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Vcsa1 Acts as a Marker of Erectile Function Recovery After Gene Therapeutic and Pharmacological Interventions

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Abstract

Purpose—We identified molecular markers of erectile function, particularly those responding to erectile dysfunction treatment.

Materials and Methods—Sprague-Dawley retired breeder rats were intracorporeally injected with pVAX-hSlo, pSMAA-hSlo or the control plasmid pVAX. One week later the intracorporeal pressure-to-blood pressure ratio and gene expression were determined by microarray analysis and quantitative reverse transcriptase-polymerase chain reaction. Rat corporeal cells were transfected in vitro with pVAX-hSlo, pSMAA-hSlo or pVAX and the change in gene expression was determined. We also determined whether *Vcsa1* expression was changed after pharmacotherapy using tadalafil.

Results—Animals treated with vectors expressing *hSlo* had significantly improved erectile function compared to that in controls, accompanied by changed expression of a subset of genes. *Vcsa1* was one of the genes that was most changed in expression (the third of approximately 31,000 with greater than 10-fold up-regulation). Changes in gene expression were different than those observed in corporeal cells transfected in vitro, distinguishing gene expression changes that were a direct effect of *hSlo* over expression. When tadalafil was administered in retired breeder rats, the *Vcsa1* transcript increased 4-fold in corporeal tissue compared to that in untreated controls.

Conclusions—Our study identifies a set of genes that are changed in response to improved erectile function, rather than as a direct effect of treatment. We noted *Vcsa1* may act as marker of the restoration of erectile function after gene transfer and pharmacotherapy.

Keywords

penis; gene expression; tadalafil; muscle; smooth; microarray analysis

The development of ED is considered a multifactorial process, involving changes in the expression of several regulators of CSM tone.[1] Recent microarray studies supports this hypothesis, demonstrating that the development of ED involves changes in the expression of multiple genes.[2–6] However, few studies have focused on which genes are changed in expression following the restoration of erectile function after treatment. Published studies describe changes in gene expression in corporeal tissue after ED treatment with PDE5 inhibitors.[5,7] These reports do not distinguish between the direct effect of the pharmacological agent on gene expression and effects that are secondary to improved erectile physiology.

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Study received Albert Einstein College of Medicine animal use committee approval.

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Although the most prescribed drugs for ED are orally administered PDE5 inhibitors, potentially the next area of advancement for ED treatment will be a gene transfer approach.[8] Intracorporeal injection of plasmids expressing the *hSlo* gene (encoding the MaxiK potassium channel) expressed from the cytomegalovirus promoter pVAX-hSlo or the smooth muscle α-actin promoter pSMAA-hSlo have been shown to restore erectile function in rat models of ED.[9] Recently completed phase I clinical safety trials of the use of pVAX-hSlo to treat patients with ED show some evidence of efficacy.10 Despite evidence of the efficacy of intracorporeal injection of plasmids expressing *hSlo* to our knowledge there have been no published investigations of the impact of gene transfer on the expression of other genes or pathways.

We sought to identify molecular markers of erectile function, rather than the genes changed in direct response to treatment. One of the identified molecular markers was the *Vcsa1* gene, which was up-regulated with the restoration of erectile function after gene transfer treatments. Subsequent experiments demonstrated that the *Vcsa1* gene was also up-regulated following PDE5 mediated restoration of erectile function.

MATERIALS AND METHODS

Animals

Nine to 10-month-old male retired breeder Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) weighing greater than 500 gm were used in these experiments. They represent a commonly used model of age related ED.[11,12] All animal protocols were approved by the Albert Einstein College of Medicine animal use committee.

Intracorporeal injection of 100 μg pVAX, pVAX-hSlo and pSMAA-hSlo into rats was previously described.[9] One week after injection the ICP/BP response to electrostimulation of the cavernous nerve was determined.

In a second set of animals 1,000 μg pVAX-hSlo were administered to 10 animals 1 month before determining erectile function and gene expression. Five of these animals were treated with 2.5 mg/kg tadalafil orally 2 hours before ICP/BP measurement. A third group of 5 animals served as untreated controls and a fourth group of 5 was treated with 2.5 mg/kg tadalafil orally 2 hours before ICP/BP measurement. Mean ± SD ICP/BP and ANOVA were calculated in each treatment group.

