

Inhibition of human-type 1 3 β -hydroxysteroid deshydrogenase/ Δ^5 - Δ^4 -isomerase expression using siRNA[☆]

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Abstract

Specific inhibition of type 1 3 β -HSD is of particular interest since it will allow us to control the formation of androgens and estrogens in peripheral target tissues without affecting type 2 3 β -HSD, which is responsible for the biosynthesis of glucocorticoids and mineralocorticoids in the adrenals. The high homology between types 1 and 2 3 β -HSD is a major difficulty in the development of specific inhibitors through classical chemical synthesis. In this report, we describe the use of small interference RNA (siRNA) to specifically inhibit human type 1 3 β -HSD. We have constructed three DNA vector-based RNAi vectors that allow us to produce three RNA duplexes of 21 nucleotides targeting three different coding regions of human type 1 3 β -HSD mRNA. The resulting constructs were co-transfected into HEK-293 cells with a vector expressing type 1 3 β -HSD. The results indicate that while the two duplexes that target sequences in the 5'-region do not have a strong inhibitory effect, the duplex that targets the 3'-region efficiently inhibits 3 β -HSD activity. Up to 98% inhibition has been observed. To our knowledge, this is the first report showing successful inhibition of steroidogenic enzymes using siRNA technology.

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1. Introduction

The enzyme 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 β -HSD) which catalyzes the transformation of pregnenolone into progesterone, 17 α -hydroxypregnenolone into 17 α -hydroxyprogesterone, 5-androstene 3 β -17 β diol into testosterone and dehydroepiandrosterone (DHEA) to 4-androstenedione [1] is a crucial enzyme responsible for the formation of all active steroid hormones, namely glucocorticoid, mineralocorticoid, progesterone, androgens and estrogens [2]. In humans, two types of 3 β -HSD were isolated [2,3]. They share very high homology (93.4% amino acids identity) and are expressed in a tissue-specific manner [4]. Type 1 is expressed in the placenta, skin and mammary gland whereas the type 2 gene

encodes an enzyme differing from type 1 in 12 amino acids and expressed predominantly in the gonads and adrenals [3].

RNA interference (RNAi) represents a new technology that brings the possibility of specific gene silencing through mRNA degradation. RNAi is a highly conserved gene silencing mechanism that uses double-stranded RNA (dsRNA) as a signal to trigger the degradation of homologous mRNA. The mediators of sequence-specific mRNA degradation are 21- to 23-nucleotide small interfering RNAs (siRNAs) [5]. These siRNA duplexes are generated by ribonuclease III cleavage either from longer dsRNAs or from a RNA hairpin resulting from DNA vector transcription. Therefore, siRNA chemically synthesized duplexes can be introduced directly to trigger specific gene silencing in mammalian cells. In cells, siRNA are unwound by a helicase activity associated with a multiprotein complex known as the RNA-induced silencing complex or RISC. The strand that is complementary to the targeted mRNA is then used as primer by an RNA-dependent RNA polymerase (RdRP) to convert the cognate mRNA into dsRNA itself. This dsRNA form of mRNA then becomes a substrate for Dicer cleavage activity, which leads to the destruction of the mRNA [6].

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Although many chemical inhibitors of 3 β -HSD activity have been described [7–11], specific inhibition of type 1 3 β -HSD could not yet be found because of the high homology between 3 β -HSD types 1 and 2. In this report, we describe the use of DNA vector-based RNAi approach, which has the potential to specifically destroy high-homology mRNAs to inhibit type 1 3 β -HSD.

