MARIANA MARTINS RIBEIRO

G-QUADRUPLEX FORMATION ENHANCES SPLICING EFFICIENCY OF *PAX9* INTRON 1

FORMAÇÃO DE G-QUADRUPLEX AUMENTA A EFICIÊNCIA DE *SPLICING* DO ÍNTRON 1 DO GENE *PAX9*

PIRACICABA
2014

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Thesis presents to the Piracicaba Dentistry School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Biology, Histology and Embriology.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Buco-Dental na Área de Histologia e Embriologia.

Orientador: Prof. Dr. Sergio Roberto Peres Line
Coorientador: Prof. Dr. Marcelo Rocha Marques

Este exemplar corresponde à versão final da tese defendida pela aluna Mariana Martins Ribeiro e orientada pelo Prof. Dr. Sergio Roberto Peres Line

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Abstract

G-Quadruplexes are secondary structures present in DNA and RNA molecules, which are formed by stacking of G-quartets (i.e. interaction of four guanines (G-tracts) bounded by Hoogsteen hydrogen bonding). Human *PAX9* intron 1 has a putative G-quadruplex-forming region located near exon 1, which is conserved among placental mammals. Using Circular Dichroism (CD) analysis, and CD melting we showed that this region is able to form highly stable quadruplex structures. Due to the proximity of the quadruplex structure to exon-intron boundary we used a validated double reporter splicing assay and real time PCR to analyze its role on splicing efficiency. The human quadruplex was shown to have a key role on splicing efficiency of *PAX9* intron 1, as a mutation that abolished quadruplex formation decreased dramatically splicing efficiency. The less stable, rat quadruplex had a less efficient splicing when comparing to human sequences. Additionally, the treatment with 360A, a strong ligand that stabilizes quadruplex structures, further increased splicing efficiency of human *PAX9* intron 1. Altogether these results provide evidences that G-quadruplex structures are involved in splicing efficiency of *PAX9* intron 1.

Key Words: G-Quadruplexes. RNA Splicing. *PAX9* transcription factor.

Resumo

G-Quadruplexes são estruturas secundárias presentes nas moléculas de DNA e RNA, os quais são formados pelo empilhamento de G-quartetos (interação de quatro guaninas (G-tratos) delimitadas por ligações de hidrogênio do tipo Hoogsteen. O intron 1 do gene *PAX9* humano tem um G-quadruplex formado na região localizada perto do exon 1, que é conservada entre os mamíferos placentários. Análises de Dicroísmo Circular (CD), e CD *melting* mostraram que estas sequências são capazes de formar estruturas quadruplex altamente estáveis. Devido à proximidade da estrutura quadruplex ao limite éxon-íntron foi utilizado um ensaio validado de *splicing* duplo repórter e PCR em tempo real para analisar o seu papel na eficiência de *splicing*. O quadruplex humano mostrou ter um papel chave na eficiência de *splicing* do íntron 1 do gene *PAX9*, já que uma mutação que aboliu a formação do quadruplex diminuiu drasticamente a eficiência de *splicing*. O quadruplex de rato, menos estável, mostrou menor eficiência quando comparado com sequências humanas. Além disso, o tratamento com 360A, um forte ligante que estabiliza estruturas quadruplex, aumentou ainda mais a eficiência de *splicing* do íntron 1 do PAX9 humano. Em conjunto estes resultados fornecem evidências de que as estruturas de G-quadruplex estão envolvidas na eficiência de *splicing* do intron 1 do gene *PAX9*.

Palavras-chave: G-Quadruplexes. RNA Splicing. *PAX9* transcription factor.

SUMÁRIO

DEDICATÓRIA

Dedico esse trabalho a minha

família por todo apoio e amor.

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INTRODUÇÃO

Sequências de ácidos nucleicos (DNA ou RNA) ricas em guanina (G) têm a capacidade de formar *G-quadruplex*, estruturas secundárias constituídas por empilhamento de G-quartetos, interação de quatro guaninas ligadas por pontes de hidrogénio do tipo *Hoogsteen*. Estas estruturas são estabilizadas por cátions monovalentes, assim, condições fisiológicas favorecem a sua formação. G-quartetos são conectados por *loops*, sequências intervenientes de nucleotídeos que não estão envolvidos em sua formação (Burge, Parkinson, Hazel, Todd, & Neidle, 2006; Gomez et al., 2004, 2010; Marcel et al., 2011). Além disso, pequenas moléculas denominadas G4 ligantes podem estabilizar ainda mais os *quadruplexes*. O ligante 360A (2,6-N, N0-methyl-quinolinio-3-yl-pyridine dicarboxamide) possui elevada afinidade por *quadruplexes* e intensa seletividade, não interagindo com DNA duplex (Granotier et al., 2005; Monchaud & P. Telaude-Fichou, 2008; Gomez et al., 2010;).

Em RNAs, os G-quadruplexes podem desempenhar diferentes funções. Quando presentes nas 5' UTR de RNAm, estas estruturas modulam a tradução (Gomez et al., 2010; Kumari et al., 2007). Quando presentes em íntrons, elas podem afetar *splicing* e padrões de expressão de genes, como hTERT(*human telomerase reverse transcriptase*), *BclxL*, *TRF2*, *FMRP* (*Fragile X mental retardation protein*) e *TP53* (Gomez et al., 2004; Didiot et al., 2008; Hai et al., 2008; Gomez et al., 2010; Marcel et al., 2011). Análises bioinformáticas mostraram um enriquecimento de sítios G-ricos *downstream* ao local de início da transcrição na fita de DNA *nontemplate*, concentrados no primeiro íntron. Estes elementos são conservados em algumas espécies, como o rato, galinha e sapo. Elementos G-ricos na extremidade 5' do intron 1 podem ser alvos estruturais a nível de transcrição ou processamento de RNA, já que duas proteínas hnRNP que estão envolvidas no processamento de RNA ligam-se a sítios que contém sequências de três ou mais guaninas, que compreendem mais do que metade da região G-rica em primeiros íntrons (J. McCullough & M. Berget, 1997; J. Mccullough & M. Berget, 2000; Eddy & Maizels, 2008).

