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Efficient *in vivo* knock-down of estrogen receptor alpha: application of recombinant adenovirus vectors for delivery of short hairpin RNA

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Abstract

Background: Adenovirus (Ad) mediated gene transfer is a well-established tool to transiently express constructs in livers of mice *in vivo*. In the present study, we determined the specificity and efficiency of Ad vectors expressing short hairpin (sh) RNA constructs to knock-down the estrogen receptor α (ER α).

Results: Two different shRNA constructs derived from the murine ER α coding sequence were designed (shER α). *In vitro*, transfection of three mouse cell lines with pSUPER-shER α constructs resulted in up to 80% reduction of endogenous ER α activity. A single mismatch in the target sequence eliminated the reduction of ER α activity, demonstrating the specificity of shER α . The subsequently generated Ad.shER α vectors were equally effective *in vitro*. *In vivo*, intravenous administration of Ad.shER α resulted in 70% reduced hepatic mouse ER α mRNA levels. Co-injection of Ad.shER α with an Ad vector containing a luciferase (luc) gene driven by an estrogen responsive element (ERE) containing promoter resulted in a significant (90% on day five) down-regulation of hepatic luciferase activity, as determined by non-invasive optical imaging. Down-regulation was sustained up to day seven post-injection.

Conclusion: Ad mediated transfer of shER α expression constructs results in efficient and specific knockdown of endogenous ER α transcription both *in vitro* and *in vivo*.

Background

Estrogen exerts various biological effects in numerous organs throughout the body and has been implicated in the pathophysiology of a number of diseases including breast cancer, osteoporosis and cardiovascular disorders. Most of the estrogenic effects are mediated via the two

known estrogen receptors, ER α and ER β . These estrogen receptors are ligand-dependent transcription factors that can modulate gene transcription directly but also indirectly. Thus far, there is a relative paucity in the description of the role of estrogen and estrogen receptors in specific organs. Most studies have been performed using