Microarray Analysis

Following ICP/BP measurement the animals were sacrificed and corporeal tissue was isolated. Total RNA was extracted from frozen tissue with TRIzol®, as previously described.[4,12,13] RNA was used to perform microarray analysis of global gene expression using the RGU-230A Affymetrix™ microarray. Quality control of RNA, labeling and hybridization was performed elsewhere according to Affymetrix protocols. Four chips each for control, pVAX-hSlo and pSMAA-hSlo treated animals were used for gene expression analysis using AffylmGUI software (http://www.bioconductor.org), as previously described.[4]

Rat CSM Cell Transfection

Rat CSM cells were isolated as described previously[14] and grown in low glucose (1 gm/l) Dulbecco's modified Eagle's medium and 10% fetal bovine serum. Cells at passage 1 or 2 at 70% confluency were transfected with pVAX (control), pVAX-hSlo or pSMAA-hSlo using FuGENE® HD transfection reagent according to manufacturer instructions with a transfection reagent-to-DNA ratio of 3:2 in μl/μg. After 48 hours RNA was extracted with an RNeasy™ Mini Kit and used for quantitative RT-PCR.

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Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described.[12,13,15] Appendix 1 lists the primers. Transcript expression was analyzed using the comparative crossing threshold (Ct) method ($2^{-\Delta\Delta Ct}$), which was applicable because primer efficiency was found to be close to that of the housekeeping gene used to normalize samples.

RESULTS

Treating Aging Rats With pVAX-hSlo or pSMAA-hSlo Restored Erectile Function

Three groups of 5 animals each received intracorporeal injections of 100 μg of plasmids expressing the hSlo gene (pVAX-hSlo and pSMAA-hSlo) or the empty backbone control (pVAX).[9] Animals treated with either plasmid expressing hSlo showed significant improvement in erectile function compared to that in pVAX controls (fig. 1).

Identification of Gene Expression Changes by Microarray Analysis

We compared gene expression in animals treated with pVAX-hSlo and pSMAA-hSlo to that in control animals treated with pVAX. In corpora treated with pVAX-hSlo 144 genes showed a statistically significant, greater than 1.5-fold change in expression compared to that in controls (B-statistic greater than 1), whereas animals treated with pSMAA-hSlo had a total of 189 genes with a greater than 1.5-fold level of change compared to that in controls. There was considerable overlap in genes that changed in expression (fig. 2), suggesting that treatment with either plasmid expressing *hSlo* triggered analogous physiological and molecular effects in vivo. Overall the changed genes represented less than 1% of the total genes (about 31,100) on the chip.

We sorted the entire list of genes changed in expression by ontological themes using the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/home.jsp). Ontological analysis indicated that the keratins, which are the intermediate filament group of genes, were up-regulated after treatment (Appendix 2). Of the down-regulated genes those involved in transcription regulation were significantly overrepre-sented (Appendix 3).

Since genes with unknown function would not be included in an ontological grouping, we also focused on the 20 most up-regulated and down-regulated genes. *Vcsa1* was up-regulated with each treatment (tables 1 and 2). This is of particular interest since *Vcsa1* has been suggested as a marker of ED because it is down-regulated in several animal models of ED.[3,12] Neither microarray analysis nor quantitative RT-PCR revealed significant changes in *Slo* gene expression (table 3). It is known that only a small number of cells uptake intracorporeally injected plasmid,[11] so that it might be expected that *hSlo* expression would not be greatly affected in the whole corpora. This confirms past observations that despite the positive physiological effect of *hSlo* expressing plasmids on erectile function there is no effect on total *Slo* levels after 1 week.[9]

We performed quantitative RT-PCR to confirm changes in expression of several of the genes identified as changed by microarray analysis (table 3). The ribosomal protein 24 gene (*RPL24*) was selected as the housekeeping gene since its expression was unchanged following treatment according to microarray analysis. Overall quantitative RT-PCR data supported the changes in gene expression identified by microarray data (tables 1 to 3). Differences in the fold change were likely due to the different methods of normalization used for quantitative RT-PCR and microarray analysis.

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Gene Expression After CSM Cell Transfection With pVAX-hSlo or pSMAA-hSlo

We hypothesized that the changes in gene expression after treatment with pSMAA-hSlo or pVAX-hSlo were the result of a physiological improvement in erectile function, rather than a direct consequence of gene transfer of the plasmids expressing *hSlo*. To test this hypothesis we transfected rat CSM cells in vitro with pVAX-hSlo or pSMAA-hSlo plasmids and compared gene expression with that in cells transfected with pVAX. In contrast to the *Slo* levels detected after gene transfer of pVAX-hSlo and pSMAA-hSlo in vivo, *Slo* expression following the transfection of CSM cells with pVAX-hSlo was increased greater than 14-fold and greater than 100,000-fold with pSMAA-hSlo. Greater expression of the *Slo* gene from pSMAA-hSlo may be facilitated by greater efficiency of the smooth muscle α-actin promoter, as we have previously observed.[9]

Using quantitative RT-PCR we analyzed genes that were up-regulated (*Vcsa1, EXPI* and *KRT1–18*) and down-regulated (*Cav, RGD:1303126, EMP1* and *Eef1a1*) after gene transfer of pVAX-hSlo and pSMAA-hSlo administered in vivo (tables 1 and 2). None of these genes was significantly changed in expression in vitro (fig. 3). Therefore, the subset of genes identified as physiological markers for the recovery of erectile function following gene transfer are distinct from changes in gene expression that may occur in direct response to treatment, ie *Slo* gene over expression (tables 1 to 3).