Estudos anteriores demonstraram que as regiões ricas em G na extremidade 5'do primeiro íntron estão presentes em 48% dos genes humanos, incluindo o gene que codifica um fator de transcrição PAX9 (Eddy & Maizels, 2008). Esta região possui diversas repetições de guanina e é altamente conservada entre as espécies, sugerindo que esta sequência possa ter um papel regulador. Neste trabalho, mostramos que estas sequências G-ricas adotam uma estrutura de RNA G-quadruplex estável, que pode estar envolvida no *splicing* do íntron 1 do gene PAX9. Mutação nesta sequência, que impede a formação de quadruplex, reduziu drasticamente a eficiência de *splicing*.

CAPÍTULO 1: G-quadruplex formation enhances splicing efficiency of *PAX9* intron 1

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Abstract

G-quadruplexes are secondary structures present in DNA and RNA molecules, which are formed by stacking of G-quartets (i.e. interaction of four guanines (G-tracts) bounded by Hoogsteen hydrogen bonding). Human *PAX9* intron 1 has a putative G-quadruplex-forming region located near exon 1, which is present in all known sequenced placental mammals. Using Circular Dichroism (CD) analysis, and CD melting we showed that these sequences are able to form highly stable quadruplex structures. Due to the proximity of the quadruplex structure to exon-intron boundary we used a validated double reporter splicing assay and real time PCR to analyze its role on splicing efficiency. The human quadruplex was shown to have a key role on splicing efficiency of *PAX9* intron 1, as a mutation that abolished quadruplex formation decreased dramatically splicing efficiency. The less stable, rat quadruplex had a less efficient splicing when comparing to human sequences. Additionally, the treatment with 360A, a strong ligand that stabilizes quadruplex structures, further increased splicing efficiency of human *PAX9* intron 1.

Altogether these results provide evidences that G-quadruplex structures are involved in splicing efficiency of *PAX9* intron 1.

Key Words: Quadruplex. Splicing. *PAX9*.

Introduction

Guanine-rich nucleic acid sequences have the ability to form G-quadruplex (G4), which are secondary structures composed by stacking of G-quartets, interaction of four guanines (G-tracts) linked by Hoogsteen hydrogen bonding. These structures are stabilized by monovalent cations; thus, physiological buffer conditions favor their formation. G-quartets are held together by loops, intervenient sequences of nucleotides that are not involved in the quartets (1–4) (Figure 1A). These structures can be arranged in different forms, the intermolecular complexes are generated by association of 4 separate strands, or by dimerization of molecules that contain two G-tracts, while the intramolecular structures are composed by folding of one single strand (5).

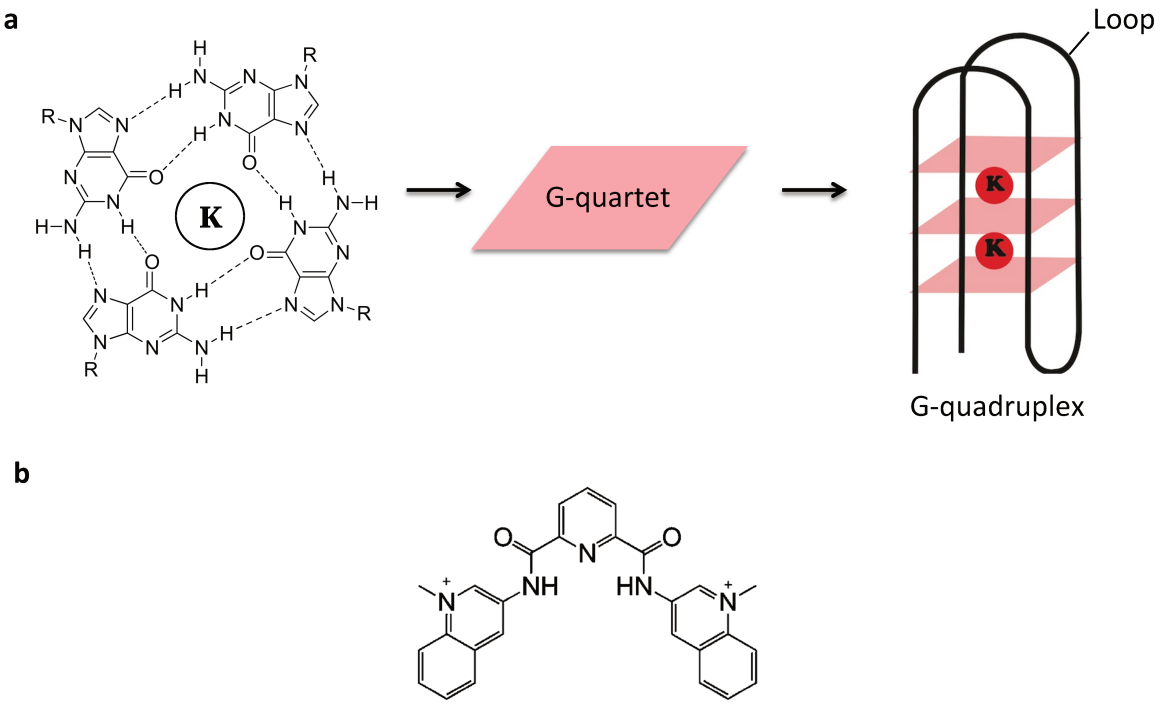

Figure 1. (a) The first panel shows the association of four guanines bonded by Hoogsteen bonding stabilized by monovalent cations composing a G-quartet (second panel). G-quadruplex are formed by the stacking of this G-quartets (third panel). **(b)** Chemical structure of the quadruplex ligand 360A.