2. Materials and methods

2.1. Plasmid construction

Three single-stranded DNA 76-mer oligonucleotides encoding, in order, a 21 nt siRNA sense, a loop, and a 21 nt siRNA antisense, were selected according to analysis of the ORF using GenScript's siRNA design center. The siRNA target finder software was used to identify unique candidate siRNA target sequences in the 3 β -HSD type 1 cDNA sequence and siRNA construct builder software to build a small hairpin insert from siRNA target for the expression vector. These oligonucleotides were synthesized by an ABI-3900 and purified by polyacrylamide gel electrophoresis. Each oligonucleotide was annealed with corresponding complementary single-strand DNA oligonucleotides. The resultant dsDNA was inserted into the *Bam*HI–*Hind*III site of pRNA-H1.1/Hygro vector (GenScript) to generate three plasmids (pRNA-H1.1/Hygro-3 β HSD1.1, pRNA-H1.1/Hygro-3 β HSD1.2 and pRNA-H1.1/Hygro-3 β HSD1.3) under control of the H1 promoter. *Escherichia coli* XL1-Blues competent cells (Stratagene) were transformed with the resultant plasmids and these plasmids were purified using QUIAGEN Plasmid Maxi Kit.

2.2. Cell culture and transfection

Human embryonic kidney (HEK-293) cells obtained from ATCC (CRL-1573) were maintained in MEM (Invitrogen Life Technologies Inc.) supplemented with 10% (v/v) FBS (Wisent Inc.) at 37 °C under 95% air:5% CO₂ humidified atmosphere. One day before transfection, cells were plated in six-well falcon flasks to 5 × 10⁵ cells/well. Transfections of siRNA plasmids were performed using EXGEN 500 (Fermentas Life Sciences) according to the manufacturer's protocol. Forty-eight hours after transfection, assay of enzymatic activity was performed.

2.3. Assay of enzymatic activity

The enzymatic activity was determined using intact cells in culture as previously described [12]. Briefly, 0.1 μ M of the [¹⁴C]-labeled DHEA (Dupont Inc., Mississauga, Ontario, Canada) was added to six-well culture plates containing 2 ml of culture medium per well. At the same time, we added Epostane (1 μ M) in one well containing steroid as control for inhibitory effect. After 2 h of incubation, steroids were extracted twice with 1 ml ethyl-ether. The organic phases

were pooled and evaporated to dryness. The steroids were then solubilized in 100 μ l dichloromethane, and separated on thin layer chromatography (TLC) Silica gel 60 plates (Merck, Darmstadt, Germany) using the toluene–acetone (4:1) solvent system. Substrates and metabolites were revealed and quantified by a PhosphoImager Storm 860 system and identified by comparison with reference steroids.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from 1 × 10⁶ cells using Tri-reagent, a mixture of phenol and guanidine thiocyanate in a monophasic solution (Molecular Research Center Inc.) according to the manufacturer's protocol. Genomic DNA contamination was removed by total RNA extraction using phenol:chloroform:IAA, 25:24:1, pH 6.6 (Ambion) and purified using RNeasy MinElute Cleanup (Qiagen). Five micrograms of total RNA was converted to cDNA by incubation at 42 °C for 2 h with 200 U SuperScript II reverse transcriptase (Invitrogen) using oligo-d(T)₂₄ as primer in a reaction buffer containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 0.5 mM dNTPs. cDNA was purified with QIAquick PCR purification Kit (Qiagen). Quantification of mRNA levels was performed using a quantitative RT-PCR. The LightCycler Realtime PCR apparatus as well as reagents were from Hoffman-La Roche Inc. (Nutley, NJ, USA) and the FastStart DNA Master SYBR green kit (Roche Diagnostics) were used as described by the manufacturer. The reaction was performed using the amount of cDNA corresponding to 20 ng of initial total RNA. The conditions for the PCR reactions were denaturation at 94 °C for 15 s, annealing at 50–65 °C for 10 s and elongation at 72 °C for 15 s. The reaction was then heated for 3 s at 2 °C lower than the melting temperature of the DNA fragment. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signal. Annealing temperature was selected based on contamination levels and melting curve results. Oligoprimers that allow the amplification of approximately 200 bp were designed with the GeneTools software (Biotools Inc., Edmonton, Alberta, Canada) and their specificity was verified by blasting in the GenBank database. To avoid errors due to RNA and cDNA preparation and handling, we performed a first correction with a housekeeping gene, subunit O of ATPase (*Atp5o*), at each assay. The resulting values of mRNA expression levels were converted in copies/ μ g total RNA. Statistical analyses were performed as indicated using the JMP statistical software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Construction of siRNA expression vectors targeting type 1 3 β -HSD