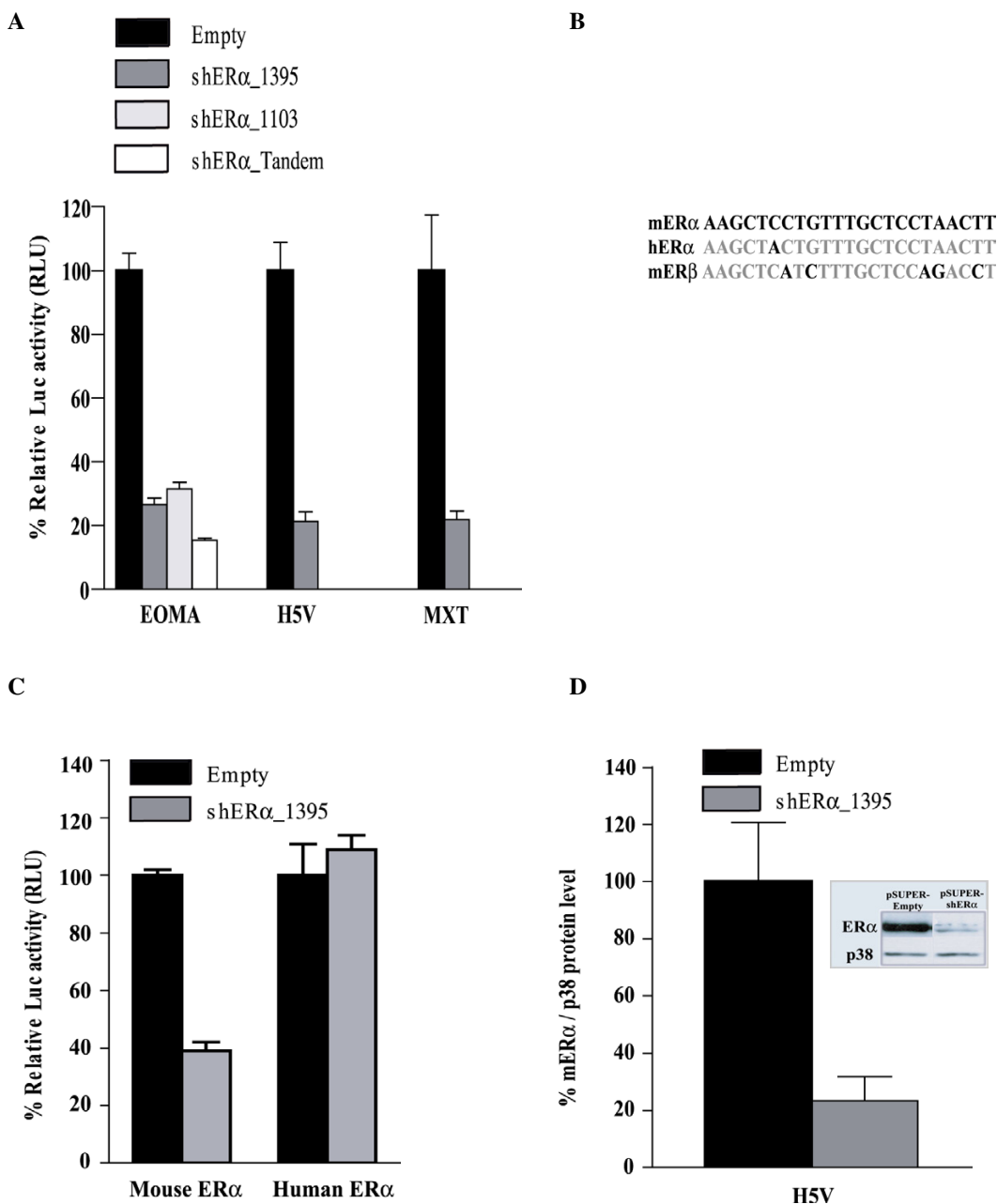


Figure 1

Affectivity and specificity of pSUPER mediated expression of shERα in mouse cell lines. (A+C) The indicated mouse cell lines were co-transfected with, ERE-Luc, CMV-LacZ, and pSUPER-empty, pSUPER-shERα₁₃₉₅, pSUPER-shERα₁₁₀₃, or pSUPER-shERα_{tandem}. Subsequently, the cells were treated 24 hours with 10⁻⁹ M 17-β-estradiol. Luciferase activity was measured 48 hours after transfection and after correction for LacZ expression, represented as the mean (n = 3) ± SD relative to the transfection with pSUPER-empty. (A) Endogenous mouse ERα mediated transcription in EOMA, H5V and MXT cells after introducing pSUPER +/- shERα. (B) The 19-nt target-recognition sequence of ERα₁₃₉₅ contains one mismatch with human ERα and five mismatches with the mouse ERβ sequence. (C) ERα mediated transcription in EOMA cells after over expression of either mouse ERα- or human ERα-expression vectors in presence of pSUPER empty or pSUPER shERα₁₃₉₅ (D) Western blot analysis of H5V cells co-transfected with pCMV-mERα and pSUPER-empty or pSUPER-shERα₁₃₉₅. The lysates were analysed by immunoblotting (insert-photo) with anti-mouse ERα and anti-p38. The intensity of the bands was quantified and normalized to cells transfected with pSUPER-empty. The relative ERα protein levels are presented (bar-diagram) as mean (n = 3) +/- SD.

non-tissue specific manipulation of ER signaling such as complete knockouts either via deletion of the estrogen receptor or via deletion of estrogen production by ovariectomy. The availability of tools to specifically address the role of ER signaling in individual tissues would thus fill a void.

Short synthetic duplexes of 21 nucleotides long RNA molecules can specifically inhibit gene expression in mammalian cells [1]. Because of their efficacy and specificity, siRNA molecules provide a powerful tool to dissect gene function. To expand the applicability of the siRNA approach, Brummelkamp and co-workers [2] have introduced vector-based siRNA expression systems. By directing the synthesis of shRNA via the polymerase-III H1 RNA gene promoter, effective siRNA molecules are formed intracellular after transfection of shRNA expression constructs. To further expand the applicability of the siRNA approach, recombinant retro- and adenoviral based vectors have been designed [3,4]. Of these, adenoviral vectors offer the advantage of highly efficient infection of a broad range of cells, independent of active cell division. Moreover, high titers can be obtained and intravenous injection results in efficient transduction of the liver.

The present study was designed to generate tools to address the role of ER α in a tissue- and time- specific manner. To this end, we have developed recombinant Ad vectors encoding shRNA's directed against mouse ER α (Ad.shER α). Introduction of shER α , either by transfection or by Ad mediated gene transfer into different murine cell lines, led to efficient sequence specific repression of ER mediated transcription. Furthermore, intravenously administration of Ad.shER α resulted in efficient reduction of hepatic ER α mRNA levels ($P < 0.005$) and ER α functionality.

Results

Efficient and specific knock-down of endogenous mER α in vitro: Transfection with pSUPER-shER α constructs

Three pSUPER-derived vectors [2] designed to drive expression shER α sequences were constructed. Two vectors contained sh sequences derived from the boundary of the DNA binding domain and the hinge region (shER α _1103), or from the ligand binding domain (shER α _1395) of mER α , respectively. A third expression vector contained both the shER α _1103 and shER α _1395 expression cassettes in series (shER α _tandem).

The efficiency of the shER α constructs for reducing endogenous ER α activity *in vitro* was determined using a luciferase reporter assay. For this purpose, the pSUPER-shER α _1395, pSUPER-shER α _1103, or pSUPER-shER α _tandem were transfected together with a reporter plasmid carrying a trimer of ERE plus TATA box upstream

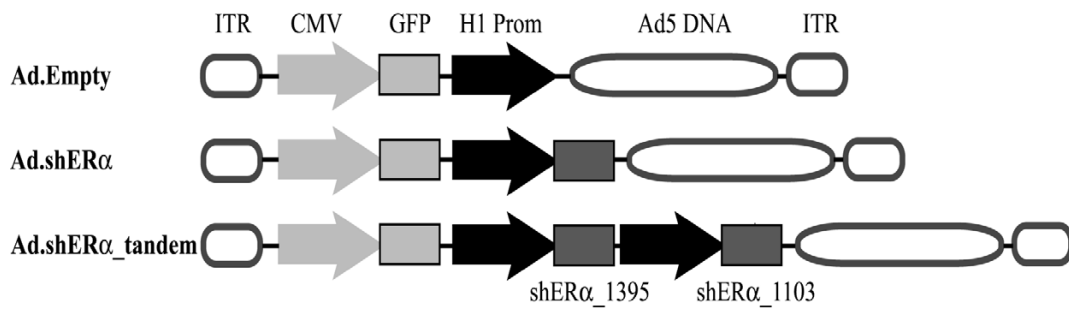
of luciferase (pERE-Luc) into endothelial cell lines (EOMA and H5V) and in mouse breast cancer cells (MXT). As shown in Figure 1A, upon transfection with shER α _1395, relative luciferase activity in lysates of all three cell lines was reduced by 70–80%. A similar result was obtained with shER α _1103 in EOMA's. In addition, the shER α _tandem expression construct proved to be more efficient than either of single shER α constructs alone in the EOMA cells, adding some 15% to the 70% reduction observed with shER α _1395 (Fig. 1A).