At the RNA level these structures may play a number of roles. In 5' UTR of mRNAs, these structures have been shown to modulate translation (3, 12). When present in introns, they can affect the splicing and expression patterns of genes such as *hTERT* (human telomerase reverse transcriptase), *BclxL*, *TRF2*, *FMRP* (Fragile X mental retardation protein) and *TP53* (2, 3, 13–15). Bioinformatics analyses have shown an enrichment of G-rich sequences downstream transcription start sites on the nontemplate DNA strand, concentrated in the first intron. G-rich elements at the 5' end of intron 1 may provide structural targets at the level of transcription or RNA processing, since two hnRNP proteins that are involved in RNA processing binds to motifs containing runs of three or more guanines, comprising more than half of G-rich in first introns (9-11).

Previous studies have shown that G-rich regions at the 5'end of first intron are present in 48% of human genes, including the gene encoding the transcription factor PAX9. This region has several guanine runs, which is present in all known sequenced placental mammals suggesting that this sequence may have a regulatory role. In this work, we show, that these G-rich sequences adopt a stable G-quadruplex RNA structure which may be involved in the splicing of *PAX9* intron 1. Mutation in this sequence that impairs quadruplex formation reduced dramatically splicing efficiency.

Material and Methods

Synthetic oligonucleotides, plasmid and compound

The F21T labeled oligonucleotide (Table 1) was synthesized by Eurofins MWG Operon (Germany). All the unlabeled oligonucleotides (Table 1) and primers for qPCR (Table 2) were synthetized by Exxtend (Brazil). The DNA sequences synthesis was carried out by Genone (Brazil). The pBPLUGA vector was provided by Dr John E.G. McCarthy from National Biotechnology Research Centre (Germany). The quadruplex ligand 360A was a kind gift from Marie-Paule Teulade Fichou from Curie Institute (France).

Table 1. Oligonucleotides used in this work

Name	Sequence
F21T	5' FAM-GGGTTAGGGTTAGGGTTAGGG-TAMRA 3'
Hum	5`GGGAGGGAGGGAGGG 3`
Hum2	5`GGGAAGGGAGGGAGGG 3`
Rat	5`GGGAGGGAGCGAGCAGGCCAGGGCTAGAGGGAGGG 3'
Rat2	5`GGGAGCGAGCAGGCGAGGGCTAGAGGGAGGG 3`
Mutated	5' GAGAAGAGAGAGAGA 3'
NHEG27	5' TGGGGAGGGTGGGGAGGGTGGGGAAGG 3'
ds26	5' CAATCGGATCGAATTCGATCCGATTG 3'

Table 2. Primers used in Real time PCR

Name	Sequence
Spliced_F	5' GAGCAATGGAGCCAGCC 3'
Spliced_R	5' CAGCGGTTCCATCCTCTAG 3'
Control_F	5' AACCAGCCATCGCCATCT 3'
Control_R	5' GAGTCGTCGCCACCAATC 3'

Circular Dichroism Spectroscopy

Circular Dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Easton, MD, USA) with the temperature controlled by a Peltier-type control system PFD 425S. The oligonucleotides (Hum1-2, Rat1-2) were used at a concentration of 10 uM in a reaction volume of 400 ul in a quartz cell of 2 mm optical path length. Spectra were collected between 220–320 nm at 100 nm/min. The average scan for each sample was subtracted from the scan of the buffer (background) (5). Data were recorded in units of ellipticity ($deg.cm^2$ $dmol^{-1}$) versus wavelength. Before CD measurement, DNA samples were annealed at 95°C for 5 min, allowed to cool down overnight. The concentration of oligomer used was 10 uM; CD data represent ten averaged scans taken at room temperature in the presence of 10 mM of lithium cacodilate pH 7.2 buffer with 100 mM potassium.

CD melting

The CD melting profiles were collected at 263 nm. The thermal stability of quadruplexes was measured by recording the CD ellipticity at 263 nm as a function of temperature. The melting experiments were carried out on DNA samples at a concentration of 10 uM in buffer containing 10 mM of lithium cacodilate pH 7.2 buffer with 5 mM potassium, 100 ul of mineral oil was added to prevent evaporation. The temperature ranged from 20 to 100°C, the heating rate was 1°C per minute. The melting curves were plotted in Originpro 8.0 (MicroCal); melting temperature (T_m) was defined as the point of inflection in sigmoidal fitting.

Fluorescence Resonance Energy Transfer Competition assay

The F21T oligonucleotide, mimicking human telomeric motifs and double-labeled with FAM and TAMRA at either end, was used for this technique (Table 1). In the presence of 360A, this oligonucleotide folds into a stable *G-quadruplex,* thus allowing fluorescence transfer to occur. The melting of F21T was recorded by measuring fluorescence emission as a function of temperature. Different unlabeled oligonucleotides (Table 1) were used as competitors, including the *G-quadruplexes* of human, rat PAX9 intron and positive (known quadruplex forming sequence), and negative (a self-complementary 26-base-long oligonucleotide of mixed base content) controls (Table 1) (16).

Fluorescence melting curves were determined in a Roche LightCycler in a total reaction volume of 25 ul. For each reaction the final labeled oligonucleotide concentration was 0.2 uM, diluted in 10 mM lithium phosphate pH 7.2, supplemented with 10mM of KCl and 90 mM of LiCl. The LightCycler has one excitation source (488 nm) and the changes in fluorescence were measured at 520 nm. The oligonucleotides were first denatured by heating to 95°C for 5 min. They were then annealed by cooling to 25°C at a rate of 0.1°C s^{-1} (the slowest heating and cooling rate for the LightCycler). After this, 1uM of 360A were added and the samples were kept at room temperature overnight. The samples were maintained at 25°C for 5 minutes to equilibrate then melted by heating to 95°C at 0.1°C s^{-}

[1]. T_m values were obtained from the maximum values of the first derivatives of the melting profiles using excel (17, 18). The reactions were carried in triplicate and three independent experiments were performed.