Because siRNA target sequences do not give rise to equally potent inhibitory effects due to secondary structure

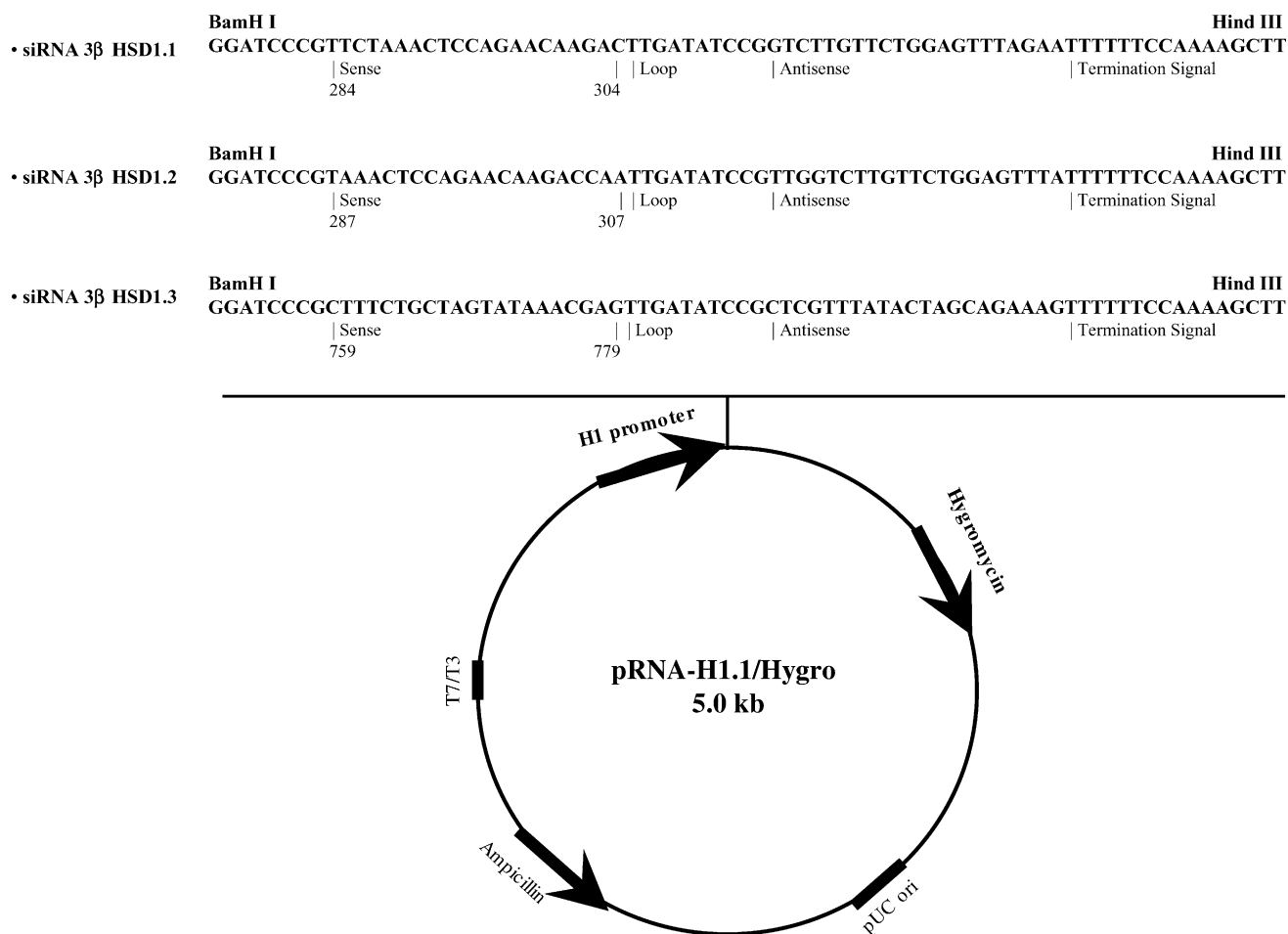


Fig. 1. Schematic representation of the structure of pRNA-H1.1/Hygro vector and sequences of type 1 3 β -HSD that will be inserted into the vector. The numbers indicate the corresponding positions of the selected 21-nucleotide sense fragments in ORF.

and other factors, we constructed three vector-based siRNA, namely pRNA-H1.1/Hygro-3 β HSD1.1, pRNA-H1.1/Hygro-3 β HSD1.2 and pRNA-H1.1/Hygro-3 β HSD1.3 that target different coding region of human type 1 3 β -HSD. Fig. 1 shows the three sequences that are selected using a program available from GenScript (www.GenScript.com). The sequences were synthesized and inserted in pRNA-H1.1/Hygro vector that is under the control of H1 promoter.

3.2. Inhibition of 3 β -HSD activity by siRNA

To assess the effectiveness of siRNA to inhibit 3 β -HSD activity, we co-transfected into HEK-293 cells a vector that expresses type 1 3 β -HSD (pCMV-3 β -HSD1) along with pRNA-H1.1/Hygro-3 β HSD1.1 and assessed the ability of the cells in culture to convert [¹⁴C]DHEA into [¹⁴C]4-androstenedione using thin layer chromatography. Cells without transfection with