Tadalafil Administration Resulted in *Vcsa1* Up-Regulation

Vcsa1, one of the genes changed as a physiological response to the recovery of erectile function, has previously been suggested to be a marker of ED in several animal models.[3,12] We determined whether *Vcsa1* expression correlated with the recovery of erectile function after the administration of the PDE5 inhibitor tadalafil alone or in association with pVAX-hSlo.

The longest visually observed erection and ICP/BP were determined (fig. 4). In rats treated with tadalafil or pVAX-hSlo the longest measured erection time was about 100 seconds longer than that in the untreated control groups, while a combination of treatments led to almost a 2-fold increase. Although all treatments produced a significant improvement in the ICP/BP response at 4 and 10 mA stimulation compared to that in control groups, combining the treatments showed a slight improvement in the ICP/BP ratio over that of the single treatment. At the lowest level of stimulation (0.75 mA) there was a significant increase in ICP/BP in animals receiving combined treatments compared to that in controls and animals receiving a single treatment. This suggests that combined treatments lower the level of stimulation needed to develop a significant increase in ICP/BP and, therefore, potentially an erection.

Improved Erection was Associated With Increased *Vcsa1* Expression

Quantitative RT-PCR was used to analyze expression of the *Vcsa1* and *hSlo* transcripts after the administration of tadalafil, pVAX-hSlo or a combination of the 2 treatments. Intracorporeal gene transfer of pVAX-*hSlo* resulted in higher but not statistically higher levels of the *hSlo* transcript after 4 weeks ($p > 0.05$, fig. 5). Quantitative RT-PCR was performed to verify whether the recovery of erection using different treatments still correlated with *Vcsa1* up-regulation. Interestingly although tadalafil was administered only 2 hours before measuring erectile function, *Vcsa1* expression was increased approximately 4-fold (figs. 4 and 5). Combination therapy seemed to have a synergistic effect on *Vcsa1* expression. The detected level of the *Vcsa1* transcript was approximately 20-fold greater than in untreated animals and 5-fold greater than after the individual treatments.

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DISCUSSION

Our study shows that a subset of genes is changed in corporeal tissue after gene transfer of plasmids that express *hSlo*, resulting in improved erectile function in an aging rat model of ED. The genes that changed in these in vivo experiments were different than the genes that changed after over expression of *hSlo* in vitro. Therefore, we conclude that the subset of genes changed in vivo following intracorporeal injection of pVAX-hSlo or pSMAA-hSlo, which resulted in improved erectile function, represent molecular markers of erectile function. The change in expression may reflect the efficacy of ED treatment, rather than a direct response to treatment.

Our group is investigating a gene transfer approach to treat smooth muscle disorders of the urogenital system. The pVAX-hSlo vector has been shown to improve erectile and bladder function in animal models,[11,16,17] and it has been evaluated in phase I clinical trials of ED treatment.[10] Recently the gene transfer vector pSMAA-hSlo, in which *hSlo* is expressed from a smooth muscle specific promoter, was also shown to be effective for treating ED in aging rats.[9] Gene transfer for ED treatment is useful for identifying markers of erectile function because 1) a single intracorporeal injection of pVAX-hSlo can improve erectile function in rats from 1 week to at least 6 months without a significant effect on the overall level of *Slo* in the penis and 2) only a small population of cells in the corpora actually take up the plasmid. [11] Therefore, resulting gene changes are unlikely to be a direct effect of *hSlo* over expression, but rather a change in the physiology of corporeal tissue as a result of improved erectile function.

We identified a number of up-regulated and down-regulated genes on microarray analysis after the administration of pVAX-hSlo or pSMAA-hSlo. The 2 plasmids expressing *hSlo* each caused similar improvement in erectile function and there is considerable overlap between the lists of changed genes. Ontological analysis showed that intermediate filaments were up-regulated in animals treated with each plasmid. These proteins are involved in mechanically integrating the various components of the cytoplasmic space in eukaryotic cells. They are important regulators of smooth muscle tone and, therefore, over expression could be physiologically relevant, representing improved erectile function following gene transfer.[18] Genes involved in transcriptional regulation are also overrepresented in the group of down-regulated genes, which may represent an adaptive response to improved erectile function.