Plasmid Construction for Splicing Efficiency assay

The possible role of *PAX9* intron 1 quadruplex in splicing efficiency was evaluated using a previously validated double reporter splicing assay (19). For this, three sequences based on PAX9 intron 1 were synthetized and cloned in the *Sal*I and *BamH*I sites of plasmid pBPLUGA, which contains the open reading frames for β-galactosidase and luciferase. pPAX9H contained the last 22nt from PAX9 exon 1, the entire intron 1 and the first 31nt from exon 2. pPAX9R had the same sequence as pPAX9H, replacing the quadruplex sequence of human by 59nt of the rat quadruplex forming sequence. pPAX9M had the 68nt human quadruplex forming sequence mutated (some guanines replaced by adenines), in order to abolish quadruplex formation (Figure 2 and Figure 2 supplementary material).

Double reporter splicing assay

In 12-well plates $1x10^5$ of Hek293 cells were transfected with 1 ug of plasmids with Calcium phosphate. After 24 hours, cells were treated with 10uM of 360A. Cells were harvested 48 hours after transfection, and β-galactosidase and luciferase activities were measured with Dual-Light®system (Applied Biosystems) and calculated using the ratio between luciferase and β-galactosidase, and normalized by dividing the ratio with the positive control. Six independent experiments were performed and the data analyzed by Student's *t*-test. The CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega) was performed with different compound concentrations (1, 5 and 10uM) in 96-well plates, each point in quintuplicate, as recommended by the manufacturer. Also, viable cells were counted using Trypan blue dye in a hematocytometer (Figure 1, supplementary material).

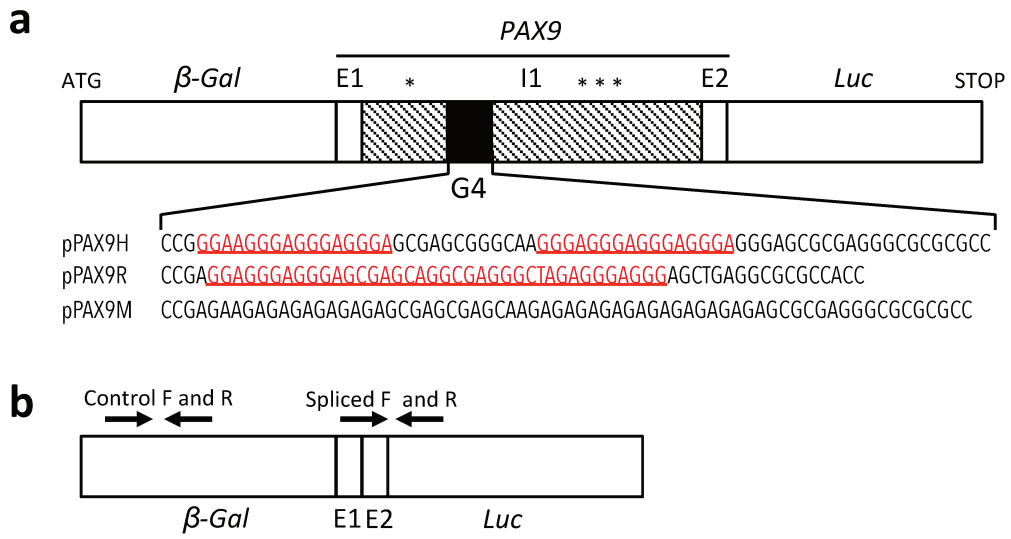

Figure 2. (a) Double reporter assay based on Nasim, 2002. The system contains reading frames for β-galactosidase (β-gal) and Luciferase (Luc) separated by several termination codons (*). Three sequences based on PAX9 intron 1 were synthetized PAX9H contained 22nt from PAX9 exon 1 (E1), the entire intron 1 (I1) and 31 nt from exon 2 (E2). PAX9R has the same sequence as PAX9H, replacing the quadruplex sequence (G4) of human by 59 nt of the rat quadruplex forming sequence. PAX9M with the 68 nt human quadruplex forming sequence mutated (some guanines replaced by adenines), in order to abolish quadruplex formation. Oligos Hum1 and 2, Rat1 and 2 are shown in red. In this system, the protein β-galactosidase is expressed constitutively, luciferase protein is only expressed if the upstream intron, that contains several stop codons, is removed. In this manner, the ratio of β-galactosidase and luciferase activities should indicate the proportion of mRNA that is spliced. Complete splicing would result the highest ratio of luciferase to β-galactosidase. **(b)** Scheme showing position of primers for qPCR analysis.

qPCR analysis

Total RNA from cells was isolated using Trizol®Reagent (Life Technologies). The RNA was treated with Turbo DNA-free™ Kit (Life Technologies) to remove any trace of DNA from samples. After this, reverse transcription was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). qPCR was performed using SYBR®Green PCR Master Mix (Life Technologies). Specific primers for spliced intron 1 PAX9 were used. The control was a set of primers that amplifies the β-galactosidase sequence. (Figure 2B, Table 2). Reactions were performed as follows, initial denaturation 3 min at 95ºC, then annealing 30 s at 95ºC, 30 s extension at 60ºC, with 40 cycles. All samples were run in triplicate on a 7300 Real-time system (Applied biosystems). Three independent

experiments were performed.

Statistical Analysis

The data were analyzed by Student's *t*-test using R (20).

Results

Characterization of G-quadruplex in intron 1 of *PAX9* pre-mRNA

The software Quadparser (21) was used for locating quadruplex sequence motifs, identifying putative G-quadruplex forming sequences in the first intron of *PAX9*. The predicted G-quadruplex sequences in intron 1 was located 29 bp downstream the 5' splice site.

To analyze whether this G-rich region could form G-quadruplexes we synthetized 4 RNA oligomers derived from human and Rat G-rich region of intron 1 (Table 1), and performed Circular Dichroísm. After this, we analyzed their thermal stability using CD melting.