pRNA-H1.1/Hygro-3 β HSD1.1 were used as control of the activity and epostane, a well-known inhibitor of 3 β -HSD [13] was used as control for inhibitory effect. As illustrated in Fig. 2, pRNA-H1.1/Hygro-3 β HSD1.1 efficiently inhibits 3 β -HSD activity similar to that of epostane.

3.3. Inhibitory effect of various siRNA constructs

To determine the most efficient siRNA sequences, the three constructs described were co-transfected with 0.5 μ g of pCMV-3 β HSD1 vector as described above. As illustrated in Fig. 3, pRNA-H1.1/Hygro-3 β HSD1.1 shows the most potent inhibitory effect of 3 β -HSD activity while pRNA-H1.1/Hygro-3 β HSD1.2 and pRNA-H1.1/Hygro-3 β HSD1.3 exerted much weaker inhibitory effects. The results also suggest that the inhibitory effect of pRNA-H1.1/Hygro-3 β HSD1.1 is highly specific.

3.4. Effect of various siRNA constructs on 3 β -HSD mRNA level

To correlate 3 β -HSD-activity inhibition with a diminution of 3 β -HSD mRNA level, we measured enzyme mRNA levels by quantitative real-time PCR (Q-RT-PCR). This method allows for the monitoring of the efficiency of the PCR amplification efficiency by calculating the efficiency coefficient of corresponding standard curves. Q-RT-PCR analysis was conducted on total RNA extracted from