The variable coding sequence protein A1, *Vcsa1*, was among the most up-regulated genes (tables 1 to 3). *Vcsa1* has previously been suggested to be a marker of ED in several animal models.[3,12] In addition, its human homologues *ProL1* and *hSMR3A/B* are down-regulated in the corpora of patients with ED.[13,19] Since we concluded that the set of genes that changed after treatment with plasmids expressing *hSlo* is indicative of restored erectile function, rather than a direct effect of treatment, we determined whether another ED treatment with a different mode of action would also change *Vcsa1* expression. We treated retired breeder rats with the PDE5 inhibitor tadalafil. Although this treatment has a different mode of action to improve erectile function, it also resulted in a significant increase in *Vcsa1* expression. Indeed, the change in *Vcsa1* expression was impressively rapid, considering that tadalafil administration occurred 2 hours before the physiological/molecular determinations. In addition, our experiments showed a correlation between the degree of improvement of erection and the level of *Vcsa1* up-regulation. Combined treatment with tadalafil and pVAX-hSlo in the animals demonstrated a significant increase in the longest observed erection time and greater sensitivity of the ICP/BP response to cavernous nerve stimulation with a corresponding 5-fold up-regulation of *Vcsa1* when each treatment was used separately.

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CONCLUSIONS

We identified a set of genes that act as molecular markers of erectile function. One of these markers, *Vcsa1*, is up-regulated in response to gene therapy and pharmacotherapy for ED. A combination of the 2 treatments causes a synergistic affect in improving erectile function, corresponding to a synergistic affect on *Vcsa1* expression. Therefore, *Vcsa1* might be useful for determining a quantitative measure of the efficacy of ED treatment. We are actively pursuing the development of immunoassays for the gene products of *Vcsa1* in rats, and *hSMR3* and *ProL1* in humans. Indeed, to develop any practical assay for a marker of erectile function it will be necessary to confirm that changes at the gene expression level are mirrored at the protein level. Identifying an easily assayable objective marker for erectile function in humans would be of great advantage for evaluating the efficacy of ED treatments.

Abbreviations and Acronyms

CSM	corporeal smooth muscle
ED	erectile dysfunction
ICP/BP	intracavernous pressure-to-systemic blood pressure ratio
PDE5	phosphodiesterase type 5
RT-PCR	reverse transcriptase-polymerase chain reaction

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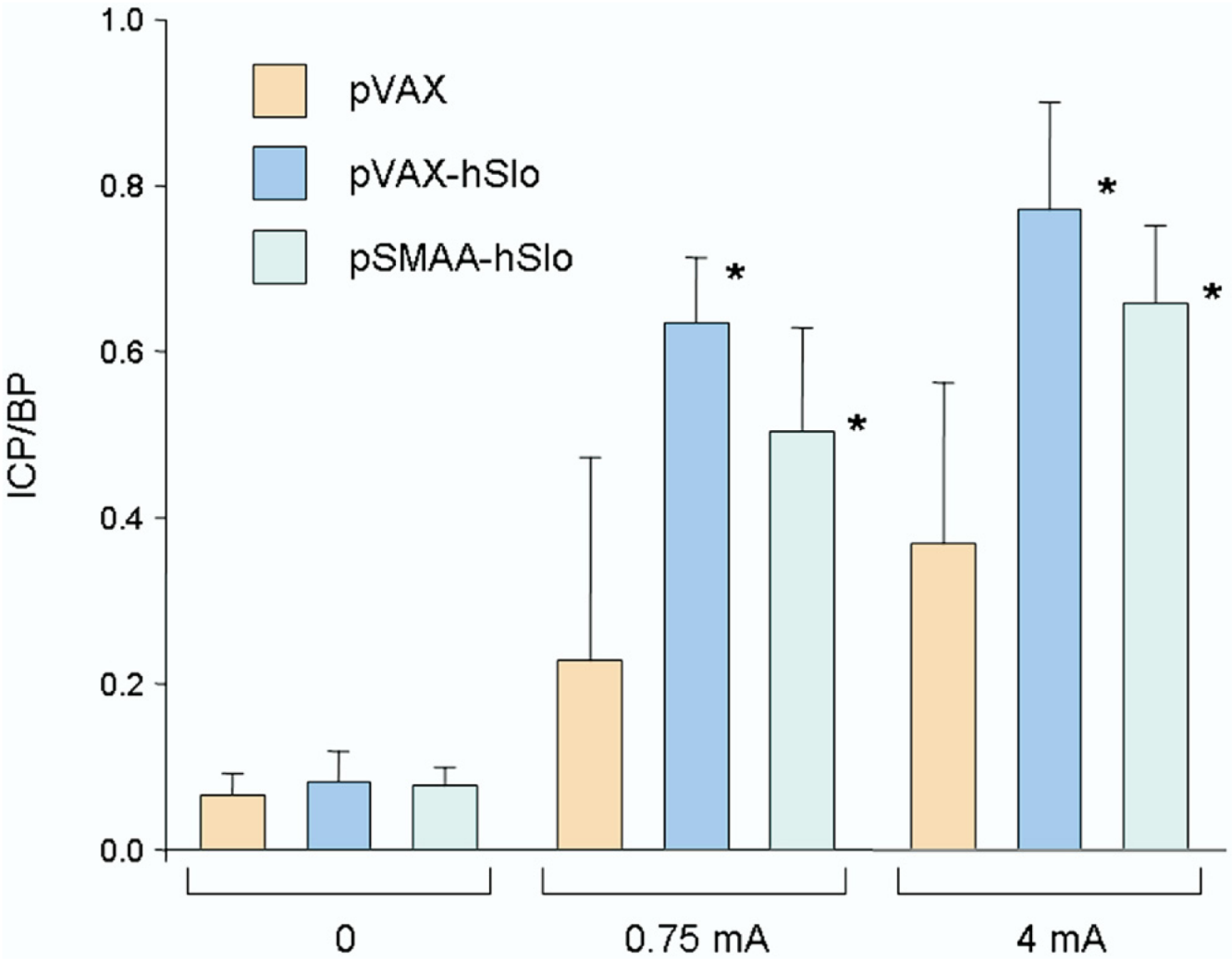