CD spectra of these sequences in the presence of KCl displays the characteristic signature for the intramolecular parallel-type of G-quadruplex, exhibiting a negative peak at 240 nm and a positive peak at 263 nm (Figure 3A).

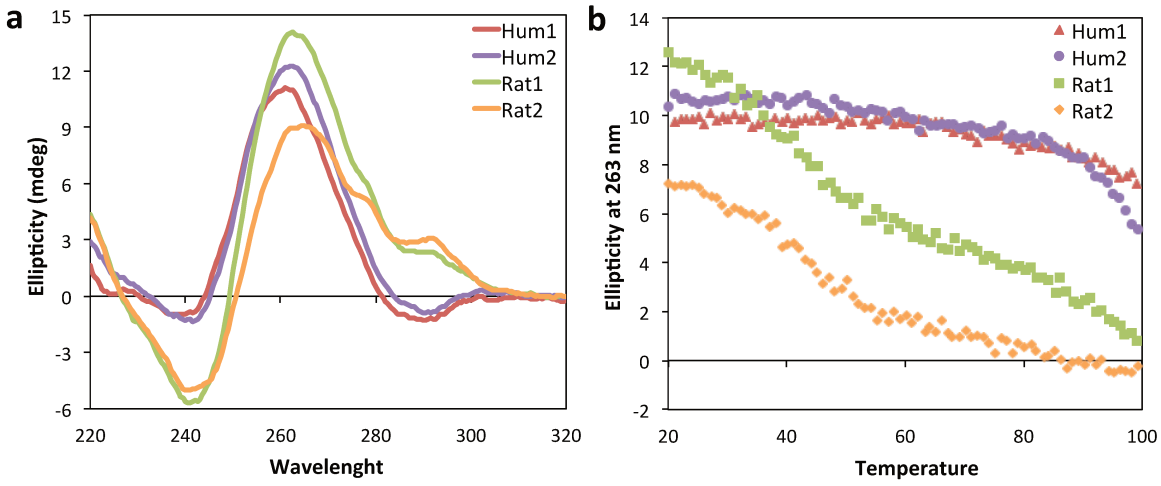

Figure 3. *PAX9* intron 1 quadruplexes characterization**. (a)** CD spectra of human and rat oligomers in the presence of 10 mM of lithium cacodilate pH 7.2 with 100 mM KCl. **(b)** CD melting of human and rat oligomers in the presence of 10 mM of lithium cacodilate pH 7.2 with 5 Mm KCl.

The melting profiles showed that these oligonucleotides adopt highly stable structures; as the human oligonucleotides (Hum1 and Hum2) were not able to melt in these ionic conditions. Rat oligonucleotides (Rat1 and Rat2) showed a T_m value of 47.79 and 42.88, respectively (Figure 3B).

All these results demonstrate that the G-rich sequences present in PAX9 intron 1 of human and rat are able to form quadruplex structures. Moreover, rat quadruplexes showed less stability than human quadruplex.

Effects of the ligand 360A on PAX9 intron 1 quadruplexes.

The compound 360 A has high affinity to G-quadruplexes showing stabilizing effects. (7). To demonstrate the affinity of 360A to PAX9 quadruplexes Fluorescence Resonance Energy Transfer (FRET) competition assay was used. (16)

An increased melting temperature (ΔTm) was observed after the addition of 360A due to the stabilization of the G-quadruplex. As the temperature rises, fluorescence emission increases due to unfolding of the G-quadruplex interrupting fluorescence transfer (the donor is distant to the acceptor fluorophore). Unlabeled oligonucleotides (3 and 10 uM strand concentration) were used as competitors to determine their capacity to displace 360A from F21T, reducing its melting temperature (2). Figure 4 shows that the positive control (27NHEG) and two human oligonucleotides were efficient competitors, dramatically decreasing F21T melting temperature in a concentration-dependent manner.

In contrast, rat sequences were less efficient as competitors. Mutated oligonucleotide and negative control (ds26) did not compete with the labeled oligonucleotide (F21T). Three independent experiments were performed. These results indicate that 360A compound could selectively bind to the quadruplexes formed in human PAX9 intron 1.

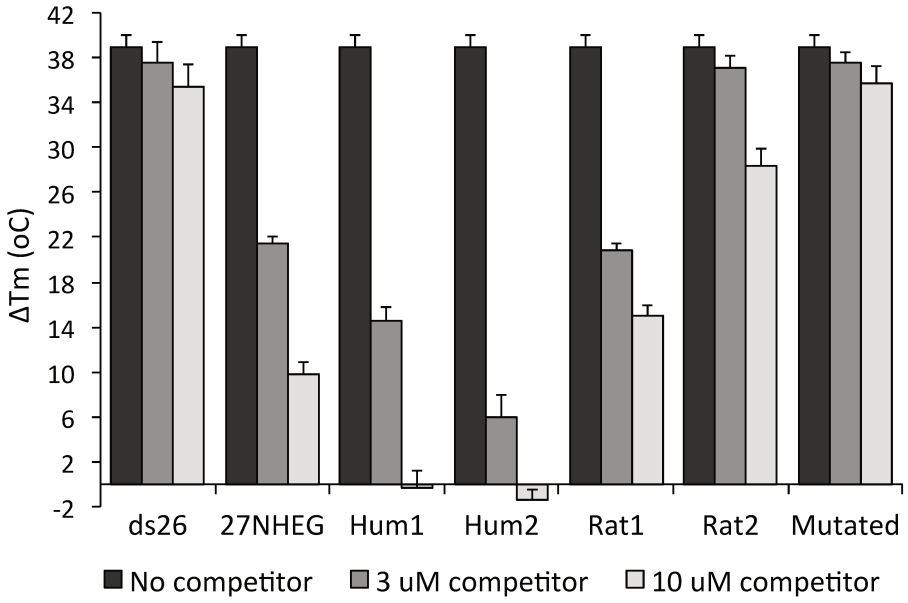

Figure 4. FRET compretition assay demonstrating the effect of G-quadruplex ligand 360A on the stability of PAX9 intron 1 quadruplexes. The ΔT_m was obtained by subtracting the melting temperature values of labeled labeled oligonucleotide F21T with or without 1uM of 360A. The sequences of labeled F21T and the competitor oligonucleotides are listed in table 1. Average of three independent experiments.