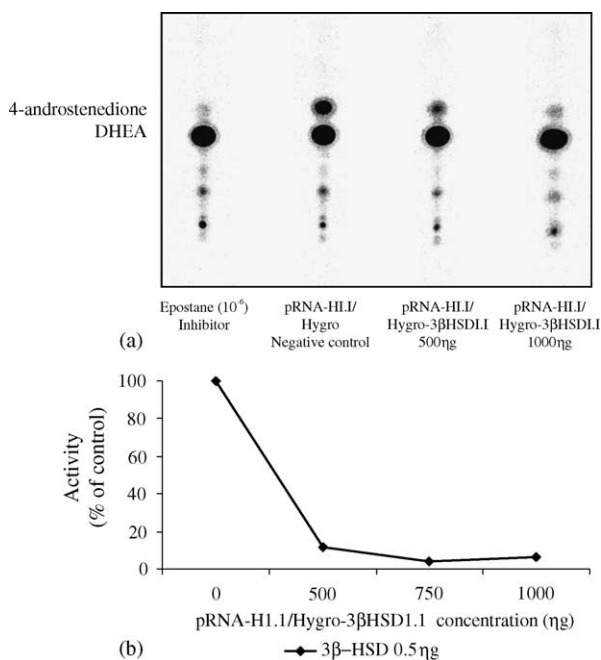


Fig. 2. Inhibition of 3β-HSD activity by transfection of pRNA-H1.1/Hygro-3βHSD1.1 in HEK-293 cells; 0.1 μM [¹⁴C]DHEA was used to assess 3β-HSD activity by TLC. (A) Substrate and product were quantified by a phosphoimager and the results are plotted in graph. (B) The activity of the control (100%) corresponds to 16 pmol/h/10⁶ cells.

HEK-293 cells co-transfected with pCMV-3β-HSD1 and pRNA-H1.1/Hygro-3βHSD1.1 or pRNA-H1.1/Hygro-3βHSD1.2 or pRNA-H1.1/Hygro-3βHSD1.3. As illustrated in Fig. 4, Q-RT-PCR analysis results show more 3β-HSD mRNA degradation with pRNA-H1.1/Hygro-3βHSD1.1 compared to pRNA-H1.1/Hygro-3βHSD1.3. These 3β-HSD mRNA levels correlate with the results obtained using thin layer chromatography (Fig. 3).

3.5. Specificity of siRNA inhibition

To evaluate the specificity of siRNA inhibition of 3β-HSD type 1 activity, we co-transfected into HEK-293 cells 1 ng of

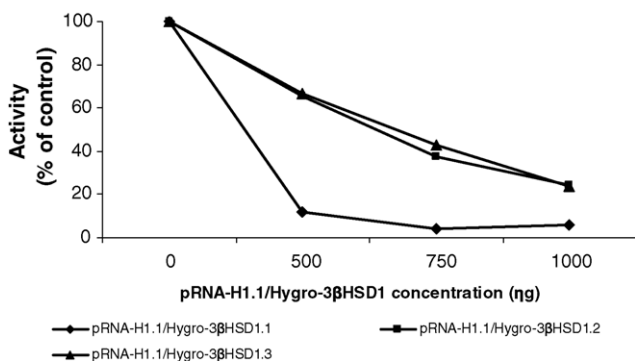


Fig. 3. Graph showing the inhibitory effect of various siRNA vectors. Inhibition of 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴-isomerase activity by pRNA-H1.1/Hygro-3βHSD1.1, pRNA-H1.1/Hygro-3βHSD1.2 and pRNA-H1.1/Hygro-3βHSD1.3 in HEK-293 cells was assessed using TLC and the results are plotted in graph as described above.

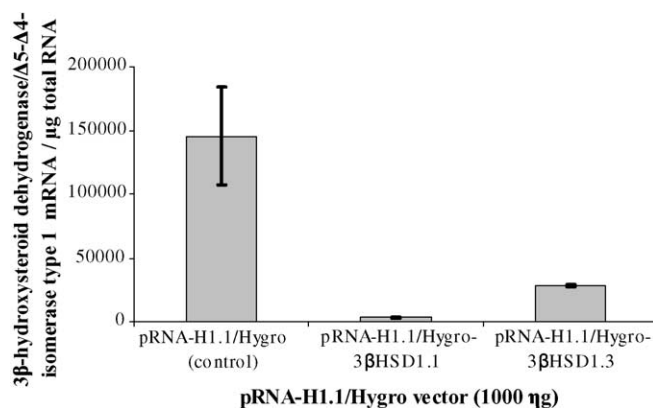


Fig. 4. Graph showing the effects of various siRNA vectors on 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴-isomerase type 1 mRNA level. The reduction of 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴-isomerase type 1 mRNA level by pRNA-H1.1/Hygro-3βHSD1.1 and pRNA-H1.1/Hygro-3βHSD1.3 in HEK-293 cells was assessed using quantitative real-time PCR (RT-PCR) and the results are plotted in graph as described above.

