA extracted from Huh7.5 cells with JFH1 treatment or not. B: fluorescence signal expressed as ratio to beacon signal from control cells.



A B