Role of quadruplex in splicing efficiency using double reporter assay

To demonstrate the ability of PAX9 intron 1 G-quadruplex to modulate splicing, a previously described double reporter splicing assay was used (19).

In this system, the protein β-galactosidase (without stop codon) is expressed constitutively. On the other hand, luciferase protein (without start codon) is only expressed if the upstream intron, that contains several stop codons in frame, is removed during the mRNA processing. In this manner, the ratio of β-galactosidase and luciferase activities should indicate the proportion of mRNA that is spliced. Complete splicing would give the highest ratio of luciferase to β-galactosidase (19).

To test the hypothesis that quadruplex can modulate splicing, we used three different sequences: The human sequence, which has highest stability, the rat quadruplex forming sequence and the human mutated sequence, which does not form quadruplex (as shown in FRET competition assay).

The measurements of β-galactosidase (Gal) and Luciferase (Luc) activities showed that cells transfected with plasmid pPAX9H and pPAX9R had much higher Luc/Gal ratios than pPAX9M (Figure 5A-C).

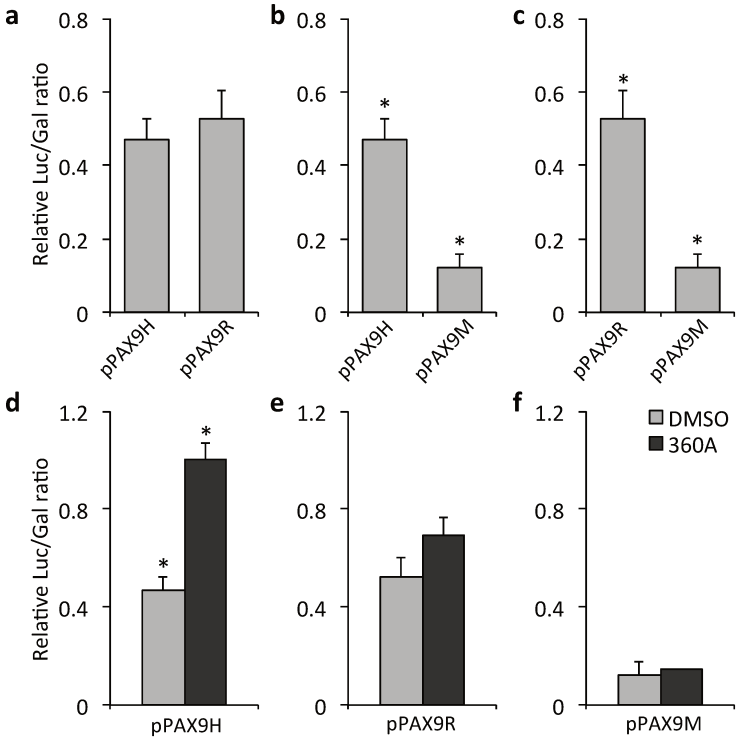

Figure 5. Analysis of reporter system to determine the splicing efficiencies *in vivo*. The reporter plasmids were transfected in HEK293 cells and the activities of β-galactosidase and Luciferase were measured and expressed as ratios normalized by positive control. The mutation in human intron 1 quadruplex decreased dramatically Gal/Luc ratio **(b,c)**. No statistical difference was observed between human and rat constructs ratio **(a)**. pPAX9H ratio increases considerable after treatment with 360A **(d)**. No significant effect of the ligand was observed in pPAX9R and pPAX9M **(e,f)**. Six independent experiments were performed. *$p < 0.05$.

Also, we investigated whether further stabilization by 360A (also showed in FRET analysis) would increase this modulation. The ligand 360A did not show toxicity at concentrations used (supplementary figure 1) however, it increased Luc/Gal ratio of pPAX9H (figure 6D). No effect on pPAX9R and pPAX9M was observed (Figure 5E,F).

qPCR analyses demonstrated that the expression of the spliced pPAX9R transcript was reduced by approximately 20% in relation to pPAX9H (Figure 6A) and 88% in pPAX9M (Figure 6B). The treatment with 360A increased splicing efficiency of pPAX9H in 60%

(Figure 6D). No effect of the ligand was observed in pPAX9R and pPAX9M.