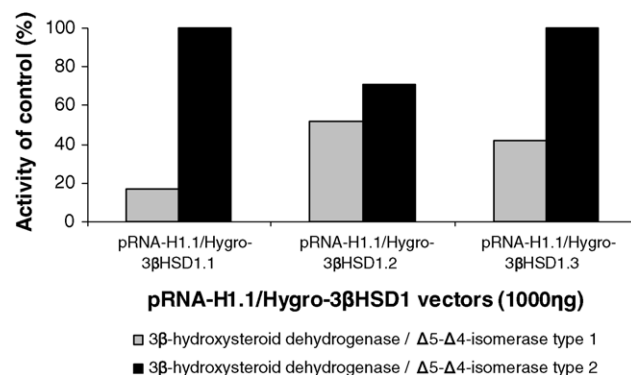


Fig. 5. Graph showing the specific inhibitory effect of siRNA vectors. Inhibition of 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴-isomerase type 1 and type 2 activities by pRNA-H1.1/Hygro-3βHSD1.1, pRNA-H1.1/Hygro-3βHSD1.2 and pRNA-H1.1/Hygro-3βHSD1.3 in HEK-293 cells were assessed using TLC and the results are plotted in graph as described above.

a vector that expresses type 2 3β-HSD (pCMV-3β-HSD2) along with the three constructs described, and assessed the ability of cultured cells to convert [¹⁴C]DHEA into [¹⁴C]4-androstenedione using thin layer chromatography as previously described. As illustrated in Fig. 5, pRNA-H1.1/Hygro-3βHSD1.1, pRNA-H1.1/Hygro-3βHSD1.2 and pRNA-H1.1/Hygro-3βHSD1.3 have no inhibitory effect on type 2 3β-HSD. These results suggest that the inhibitory effect of siRNA targeting of different coding regions in human type 1 3β-HSD is specific to 3β-HSD type 1.

4. Discussion

Using a DNA vector-based siRNA approach, we showed that type 1 3β-HSD activity could be efficiently inhibited by a construct that targets the sequence TTCTAAACTCCAGAA-CAAGAC while two other constructs that target the sequence

TAAACTCCAGAACAAGACCAA and CTTTCTGCTAG-TATAAACGAG show very low inhibitory effects. These sequences represent optimum siRNA target sequences that have been selected by the program available from GenScript. The specific reasons explaining the efficiency of the first sequence in inhibiting type 1 3β -HSD mRNA expression is still unknown. However, we observed that this sequence is found in the 5'-region while the others are located in the 3'-region. It is worth noting that much higher amount of pRNA-H1.1/Hygro- 3β HSD1.1 should be used as compared to pCMV- 3β HSD1. This could be due to the difference in power of the two promoters, since it is well known that pCMV is a very strong promoter. However, the general rule, by which much higher amount of siRNA mRNA as compared to the target mRNA is required for an efficient inhibition, could not be avoided.

Inhibition of type 1 3β -HSD gene expression is of particular interest because it is highly inducible by interleukin and is specifically expressed in many peripheral target tissues. It could, thus, control the intracrine formation of active hormones in these tissues. In the placenta, type 1 3β -HSD converts pregnenolone into progesterone and DHEA into 4-androstenedione, which is subsequently converted into estrone by aromatase, and then to estradiol by type 17 β -HSD. Progesterone and estradiol will participate in the cascade of events that initiates labor in humans [14]. In addition to placenta, type 1 3β -HSD is involved in the formation of androgens and estrogens in other human peripheral tissue, such as the skin, prostate and mammary glands. Specific inhibition of type 1 3β -HSD may have a potential to be used in the treatment of androgen- and estrogen-sensitive diseases, such prostate cancers, breast cancers as well acne and hirsutism. To our knowledge, this is the first report showing successful inhibition of steroidogenic enzymes using siRNA technology. Our results open a new field for studying inhibitory effects of steroidogenic enzymes using the promising siRNA technology.