To evaluate the specificity of the shER α construct, shER α _1395 was introduced into EOMA cells overexpressing either mouse ER α or human ER α . The ER α _1395 target sequence contains only a single mismatch with the human ER α (Fig. 1B). Significant suppression of ER α mediated transcription was only observed in lysates of cells that were transfected with mouse ER α but not with human ER α (Fig. 1C). Thus, the observed effects of shER α _1395 are specific for mouse ER α . Moreover, changing a single nucleotide in shER α _1395 completely abolished the silencing effect (data not shown). By western blotting, the effect of shER α on ER α protein expression was studied (Fig. 1D). In the presence of shER α _1395, ER α protein levels were reduced to 33% as compared to control transfected cells. This reduction correlated well with our findings in the luciferase reporter assay. Thus, the observed inhibition of luciferase activity upon treatment with shER α _1395 or shER α _1103 is caused by reduced accumulation of mER α protein. All together, the shER α _1395 and shER α _1103 expression vectors are effective and specific in repression of murine ER α expression.

Knock-down of hepatic ER α expression in vivo: using Ad.shER α vectors

To repress ER α activity *in vivo*, Ad vectors expressing either shER α _1395 (Ad.shER α _1395), shER α _1103 (Ad.shER α _1103) or both (Ad.shER α _tandem) were generated (Fig. 2A). The H1 RNA promoter plus shER α expression cassettes were sub-cloned from the corresponding pSUPER into pAdTrack [5], which is engineered to co-express GFP enabling the tracking of infected cells. In addition, we constructed a control AdTrack plasmid, carrying only the H1 RNA promoter, which allowed for the generation of Ad.Empty. Prior to the evaluation of recombinant Ad vectors *in vivo*, we tested the functionality of the vectors *in vitro*. EOMA and MXT cells were transfected with pERE-luc, and subsequently infected with Ad.Empty or the Ad.shER α vectors. Fluorescence analysis indicated a near 100% infection percentage. The luciferase experiments (Fig. 2B) were comparable to those obtained with transfection of the pSUPER constructs (Fig. 1A): both Ad.shER α vectors repressed luciferase reporter activity up

A



B

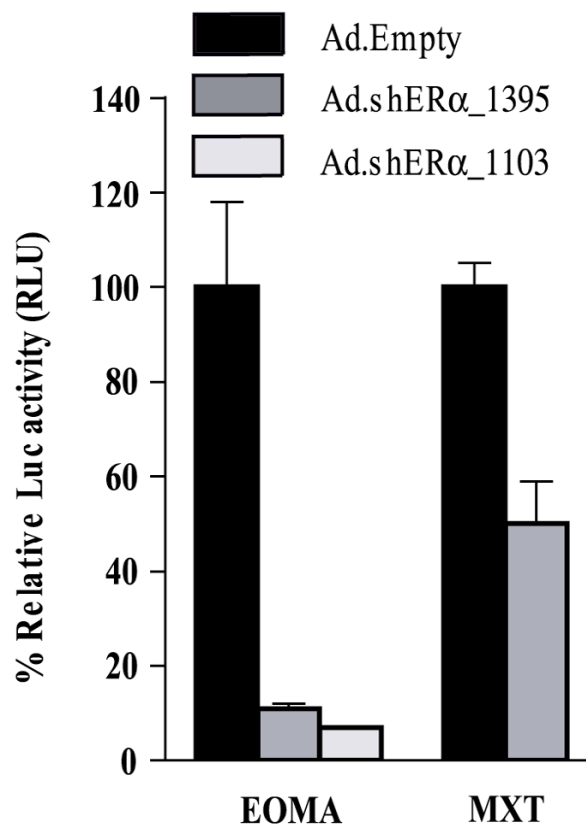


Figure 2

ER-mediated luciferase activity after Ad-mediated transfer of shERα *in vitro*. (A) Schematic representation of the recombinant Ad vectors, carrying GFP and shERα expression cassettes that were used in this study. (B) EOMA and MXT cells were co-transfected with pERE-Luc and pCMV-LacZ and then infected either with Ad.Empty, Ad.shERα_1395, or Ad.shERα_1103. 10⁻⁹ M Estrogen was administered for 24 hours. Luciferase activity was measured 48 hours after infection. Data represented as mean ± SD relative to infection with Ad.Empty.

