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MODELING GENE EXPRESSION IN THE MOUSE UTERINE HORN BY *IN VIVO* ADENOVIRUS-MEDIATED GENE DELIVERY

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1. ABSTRACT

Endometrium is a unique tissue that is prepared for implantation of blastocyst during each menstrual cycle by expression of genes during a defined period of endometrial receptivity. Induction of gene over-expression in endometrium allows gaining insight on the role that genes play in endometrial function. Here, we show that induction of a state of gene over-expression in endometrium is feasible by *in vivo* gene delivery by transduction with adenovirus. Analysis of endometrium following adenoviral transduction of LacZ showed increased beta-galactosidase activity in endometrial glands as early as 24 hours following *in vivo* gene transfer. By 72 hour, the expression was uniformly strong throughout the uterine horn. Viral transduction was efficient in the range of 0.24-24x10^8 pfu (4.8-480x10^8 particles) in normal, pregnant and decidualized mouse uterine horns. These findings show that induction of a state of gene over-expression can be successfully attained in endometrium by adenoviral gene delivery.

2. INTRODUCTION

Endometrium is a unique tissue that is prepared for embryo implantation. In humans, in case that implantation does not take place or fails, the tissue is lost during menstruation. It is believed that endometrial receptivity and tissue shedding in endometrium is due to a sequential programmatic gene expression. Proving the role of the genes thought to be involved in these processes, require induction of gene expression in a timely manner. The level of endometrial gene expression can be increased *in vivo* by three different approaches, namely, introduction of oligonucleotide, transfection of DNA, or viral transduction. Despite our success using transfection of DNA or retroviral transduction in pregnant mouse uterine horns, the efficiency of DNA transfection was low and because retroviruses only enter dividing cells, the increase in gene expression in mouse uterine horn was not high (1). For these reasons, alternative approaches are required to efficiently transduce genes *in vivo*. Somatic gene transfer by replication-deficient adenoviral vectors *in vivo* is an

appealing option because adenoviruses can enter both dividing and non-dividing cells, and can infect a diverse array of cell types that express Coxsackie adenovirus receptor (CAR) as well as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. These vectors have been shown to efficiently transduce genes in several animal models (2-4). We have tested whether this approach would permit specific gene delivery to the endometrial epithelium by injecting adenovirus encoding the LacZ reporter gene into the mouse uterine lumen.

3. MATERIALS AND METHODS

3.1. Materials

Reproductive age, *Albino*, non-pregnant and time pregnant CD1 female mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All chemicals were from Sigma-Aldrich company (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). The purified mouse monoclonal antibody to β-gal was from Promega (Madison, WI). The rabbit polyclonal antibody to actin was from Abcam, Inc. (Cambrige, MA). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Purified *E coli* β-galactosidase was obtained from Worthington Biochemical Corporation (Lakewood, NJ). Pre-stained protein ladder was from Life Technologies, Inc. (Rockville, MD). The BetaBlue staining Kit was purchased from Novagen, Inc. (Madison, WI).

3.2. Induction of decidualization in mouse uterine horn

Decidualization was induced in ovariectomized mice, 4-5 days after ovariectomy. Mice received three subcutaneous injections of 17-β estradiol (E2, 100 ng) daily. After three days of rest, animals received three additional daily subcutaneous injections of E2 (10 ng) as well as subcutaneous injections of progesterone (P, 1 mg). Both E2 and P were delivered in 0.1 ml of Sesame oil. On the last day of treatment with E2 plus P, animals received the decidualizing signal which was trauma. After anesthesia, an incision was made in the left flank and the left uterine horn was exposed. Trauma was produced by insertion of a dull, 25 gauge needle, seven times into the horn lumen. Animals received three additional daily subcutaneous injections of P (1 mg). 72 hr later, both uterine horns were removed.

3.3. Construction and preparation of Ad-B-galactosidase recombinant virus

An Ad5 E1-replacement virus was constructed by removing Ad5 nt 450-3330 and replacement with an RSV LTR promoter/enhancer fused to β-galactosidase coding sequences. This manipulation was originally performed in a recombinant DNA plasmid that contained the left 5800 nt of Ad5 and then transferred into infectious virus by homologous recombination. Recombinant virus was plaque purified and amplified on 293 cells, and confirmed by DNA sequencing. Virus particles were purified by two successive rounds of cesium chloride equilibrium centrifugation and quantified by Absorbance at 260 nm where 1 O.D. = 1 x 10^{12} virus particles/ml. All manipulations for the construction of virus were performed as described (5). Immediately prior to use, adenovirus particles were purified by two successive spin colums (Centri-Sep, Princeton Separations) into phosphate buffered saline (PBS) to remove CsCl.