Figure 1.

Mean ICP/BP in 5 retired breeder rats treated with 100 μg pVAX, pVAX-hSlo and pSMAA-hSlo, respectively. Animals treated with pVAX-hSlo or pSMAA-hSlo showed significant ICP/BP increase in response to cavernous nerve stimulation with 0.75 or 4 mA compared to that in controls treated with empty plasmid vector pVAX. Asterisk indicates $p < 0.05$.

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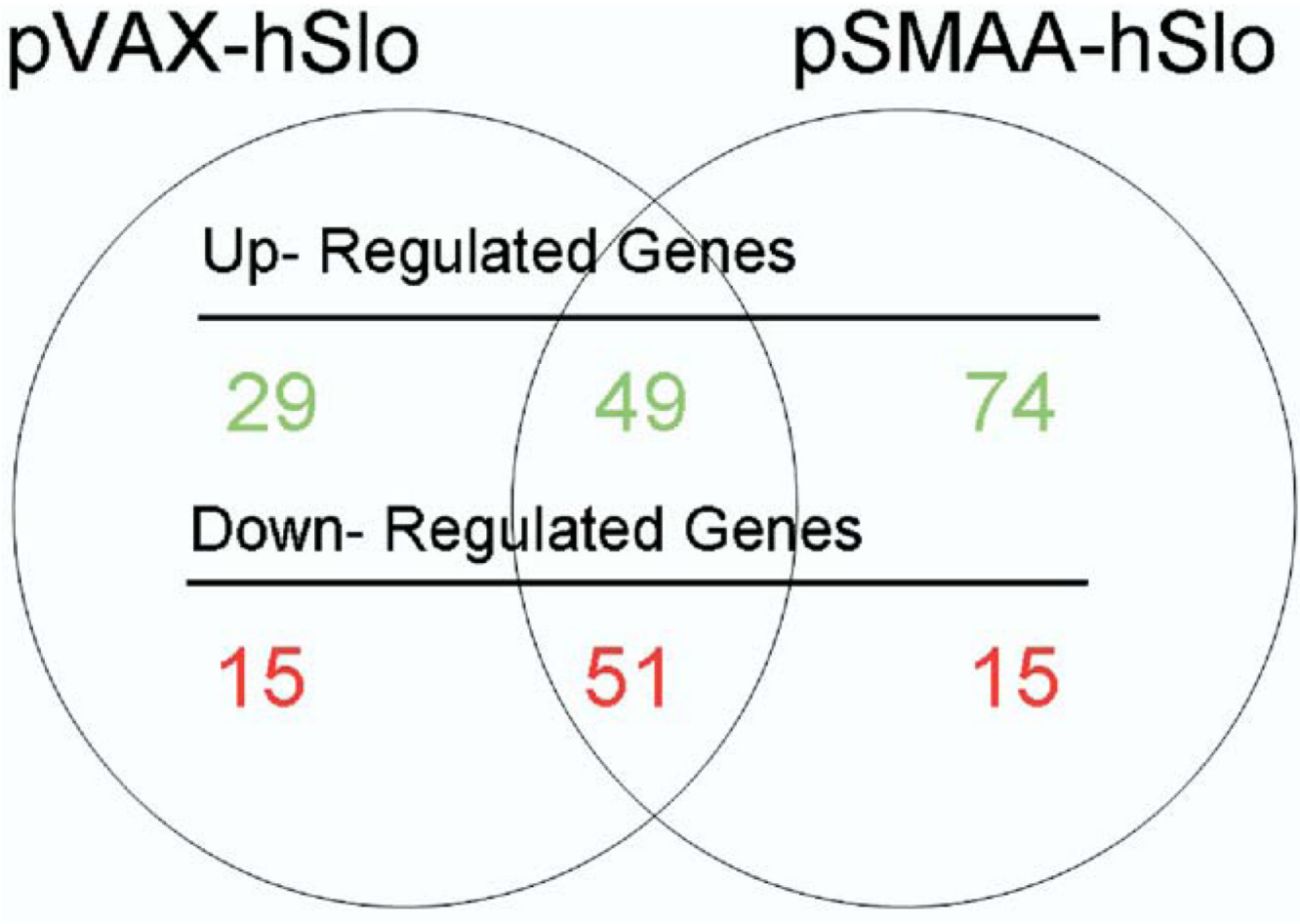