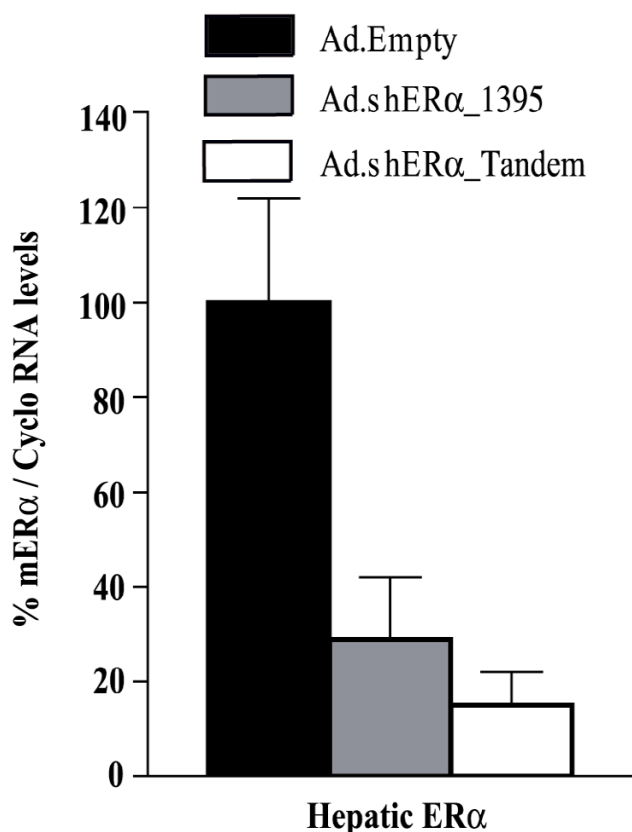


Figure 3
Hepatic ER α mRNA levels after Ad-mediated transfer of shER α *in vivo*. Male C57Bl/6 mice (n = 5) were injected with 4×10^9 pfu Ad.Empty, Ad.shER α _1395 or Ad.shER α _tandem. Livers were harvested Four days after Ad. administration and subjected to taqman analysis. The cyclophilin gene was used as internal standard. Data represented as mean \pm SD.

to 90%. Thus, Ad.shER α vectors were found to be fully functional with respect to repression of mER α activity.

We then proceeded with the application of our vectors *in vivo*. The Ad vectors (Ad.Empty, Ad.shER α _1395, or Ad.shER α _tandem) were injected in the tail vein of C57Bl/6 mice. This allowed examination of inhibition by shER α of endogenous hepatic mER α . Four days post-injection, animals were sacrificed, and the livers were studied for GFP expression and ER α mRNA level. Similar GFP expression patterns were observed in all groups, indicating equally efficient transduction (data not shown). ER α mRNA levels were studied by real time PCR analysis (Fig. 3). Administration of Ad.shER α _1395 reduced ER α mRNA levels 70%, whereas hepatic expression of shER α _tandem resulted in an 85% reduction.

Subsequently, we sought to examine the extent of shER α -mediated repression of hepatic mER α transcription activity. For this purpose, we constructed an Ad vector carrying the estrogen responsive luciferase reporter gene (Ad.ERE-Luc). First the estrogen-responsiveness of this vector was determined *in vivo* (Fig. 4A). Five days post-injection of 8×10^8 pfu Ad.ERE-Luc, the mice were injected s.c with increasing concentrations of estrogen, ranging from 0 to 50 μ g/kg. As shown in Fig 6, 6 hours post-injection, estrogen induced hepatic luciferase activity in a dose-dependent manner. Maximal stimulation was reached after applying 25 μ g/kg estrogen. Then, we determined to what extent Ad.shER α down-regulates the transcriptional activity of hepatic ER α . Ad.shER α together with Ad.ERE-Luc reporter vector was administered intravenously to C57Bl/6 mice. Luciferase expression was detected by a CCD camera in living mice. Without estrogen treatment, all mice exhibited the same basal expression of the reporter construct (data not shown). Administration of 5 μ g/kg estrogen, three and seven days after transduction with Ad.shER α _1103, resulted in a significant repression of hepatic ER α -mediated luciferase activity (Fig. 4B). These data were confirmed by measuring luciferase activity in liver extracts of mice that received estrogen (5 μ g/kg, sc) five days post-injection with Ad.ERE-Luc plus Ad.Empty or Ad.shER α _1103 (Fig. 4C).

We conclude that Ad-mediated introduction of shER α *in vivo* results in an almost complete repression of hepatic mER α mRNA levels, as well as mER α -mediated transcription activity.

Discussion

In this paper, we demonstrate that efficient silencing of mouse ER α can be achieved *in vitro* as well as *in vivo* by use of Ad-mediated transfer of shRNA molecules that target the ER α mRNA. Two independent shER α plasmid and Ad vector expression constructs were generated and shown to be effective in repressing endogenous ER α activity up to 80% in several different cell lines and *in vivo* (Fig. 1A, 2B and 3). In addition, a construct was made expressing both shER α sequences simultaneously. *In vitro* as well as *in vivo*, this construct was shown to be more effective (Fig. 1A and 3) than either of the two shER α constructs alone. Non-invasive optical imaging of living mice, allowed us to quantify shER α activity *in vivo*. Significant reduction of mouse ER α transcription levels were observed up to seven days post-transduction (Fig. 4B).