3.4. *In vivo* gene delivery

Mice were anesthetized by intraperitoneal injection of 250 µl of 5% xylazine/10% ketamine mixture and an incision was made in the left flank to expose the left uterine horn. Adenovirus was introduced into the mouse uterine lumen using a 28 gauge needle in a total volume of 30 microliters of PBS. The uterus was eased back into the body and the skin was closed with autoclips.

All experiments were conducted in accordance with an approved protocol from the SUNY Animal Care and Use Committee.

3.5. SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

The tissue lysates (250 µgprotein/lane) were fractionated in a 6% denaturing gel together with pre-stained protein ladder and were subsequently blotted onto PVDF membrane in a Mini-Trans-Blot apparatus (Bio-Rad Laboratories, Hercules, CA). The blots were stained with the monoclonal antibody to β-gal at a concentration of 2 µg/ml. The secondary antibody used was anti-mouse IgG-HRP (Santa Cruz Biotechnology, CA). Bands were detected by chemiluminescence as described by the manufacturer.

3.6. Whole-mount β-gal staining

Uteri were fixed for 30 min at 4^{o}C with 2% paraformaldehyde in phosphate-buffered saline (PBS) containing 2 mM $MgCl_2$, 5 mM EGTA (pH 8.0), and 0.02% NP-40. Samples were then washed and stained for 16 h at 37^{o}C for β-galactosidase in staining buffer containing 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% SDS, and 0.02% NP-40. Uterine horns were photographed and then postfixed in 10% buffered formalin overnight, and embedded in paraffin. Serial sections of horns were viewed without staining and after staining with Hematoxylin and Eosin.

4. RESULTS AND DISCUSSION

In this report, three sets of animals were used, 5-month-old female mice in different phases of the estrous cycle, mice with decidualized uterine horns and pregnant mice. Adenoviral particles were prepared on the day of injection and were introduced into the left uterine horn. The first day of pregnancy was the day that vaginal plug was observed after mating. When the horn was decidualized, the adenovirus was introduced into the uterine lumen following trauma. Injection of adenoviral particles was performed on day 3 of pregnancy. To insure that the results were reproducible, in each experiment, six animals were included.

In each of the experiments, we used three viral titres, $0.24x10^8$ pfu ($4.8x10^8$ particles), $2.4x10^8$ pfu ($48x10^8$ particles) and $24x10^8$ pfu ($480x10^8$ particles). In non-pregnant mice, uterine horns were removed 24 and 72 hr

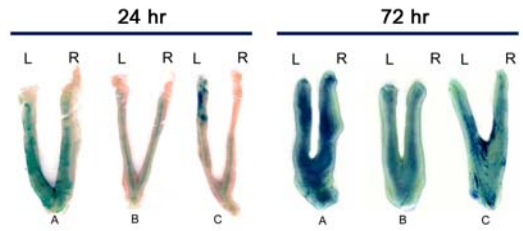

Figure 1. β-galactosidase activity in non-pregnant mouse uterine horns after adenoviral transduction. Adenoviral particles were injected into mouse uterine horns at A: $0.24x10^8$ pfu, B: $2.4x10^8$ pfu and C: $24x10^8$ pfu. Horns were removed 24 and 72 hr later and stained for β-gal by the BetaBlue staining kit.

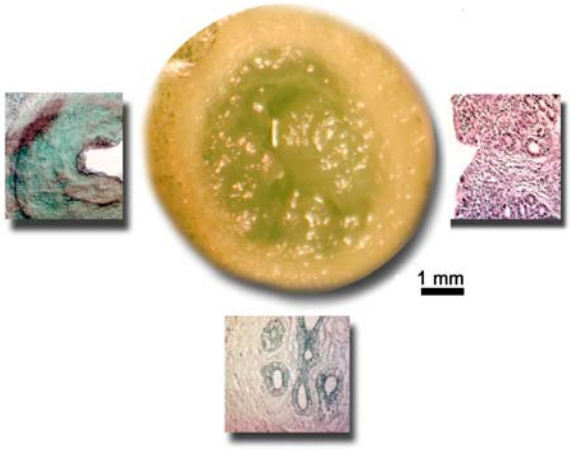

Figure 2. β-gal activity in non-pregnant mouse uterine horn after adenoviral transduction. Adenoviral particles were injected into mouse uterine horn at $24x10^8$ pfu. Center panel shows β-gal activity in endometrium. Myometrium does not show staining. Speckled staining on serosal surface is likely due to leakage of adenoviral particles from injection site seeding the surface. Left panel: a thick (100 micrometer) section shows confinement of the β-gal to endometrium. Right panel shows lack of inflammatory cells in a hematoxylin and eosin stained section. Lower panel shows β-gal in endometrial glands.