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References

- [1] C. Readhead, R.A. Lobo, O.A. Kletzky, The activity of 3 beta-hydroxysteroid dehydrogenase and delta 4-5 isomerase in human follicular tissue, *Am. J. Obstet. Gynecol.* 145 (1983) 491–495.
- [2] V. Luu The, Y. Lachance, C. Labrie, G. Leblanc, J.L. Thomas, R.C. Strickler, F. Labrie, Full length cDNA structure and deduced amino acid sequence of human 3 beta-hydroxy-5-ene steroid dehydrogenase, *Mol. Endocrinol.* 3 (1989) 1310–1312.
- [3] E. Rheaume, Y. Lachance, H.F. Zhao, N. Breton, M. Dumont, Y. de Launoit, C. Trudel, V. Luu-The, J. Simard, F. Labrie, Structure and expression of a new complementary DNA encoding the almost exclusive 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase in human adrenals and gonads, *Mol. Endocrinol.* 5 (1991) 1147–1157.
- [4] Y. Lachance, V. Luu-The, H. Verreault, M. Dumont, E. Rheaume, G. Leblanc, F. Labrie, Structure of the human type II 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD) gene: adrenal and gonadal specificity, *DNA Cell Biol.* 10 (1991) 701–711.
- [5] S.M. Elbashir, J. Harborth, K. Weber, T. Tuschl, Analysis of gene function in somatic mammalian cells using small interfering RNAs, *Methods* 26 (2002) 199–213.
- [6] G. Hutvagner, P.D. Zamore, RNAi: nature abhors a double-strand, *Curr. Opin. Genet. Dev.* 12 (2002) 225–232.
- [7] A.M. Neville, L.L. Engel, Inhibition of alpha- and beta-hydroxysteroid dehydrogenases and steroid delta-isomerase by substrate analogues, *J. Clin. Endocrinol. Metab.* 28 (1968) 49–60.
- [8] G.O. Potts, J.E. Creange, H.R. Hardomg, H.P. Schane, Trilostane, an orally active inhibitor of steroid biosynthesis, *Steroids* 32 (1978) 257–267.
- [9] P. Komanicky, R.F. Spark, J.C. Melby, Treatment of Cushing's syndrome with trilostane (WIN 24, 540), an inhibitor of adrenal steroid biosynthesis, *J. Clin. Endocrinol. Metab.* 47 (1978) 1042–1051.
- [10] M. Takahashi, V. Luu-The, F. Labrie, Inhibitory effect of synthetic progestins, 4-MA and cyanoketone on human placental 3 beta-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase activity, *J. Steroid Biochem. Mol. Biol.* 37 (1990) 231–236.
- [11] V. Luu-The, M. Takahashi, Y. de Launoit, M. Dumont, Y. Lachance, F. Labrie, Evidence for distinct dehydrogenase and isomerase sites within a single 3 beta-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase protein, *Biochemistry* 30 (1991) 8861–8865.
- [12] V. Luu-The, Y. Zhang, D. Poirier, F. Labrie, Characteristics of human types 1, 2 and 317 beta-hydroxysteroid dehydrogenase activities: oxidation/reduction and inhibition, *J. Steroid Biochem. Mol. Biol.* 55 (1995) 581–587.
- [13] N.S. Pattison, M.A. Webster, S.L. Phipps, A.B. Anderson, M.D. Gillmer, Inhibition of 3 beta-hydroxysteroid dehydrogenase (3 beta-HSD) activity in first- and second-trimester human pregnancy and the luteal phase using Epostane, *Fertil. Steril.* 42 (1984) 875–881.
- [14] W.E. Rainey, B.R. Carr, H. Sasano, T. Suzuki, J.I. Mason, Dissecting human adrenal androgen production, *Trends Endocrinol. Metab.* 13 (2002) 234–239.