Figure 2.
Microarray analysis showed that approximately 144 and 189 genes were up-regulated and down-regulated in corpora of animals after treatment with pVAX-hSlo and pSMAA-hSlo, respectively. Intersection of circles indicates number of genes that 2 treatments shared in common.

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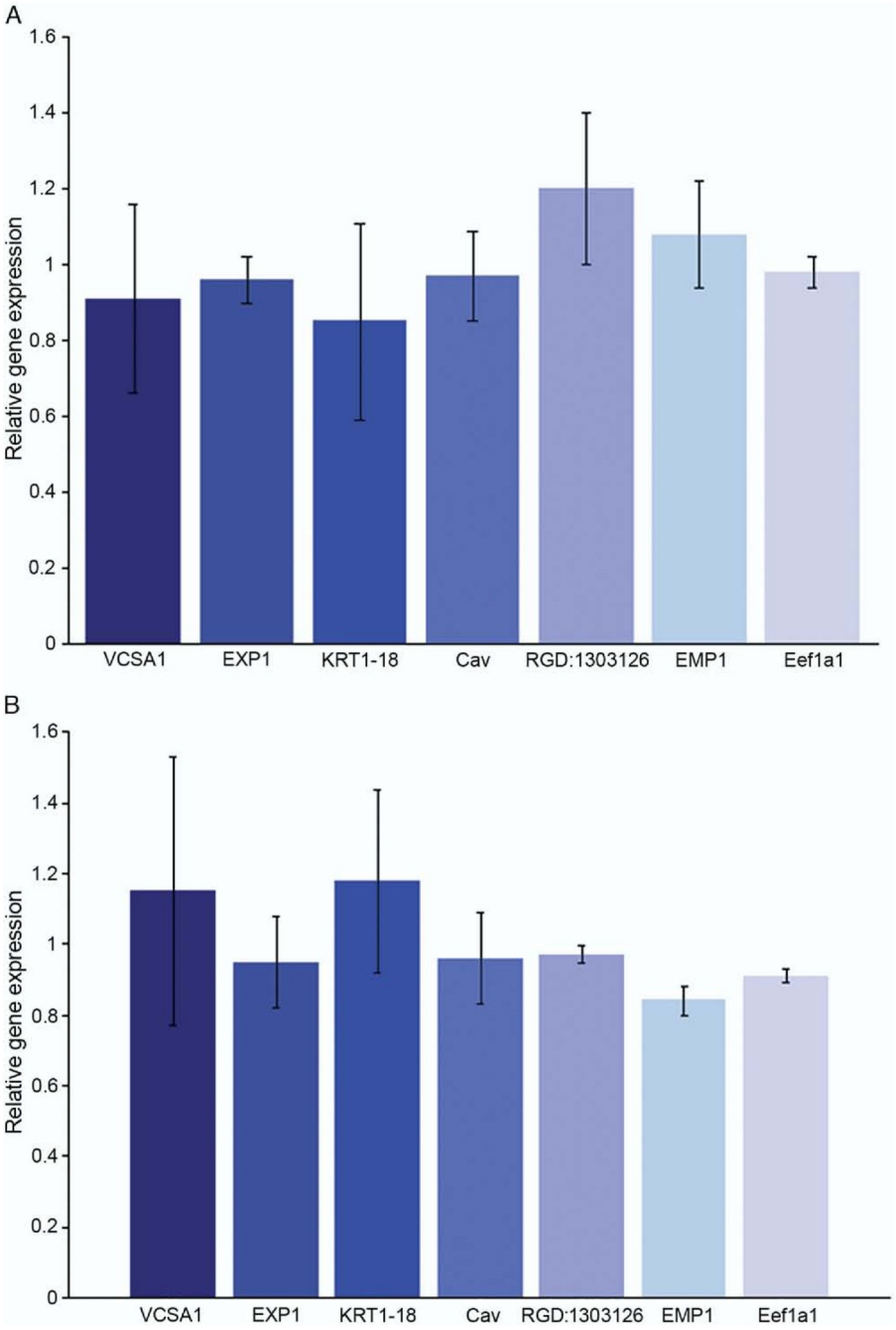

Figure 3.