Thus far, bystander effects caused by shRNA constructs targeted to an unrelated gene have not been reported, and the specificity of the shER α _1395 construct was verified by the observation that human ER α , which has a single mismatch with the murine ER α target sequence, is not down-regulated (Fig. 1C). The number of mismatches

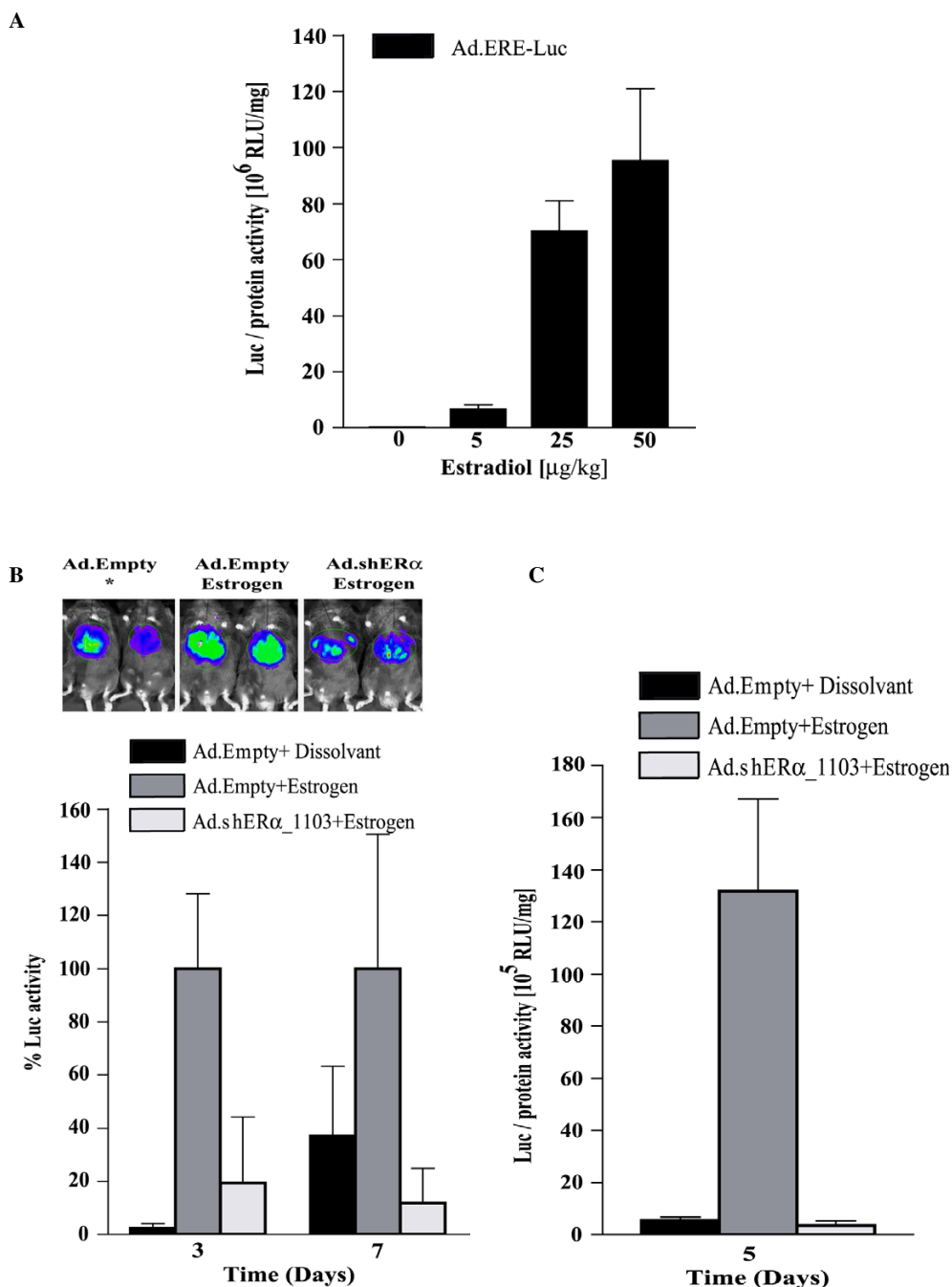


Figure 4

Hepatic ER α activity. (A) Male wt/ C57Bl/6 mice, pre injected with 5×10^8 pfu Ad.LacZ, were injected with 8×10^8 pfu Ad.ERELuc. Five days later, the recipients were treated for 6 hours with increasing amounts of estrogen (0–50 $\mu\text{g/kg}$, s.c). Then, the mice were sacrificed, and the livers were processed for luciferase assays. Luciferase activity is expressed as relative luciferase units (RLU) per mg total liver protein. (B) Male C57Bl/6 mice ($n = 5$) were injected with Ad.ERE-Luc (5×10^8 pfu) plus Ad.Empty or Ad.shER α _1103 (3×10^9 pfu). Three or seven days post-infection, the mice were injected with 5 $\mu\text{g/kg}$ estrogen. The (inset) photo shows the result of optical imaging of the bioluminescence at day three, the bar-diagram is a quantitative representation of hepatic luciferase activity at day three or day seven. (C) Male C57Bl/6 mice ($n = 5$) were co-injected with Ad.ERELuc (5×10^8 pfu) + Ad.Empty or Ad.shER α _1103 (3×10^9 pfu). Five days later, the mice received 0 or 5 $\mu\text{g/kg}$ estrogen. After 6 hours, the animals were sacrificed, and hepatic luciferase activity was determined. Luciferase activity is expressed as relative luciferase units (RLU) per mg total liver protein. Data represented as mean \pm SD.

with the murine ER β sequence totals five, making it unlikely that the shER α _1395 construct would affect expression of ER β . Similarly, the shER α _1103 construct has three mismatches with the human ER α and nine mismatches with murine ER β , making it unlikely that the shER α _1103 construct would interfere with either of them. A single mismatch in the shER α _1395 sequence did render the construct ineffective in down-regulating murine ER α (data not shown). Thus, the two independent shER α constructs described here are exquisitely suited to demonstrate that a specific effect is mediated by down-regulation of ER α expression and not by down-regulation of a related sequence.