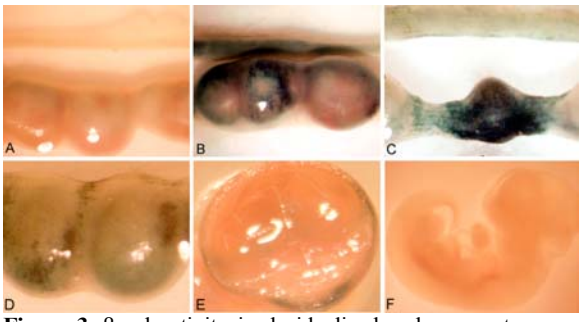

Figure 3. β-gal activity in decidualized and pregnant mouse uterine horn after adenoviral transduction. Adenoviral particles were injected to the left uterine horn at A-C: A: 0, B: $2.4x10^8$ pfu and C: $24x10^8$ pfu. Horns were removed 6 and 10 days after transduction, respectively, and stained for β-gal. In each panel, the right horn is on the top and the left horn at the bottom of the figure. Some β–gal staining is seen in the right horn due to trans-uterine flow of viral particles. D-E: Adenoviral particles ($24x10^8$ pfu) were injected to the pregnant left mouse uterine horn on day 3 of pregnancy. D: uterine horn , E: Placenta, F: Embryo stained for β-gal.

after injection. In mice with decidualized uterine horn, horns were removed 6 days after injection. In pregnant mice, the horns were removed on day 13 of pregnancy.

Following removal, the uterine horns were stained for β-gal. β-gal was evident in both uterine horns in non-pregnant mice at each of the three concentrations of the virus both at 24 and 72 hr after injection (Figure 1). Cross sections of the stained uterine horns showed the staining to be confined to the endometrium. Thick sections showed staining in the endometrial glands and thin sections confirmed presence of staining in glandular epithelium (Figure 2).

One potential drawback of the adenoviral transduction in clinical trial has been innate response to viral particle or capsid. This response results in inflammation of transduced tissues and loss of vector genomes (5). To detect such a response, tissues were embedded in paraffin and sections were stained with hematoxylin and eosin. Analysis of sections failed to show accumulation of inflammatory cells in the endometrium within the timeline of the study (Figure 2).

Similar results were obtained when adenoviral transduction was carried out in decidualized mouse uterine horn and during pregnancy (Figure 3). There was more β–gal activity in the left horn as compared with the right horn. Some staining was observed in the right horn due to transuterine flow of fluid to the right horn (Figure 3B). In pregnant horns, the staining was patchy and local and was not as intense as normal uterine horns or decidualized horns (Figure 3D). The reason for this is likely due to two factors. First, the horns were removed 10 days rather 1 or 3 days after transduction. Second, the glandular structures which are the main sites for the β–gal staining are atrophied or displaced by the growing decidua and embryo. This can be seen in figure 3E and F showing that the staining is confined to structures that surround placenta and not in placenta or embryo.

Tissue lysates of transduced mouse uterine horns were subjected to Western blot analysis (Figure 4). A band which co-migrated with purified β–gal protein was found in the transduced uterine horns. A similar band was not present in the non-transduced control mouse uterine horns. This band was present in the uterine horns of normal mice that were transduced with $24x10^8$ pfu and not with lower doses. This is likely due to the sensitivity of the Western blot analysis not allowing detection of lower quantitites of induced β–gal. However, the β–gal protein band was detected in the decidualized uterine horns transduced both with low and high doses of adenovirus. More β–gal was found in the horn transduced with $2.4x10^8$ pfu as compared to that induced by the lower dose of $0.24x10^8$ pfu indicating that the level of protein correlates with the number of the particles introduced in the uterine horn.

Taken together, these results show that adenovirally directed gene expression can be successfully induced in non-pregnant, decidualized and pregnant mice uterine horns. Effective dose range of the virus is 0.24-

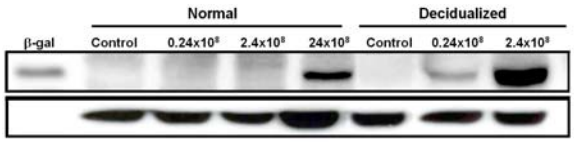

Figure 4. Western blot analysis of beta-gal in mouse endometrium transduced with adenovirus encoding beta-gal. Adenoviral particles were injected to the left uterine horn of normal mice (normal) and mice with decidualized horn (decidualized) at 0, $0.24x10^8$pfu, $2.4x10^8$ pfu and $24x10^8$ pfu. Horns were removed 72 hr (normal) or 6 days (decidualized) after transduction. Tissue lysates of left uterine horn were subjected to Western blot analysis for beta gal (upper panel) along with purified beta gal (β-gal). Blot was stripped and re-probed with antibody to actin (lower panel) to validate equal loading.

$24x10^8$ pfu ($4.8\text{-}480x10^8$ particles). There is a correlation between the dose of the adenovirus and the level of protein detected in the horn with the high dose significantly increasing the level of transduced protein. Transduced protein accumulates within the glandular structures within 24-72 hr after transduction.

5. ACKNOWLEDGMENT

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