Transfection of rat CSM cells with pVAX-hSlo and pSMAA-hSlo did not affect expression of genes that were up-regulated or down-regulated after plasmid intracorporeal gene transfer. Change in gene expression was determined by quantitative RT-PCR, compared with that in control cells transfected with pVAX and averaged for 5 experiments. No significant change in gene expression was detected. *A*, transfection with pVAX-hSlo. *B*, transfection with pSMAA-hSlo. Error bars represent SE.

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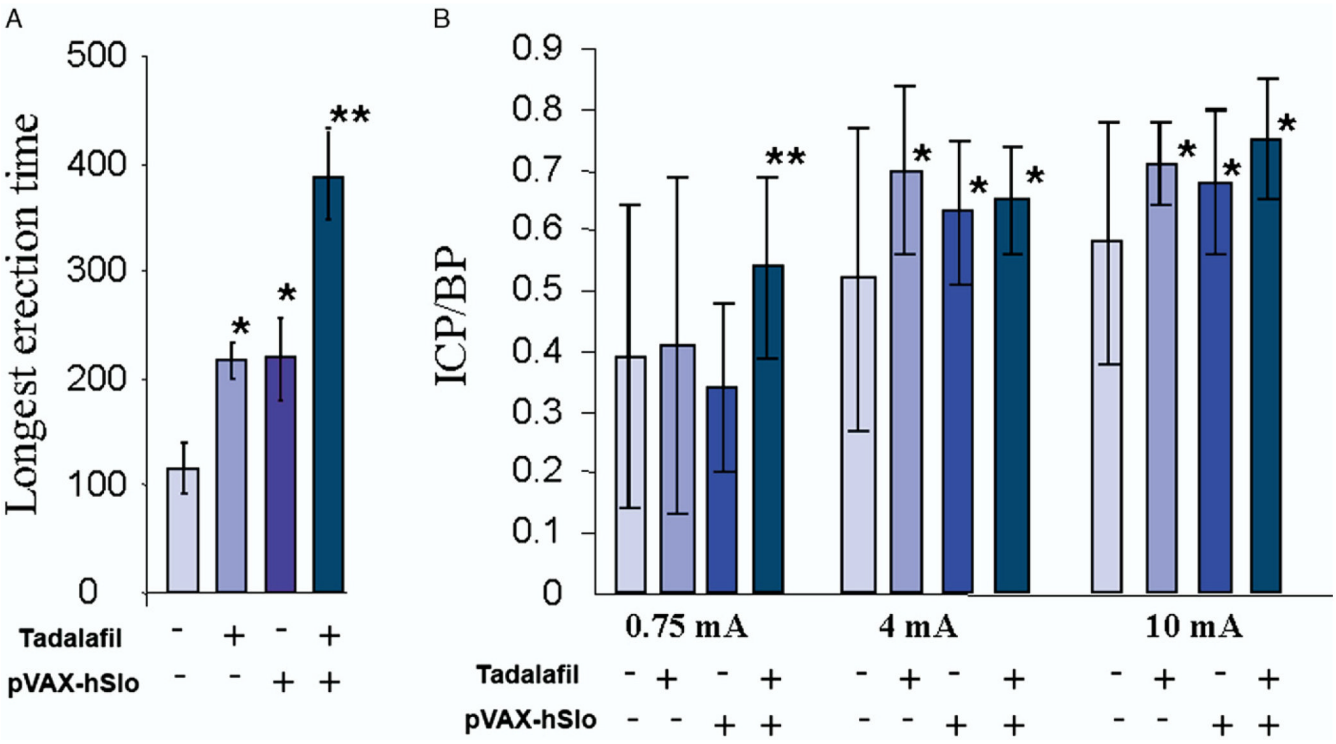

Figure 4.

Treatment with pVAX-hSlo and/or tadalafil improved erectile function. *A*, time of longest visually observed erection in seconds. *B*, ICP/BP response after electrostimulation at 0.75, 4 and 10 mA following various treatments to improve erectile function. Plus sign indicates 1,000 μg pVAX-hSlo intracorporeally injected 1 month before experiment and/or 2.5 mg/kg tadalafil administered orally 2 hours before erectile function evaluation. Error bars indicate animal with greatest ICP/BP difference from average in 5 animals. Single asterisk indicates significantly different vs control ($p < 0.05$). Double asterisks indicate that tadalafil plus pVAX-hSlo was significantly different from pVAX-hSlo alone ($p < 0.05$).