A key challenge in the application of an shRNA based approach is efficient delivery of the shRNA constructs to target cells *in vitro* and *in vivo*. For application of shRNA *in vivo*, the sh oligopair, driven by H1 RNA polymerase [2] or U6 promoter [6], can be cloned in viral vectors. Here, the Ad vector was chosen as delivery vector, because of the relative ease of generation and amplification. Moreover, the natural tropism of Ad vectors for the liver enables the rapid analysis of the hepatic knock-down phenotype. Since Ad vectors predominantly infect the parenchymal cells [7,8], it is important to note that most abundant hepatic ER α expression was detected in parenchymal cells while ER α expression was barely detected in hepatic endothelial cells or kupffer cells (data not shown). This supported the rationale for application of shER α Ad vectors *in vivo*. Another interesting observation was that upon administration of 4×10^9 pfu Ad.shER α , an 85% reduction of ER α mRNA levels was obtained (Fig. 5), whereas co-injection of 3×10^9 pfu Ad.shER α _1103 with 5×10^8 pfu Ad.ERE-Luc resulted in an almost complete absence of luciferase activity (Fig. 4C). The ratio of Ad.ERE-Luc *versus* Ad.shER α _1103 (1:6) should ensure that all cells that were transduced by Ad.ERE-Luc also received Ad.shER α _1103. Thus, the remainder of ER α expression determined by real-time PCR likely reflects ER α expression in non-parenchymal and non-infected cells.

Thus far, relative few reports describe the application of Ad vectors as delivery system for RNAi *in vitro* [9-12]. Similarly, relative few studies on effective RNA interference *in vivo* using Ad mediated gene transfer have been reported [13-15]. One potential explanation for this relative paucity in the application of Ad mediated gene transfer for shRNA expression constructs could lie in the recent observations of Lu and Cullen [16], that VA1 non-coding RNA, expressed by wild type adenovirus is a potent inhibitor of RNA interference. However, replication-incompetent adenovirus vectors such as the vectors used in our study have been reported to express low levels of VA1 [16]. Moreover, in our hands the effect of the pSUPER shRNA construct shER α _1935 on reduction of ER α activity *in vitro* was not

affected by super-infection with the Ad.empty vector (data not shown). Thus, the Ad vectors applied in this study seem to have no or a minor inhibitory effect on the RNAi response *in vitro* and *in vivo*. Whether this effect is also insert specific and/or depends on the particular target gene remains to be investigated.

The strongest evidence for efficient reduction of endogenous hepatic ER α RNA levels *in vivo* was obtained by co-injection of Ad.ERE-luc and advanced non-invasive *in vivo* optical imaging. Administration of Ad.ERE-luc led to readily detectable levels of luciferase activity from day 3 up to day 7 and disappeared at day 10 (data not shown). In agreement with this, the Ad.shER α mediated knock-down effect was present at day three, five, and seven post-injection (Fig 4B). This represents a 4 to 5-day window of expression to determine the phenotypic effects of hepatic shRNA-mediated reduction of mRNA levels.

Conclusion

We have shown significant repression of hepatic ER α activity in mice utilizing Ad.shER α vectors. In addition, using advanced non-invasive optical imaging technology, the dynamics of the knock-down effect *in vivo* have been demonstrated. Thus, our data confirm that application of shRNA represents a powerful tool for targeted gene silencing. We conclude that Ad-mediated delivery of shER α constructs represents an elegant tool to gain more insight in the role of the hepatic ER α .

Methods

Plasmids

Two oligonucleotide pairs (mER α _1395: 5'-gatccccgctcctgtttgctcctaactcaagagagtaggagcaaacag-gagcttttggaaa-3' and 5'-agcttttccaaaagctcctgtttgctcctaactccttgaagtaggagcaaacag-gagcggg-3', mER α _1103: 5'-gatccccgaatagcctgtccttcaagaggacaagggcattc ttttggaaa-3' and 5'-agc ttttccaaaagaatagcctgtcctgtcctccttgaaggacaagggcattc-tcggg) were ordered (Eurogentec, United kingdom). The bold nucleotides correspond to nucleotides 1395–1418 and 1103–1120 of the mRNA mER α sequence (GenBank accession number NM_007956). The underlined nucleotides represent a *Bgl*III and a *Hind*III site. These oligo's were annealed and ligated between the *Bgl*III and *Hind*III sites of pSUPER-H1prom [2]. The pSUPER-shER α sequences were verified by restriction and sequence analysis (ABI 3700, LGTC, Leiden).

The H1prom plus or minus shER α were cloned from the pSUPER into the promoter less pAdTrack vector [5] by use of *Xba*I and *Xho*I restriction sites. The Ad.shER α _tandem construct was generated by ligation of H1prom-shER α _1103 between the *Not*I and *Kpn*I sites of pTrack-H1prom-shER α _1395.