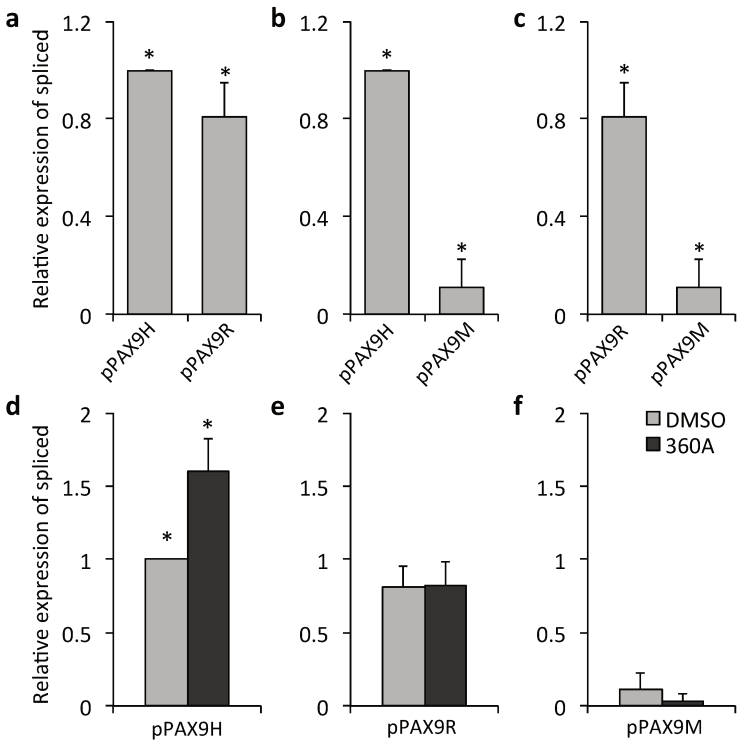

Figure 6. Real time PCR analysis of mRNA extracted from transfected HEK 293 cells. Cells were treated with 10 uM of 360A. The expression of spliced pPAX9R transcript was reduced by approximately 20% in relation to pPAX9H **(b)** and 88% in pPAX9M **(c)**. The treatment with 360A increased splicing efficiency of pPAX9H in 60% **(d)**. No significant effect of the ligand was observed in pPAX9R and pPAX9M **(e,f)**. Three independent experiments were performed. *$p < 0.05$.

Discussion

Several studies have shown that RNA as G-quadruplexes structures may have important roles in regulating splicing (4, 10, 11, 2). G-quadruplex structures in introns affect the splicing and expression patterns of genes such as hTERT, BclxL, TRF2, FMRP, and TP53 (4, 3, 14, 15, 2). In this work we show that the G-rich sequence of *PAX9* intron 1 is able to form a stable quadruplex.

Furthermore, we used a double reporter splicing assay to demonstrate the involvement of these structures in correct splicing. Using this system we could show that the human G-quadruplex is strongly related to the splicing efficiency of intron 1 PAX9, due to the increase of Luc/Gal ratio. qPCR analyzes showed that the less stable rat G-quadruplex had a smaller splicing efficiency than human sequences. The mutated human quadruplex, which abolished quadruplex formation, decreased dramatically splicing efficiency of *PAX9* intron 1. Finally, we used a potent G-quadruplex ligand to test whether this ligand could bind to these structures thus, increasing the effects observed. Using FRET competition assay, it was possible to observe that 360A could bind to human and rat quadruplexes, having more effect in human quadruplex, and no effect in mutated quadruplex. In addition, Luciferase-β-Galactosidase double reporter assay has shown that the ligand 360A increased the expression of spliced *PAX9* intron 1, this result, and FRET competition assay results, provide evidences that this ligand could bind to this quadruplex increasing its stability, thus increasing splicing efficiency of *PAX9* intron 1.

Previous studies have shown that structure and stability of intramolecular quadruplex is strongly influenced by the length and composition of the loops (5). Although these studies (22, 5) have shown that Telomeric Quadruplex forming sequences with TTA loops replaced by AAA caused a large decrease in stability of G4 structure. In this work we have demonstrated that, quadruplexes formed by loops with C were less stable than quadruplexes with A loops. This was observed in both CD melting and FRET experiments. Additionally, the Hum2 sequence demonstrated more affinity for the ligand 360A, this may be caused by an increase of stability by an extra adenine present in the looping (Table

1). It is worth mentioning that has been shown that adenine is the most frequent base in quadruplex loops composition in the human genome (1). Furthermore, Rat1 was able to displace the ligand 360A from the labeled oligonucleotide more efficiently than Rat2; this may be caused by an increase of stability of Rat 1 that has an extra G-tetrad (5 tetrads). The quadruplex Rat2 is composed by 4 tetrads rather than Rat1 composed by 5, loop sizes are basically the same (Table 1). This increasing effect in quadruplex stability by an extra G-tetrad has been previously noticed (23).

A previous study used tritiated-360A to investigate the behavior of this compound in various cell types, showing that this compound has a very potent selectivity toward G-quadruplex structures of the telomeric G-overhang (7). The ligand 360A (2,6-N,N0-methyl-quinolinio-3-yl-pyridine dicarboxamide), a member of a new family of pyridine derivatives that interact highly selectively in vitro with G-quadruplexes compared with double-stranded DNA (7, 6, 24), has been shown to inhibit telomerase activity (25, 26). This quadruplex ligand induces delayed growth arrest followed by massive apoptosis in various immortalized cell lines in direct correlation with telomeric instability (27). These results indicate that 360A has a preference over Telomeric G-quadruplexes. In the present work we provide evidences that the ligand could bind to *PAX9* intron 1 quadruplex sequences, extending the range of G-quadruplexes sequences stabilized by this ligand. Further, we showed that the induced stabilization by the ligand was strongly maintained in presence of excess of duplex DNA ds26 (negative control) and Mutated G-quadruplex sequence that could not form G-quadruplex structures. Non-displacement of the ligand from its fluorescent target was noticed with these sequences after FRET analysis, showing the extreme ligand selectivity for quadruplex structures, confirming earlier studies. (7, 6, 24, 3)

Splicing occurs in a complex composed by 5 small nuclear ribonucleoproteins (snRNP) and several protein factors called spliceosome (11). This complex assembles on the pre-mRNA. The correct splice site recognition is crucial for the correct splicing of introns and may

require *cis*-acting elements. Computer analyses were performed to search for these elements involved in splice site recognition revealed that the first 50 nt of human introns are rich in guanine and cytosine residues (28). Several studies have shown the involvement of G rich regions near 5' end of introns in splicing regulation (29, 13, 10, 11, 30, 2). β-tropomyosin intron 6 exhibits 6 repetitions of motif (A/U)GGG, that acts as an enhancer of the splicing process. Mutation in these motifs decreases splicing efficiency in ~75% (29). The second intron of the human alpha-globin gene also contains multiple G triplets near 5'end. Mutation of this triplets reduced in vivo splicing of the alpha-globin intron from near 100% to about 45%. Human *PAX9* intron 1 has eleven (A/U)GGG repeats. Replacing human for the rat sequence, which has only 5 GGG repeats caused a decrease of 20% in expression of spliced *PAX9* intron 1. This result may be due to the fact that Rat quadruplexes have lower stability than human quadruplex. This result confirms what has been seen in FRET competition assay, where the ligand showed less interaction with Rat1 and 2 quadruplex sequences compared to Hum1 and 2. Replacing human G-quadruplex intron 1 structure by mutated sequence where the guanines were changed by adenines abolishing quadruplex formation decreased dramatically (88%) the expression of spliced *PAX9* intron 1. It is well known that sequences containing stretches of guanines have the potential to fold into G-quadruplex structures. 360A, a ligand that selectively binds to quadruplex was used to demonstrate that this regulation is dependent on the quadruplex formation. This treatment has increased the splicing efficiency of *PAX9* intron 1 even further (60%). 360A treatment of Rat and Mutated sequences had no influence in splicing efficiency.

In conclusion, these results provide evidences that G-quadruplex structures formed in this intron are involved in splicing efficiency and the ligand 360A has higher affinity and selectivity for human G-quadruplex sequences, modulating human PAX9 intron 1 splicing.

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CONCLUSÃO

Os resultados demonstram que as regiões G-ricas localizadas próximas a extremidade 5' do íntron 1 do gene *PAX9* são capazes de formarem quadruplexes estáveis que interagem com o ligante 360A. Além disso, foi demonstrado que essas estruturas são capazes de aumentar a eficiência de *splicing* do íntron 1 do gene *PAX9.*

APÊNDICE

Supplementary material

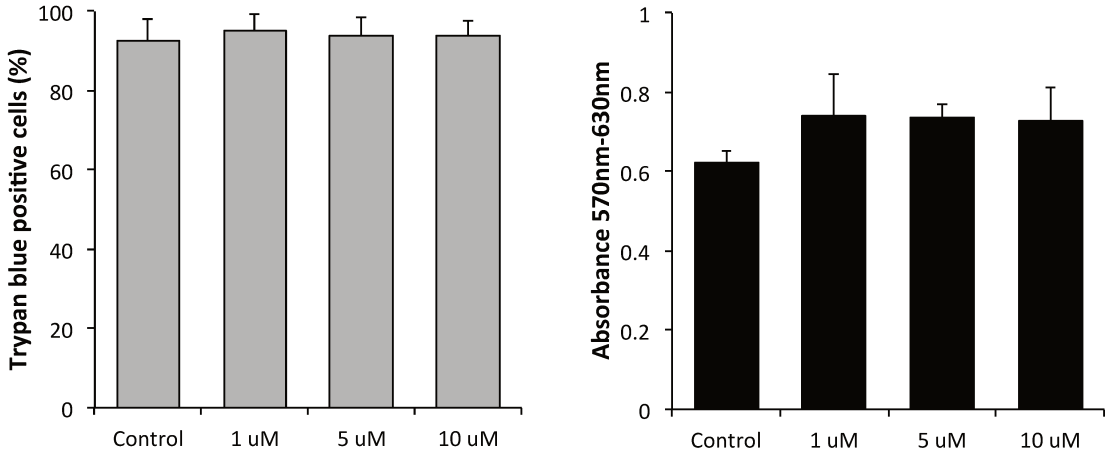

Figure 1. Cell viability under 24 hours 360A ligand treatment (a) Cell viability analysis of HEK293 cells by trypan blue in response to 24 hours of 1 – 10 uM of 360A ligand. (b) MTT assay measured by absorbance using celltiter 96® Non-Radioactive cell proliferation Assay (Promega). These two analyses indicate that 360A has no citotoxic effect on HEK293 cells at these concentrations

a) pPAX9H

GGTGTACTGCTCGGAGCAATGGgtgagtggcggcggggactctgtcagagccgggaagggagggagggagcg
agcgggcaagggagggagggagggagggagcgcgagggcgcgcgccactaggcgctcacattctctattgact
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ccccggtgcgtagggaggcccagtggcaggcagctgtcccaagcagcgggtgcgcgtccctgcgcgctgtgtg
ttcattttgcagAGCCAGCCTTCGGGGAGGTGAACCAGCTGGG

b) pPAX9R

GGTGTACTGCTCGGA**GC**AATGGgtgagtggcggcggggactctgtcagagccgaggagggagggagcgagca
ggcgagggctagagggagggagctgaggcgcgccaccactaggcgctcacattctctattgactttgctcgtg
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gtagggaggcccagtggcaggcagctgtcccaagcagcgggtgcgcgtccctgcgcgctgtgtgttcattttg
cagAGCCAGCCTTCGGGGAGGTGAACCAGCTGGG

c) pPAX9M

GGTGTACTGCTCGGAGCAATGGgtgagtggcggcgggggactctgtcagagccgagaagagagagagagagcg
agcgagcaagagagagagagagagagagagagcgcgagggcgcgcgccactaggcgctcacattctctattgact
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cccggtgcgtagggaggcccagtggcaggcagctgtcccaagcagcgggtgcgcgtccctgcgcgctgtgtgt
tcattttgcagAGCCAGCCTTCGGGGAGGTGAACCAGCTGGG

Figure 2. Constructions used in this work. Exons are in blue, quadruplex forming sequences are highlighted in yellow. **(a)** pPAX9H contained the last 22nt from PAX9 exon 1, the entire intron 1 and the first 31nt from exon 2. **(b)** pPAX9R has the same sequence as pPAX9H, replacing the quadruplex sequence of human by 59nt of the rat quadruplex forming sequence. **(c)** pPAX9M has the 68nt human quadruplex forming sequence mutated (some guanines replaced by adenines), in order to abolish quadruplex formation

ANEXO 1

DECLARAÇÃO

As cópias de artigos de minha autoria ou de minha coautoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada "Formação de DNA quadruplex aumenta a eficiência de *splicing* do íntron 1 do gene *PAX9*", não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

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ANEXO 2

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