The (ERE)₃TATA-Luc was cloned from pGL₃-basic as a *Clal*-blunt/ *KpnI* fragment in *EcoRV*- and *KpnI*- digested promoter less Shuttle vector (pShuttle) (He et al. 2509–14). The functionality of this construct was verified by transfection. hER α was cloned from pCMV5 (pCMV5-hER α) [17] as a *BamHI* fragment in the *BglII* digested pShuttle-CMV vector. The pcDNA3.1-mER α expression vector was provided by Larry Jameson [18] and subcloned as a *EcoRI*-blunt fragment in the *EcoRV* digested pShuttle-CMV vector.

Cell culture

The MXT⁺ (murine breast cancer) cell line was generously provided by Dr. Bernards. H5V (a murine endothelial cell line derived from heart), EOMA (murine hemangioma-derived micro vascular cell line) and MXT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum, 100 units/ml Penicillin, 100 μ g/ml Streptomycin and glutamax (Invitrogen) (Complete DMEM). PERC6 cells [19] were maintained in complete DMEM supplemented with 10 mM MgCl₂⁺. For large-scale production of recombinant Ad in PERC6 cells (Crucell, Leiden, he Netherlands), complete DMEM with 2% horse serum (Gibco) was used.

Luciferase reporter assays

Transient transfections were performed in triplicate in 12-wells plates (1.10⁵ cells per well) using Lipofectamine (Invitrogen). The effect of shER α on ER α mediated transcription regulation was determined by co-transfecting the cells with 100 ng of reporter construct (ERE)₃TATA-LUC and 500 ng expression vector pSUPER-shER α or an empty pSUPER control vector together with 100 ng pCMV-LacZ. After 24 hours, the cells were stimulated with complete DMEM containing 10⁻⁹M Estrogen for an additional 24 hours. The cells were lysed with reporter lyses buffer (Promega) and after centrifugation of 2 min, supernatant was used for determining β -galactosidase normalized luciferase activity by adding 100 μ l luciferyl-CoA (Promega) to 20 μ l of cell extract in a monolight luminometer (BD Biosciences). β -galactosidase was measured in a 96-well microtiter plate using the β -Galactosidase Enzyme Assay System in reporter lyses buffer (Promega). Absorbance at 450 nm was determined in a microplate reader. Luciferase activities were normalized for transfection efficiency with the β -galactosidase activity and expressed as a percentage relative to expression levels induced by endogenous estrogen receptor (ER). Expression of endogenous ER α in those cells was verified by real time PCR.

Western blot analysis

Immunoblotting procedures were as described previously [20]. H5V cells seeded in triplicate in 12-wells plate were

co-transfected with 20 ng pCMV-mER α and 500 ng expression vector pSUPER-shER α or an empty pSUPER control vector as described above. 28 hours post-transfection, the cells were lysed in 200 μ l of RIPA buffer (1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM EDTA) containing protease inhibitor (40 μ l/ml, Roche). Extracts were cleared by centrifugation (4°C, 14 000 g, 5 min), and protein content was determined using the BCA kit (Pierce). Protein samples were denatured (5 min, 90°C) and separated on SDS/PAGE by use of 8% gradient gels and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). Blots were stained with Ponceau S before blocking to verify equal loading and appropriate protein transfer. Membranes were blocked for 90 min in PBS, pH 7.4, containing 0.05% Tween 20 and 10% milk powder. Thereafter, membranes were incubated for 16 h at 4°C with abMC20, 1:1000 (mER α rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). After extensive washing with blocking buffer without milk powder or BSA, membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:5000 (Promega). Membranes were again extensively washed and bound peroxidase conjugates were visualized by enhanced chemiluminescence (ECL, Amersham) on a Lumilimager workstation. Additionally, filters were stripped by a 30 min incubation in 100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8 at 50°C, to proceed with the whole procedure as described above. However, now membranes were incubated for 16 h at 4°C with p-38 ab, 1:1000 (N-20, cs-728, rabbit polyclonal antibody, Santa Cruz Biotechnology, CA). Immunoblots were quantified using LUMIANALYST software on a Lumilimager (Boehringer-Mannheim).

Adenoviral vectors

Recombinant adenoviral plasmids were generated by homologous recombination of pAdtrack or pShuttle vectors with pAdEasy1 in BJ5183 cells as described previously [5]. Correct clones were propagated in DH5 α cells (Life Technologies). For the generation of the Ad.shER α vectors, Ad.Empty and Ad.ERE-Luc, PERC6 cells were transfected with 4 μ g Pac-I-linearized adenoviral construct using LipofectAMINE PLUS (Life Technologies). After 16 hours transfection medium was replaced by growth medium. Transfected cells were harvested at day seven post-transfection and after three freeze-thaw cycles the lysate was used for large-scale production of Ad vectors in PERC6 cells. Virus was purified by double CsCl centrifugation and subsequently dialysed as described previously [21]. Final yields as assessed by plaque assays on 911 cells were approximately 2 \times 10¹⁰ plaque forming units (pfu)/ml. The control virus (Ad.Empty) carries the green fluorescent protein (GFP) under control of cytomegalovirus promoter (CMV) and contained the H1prom.

Ad.shER α _1395 and Ad.shER α _1103 carry GFP under control of CMV and shER α _1395 or shER α _1103 under control of H1prom. Ad. shER α _tandem carries both shER α _1395 and shER α _1103 under control of their own H1prom. Ad.ERE-Luc does not contain CMV-GFP and its functionality was verified *in vitro* and *in vivo*.

Infection cells

24 hours before transfection, 1.10^5 cells per well were seeded into 12 wells-plate. Cells were transiently transfected by use of lipofectamine with a total of 450 ng of DNA per well (150 ng of reporter plasmid (ERE)₃TATA-LUC and 300 ng pCMV-LacZ). After 4 hours cells were infected with either Ad.shER α or control Ad.Empty (MOI 5.000). Additionally, they received 10^{-9} M estrogen for 24 hours. Cells were lysed in 300 μ l reporter lyses buffer. β -galactosidase and luciferase activity was determined as described above.

Animals and Ad Injection

The Ethics Committee for Animal Experiments of the Leiden University approved all animal work and the experimental protocols complied with the national guidelines for use of experimental animals. Male C57BL/6Jlco (Charles river, The Netherlands) were given a standard diet Chow (Hope Farms, Woerden, NL) and housed under standard conditions in conventional cages with free access to water and food.

Recombinant Ad, with a maximum of 4×10^9 pfu in 200 μ l of PBS, were administered by injection into the tail vein of mice at the age of 14 weeks. Within five days post-infection, mice were sacrificed; liver pieces were removed and immediately deep-frozen in liquid nitrogen and stored at -80°C .

Pharmacological treatment

The experiment was carried out in 12-wks old C57BL/6 male mice. To prevent sequestration of low doses of Ad.ERE-Luc by liver Kupffer cells, mice were pre-injected with Ad.LacZ (5×10^8 pfu) 4 hours before administration of 8×10^8 pfu Ad.ERE-Luc. 17β -estradiol (Sigma, E8875) was dissolved in sesame oil (Sigma). In the dose-response experiment, five days post-injection of Ad.ERE-Luc, 0, 5, 25 and 50 $\mu\text{g}/\text{kg}$ 17β -estradiol was injected for 6 hours. Then liver pieces were rapidly dissected and immediately deep-frozen in liquid nitrogen and stored at -80°C for further analysis.

Bioluminescent reporter imaging

The experiment was carried out in 12 wks old C57BL/6 male mice co-injected with Ad.ERELuc (5×10^8 pfu) plus either Ad.Empty or Ad.shER α (3×10^9 pfu). Bioluminescent signals (BLS) were performed at time 0 and at several days after 6 and 24 hours s.c injections of 5 $\mu\text{g}/\text{kg}$ 17β -

estradiol with the Xenogen IVIS imaging system (IVIS 100). The living mice were intraperitoneal (ip) injected with the luciferase substrate, luciferin, at a dose of 150 mg/kg body weight approximately 5 minutes before imaging. The mice were anaesthetized with isoflurane/oxygen and placed on the imaging stage. Total photon emission of each animal was acquired for 1 minute. Captured images were then quantified by using the Living Image software (Xenogen Corp, Alameda, CA) and the IGOR software (WaveMetrics Corp, Lake Oswego, OR). BLS from the region of interest (ROI) was expressed using the pseudo colour scale (Red most intense and Blue least intense luminescence) and the data were presented as the cumulative photon counts collected within each ROI. Because layers of tissue may limit photon emission from inner organs, the experiment was repeated. Of these mice the livers were rapidly dissected at day 5, 6 hours after 17β -estradiol administration, verifying the results from the bioluminescent reporter imaging by determining the luciferase activity in liver lysates

Luciferase enzymatic assay

The liver extracts were prepared by homogenisation with the minibead beater in reporter lyses buffer (Promega), two cycles of freeze-thawing and 2 min. of centrifugation at maximum speed. Supernatants were used for determining protein-normalized luciferase activity by adding 100 μ l luciferyl-CoA (Promega) to 20 μ l of liver extract in a monolight luminometer (BD Biosciences). Protein content was measured in a 96-well microtiter plate using the BCA protein assay kit (Pierce). Absorbance at 562 nm was determined in a microplate reader.

Real time quantitative PCR analysis

Total RNA was extracted from liver using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/2 μg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier (Hoekstra et al. 25448–53). PCR primer sets (Cyclophilline, Fw: AAAAGGAAGACGACGGAGCC Rev: TCGGAGCGCAATATGAAGGT and mER α , Fw: CTAGCAGATAGGGAGCTGGTTCA, Rev: GGAGATTCAGTC-CCCAAAGC) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. Cyclophilline was used as a control.

Data Analysis – The significance of differences in relative gene expression numbers C_t ($C_{t((Cyclo))} - C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a two-tailed Student's *t* test. Probability values less than 0.05 were considered significant.

Abbreviations

Ad5, Human Ad serotype 5; Ad.shER α , Ad mediated expression of shER α ; Ad.ERE-Luc, Ad mediated expression of ERE-Luc; ArKO, Aromatase knockout; CCD, Charged coupled device; DMEM, Dulbecco's modified Eagle's medium; ER, Estrogen Receptor; ERE, Estrogen Responsive Element; GFP, Green Fluorescent Protein; Ip, Intra-peritoneal; Luc, Luciferase; Pfu, Plaque forming units; S.c, Subcutaneous; siRNA, small interfering RNA; shRNA, short hairpin RNA; shER α , shRNA specific for mouse ER α ; Wt, wild type

Authors' contributions

Y.K carried out the studies described in this paper and drafted the manuscript. F.F participated in the concept of designing shER α . I.Q and C.L. contributed to the imaging experiments shown in Fig 4B. K.WvD participated in the design and coordination of this study and provided expert input for writing the manuscript. All authors read and approved the final manuscript.

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