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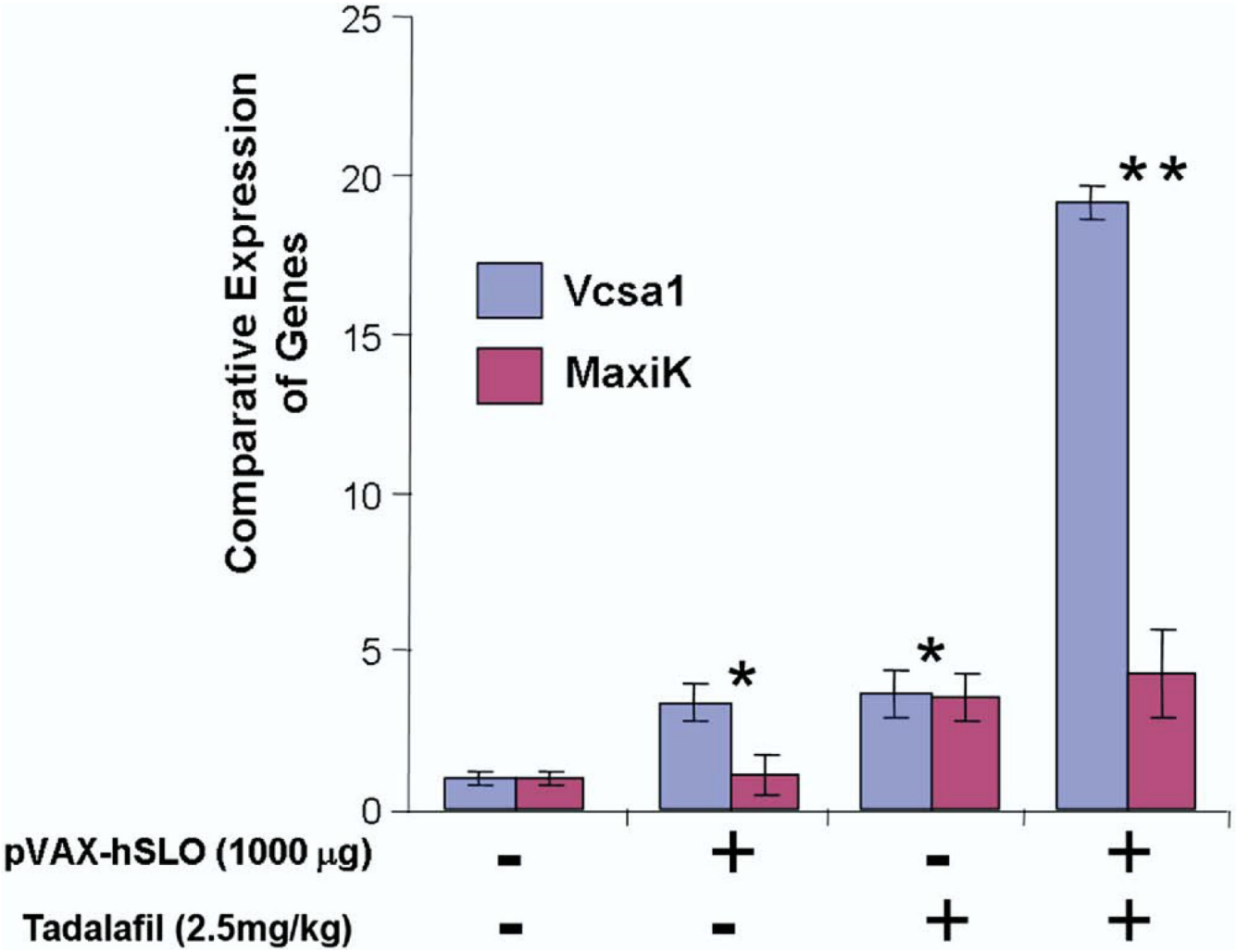

Figure 5.

Amount of *Vcsa1* and MaxiK expression was measured by RT-PCR after erectile dysfunction treatment with pVAX-hSlo and/or tadalafil. After pVAX-hSlo or tadalafil *Vcsa1* was up-regulated when erectile function was recovered, and combined treatment further enhanced *Vcsa1* up-regulation. Single asterisk indicates significant increase in *Vcsa1* expression in animals receiving pVAX-hSlo or tadalafil gene transfer vs that in untreated animals ($p < 0.05$). Double asterisks indicate significant increase in *Vcsa1* expression in animals with pVAX-hSlo plus tadalafil gene transfer vs that in animals receiving only 1 treatment type ($p < 0.05$).

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Table 1

After pVAX-hSlo and pSMAA-hSlo treatment in rats 20 most up-regulated genes, respectively

		Gene Expression Change	
Gene Symbol	**Gene**	**Log-Fold**	**Fold**
pVAX-hSlo:			
LOC362442*	Not available	6.68	44.59
Muc10*	Mucin 10, submandibular gland salivary mucin	6.40	40.99
RGD:708577*	Common salivary protein 1	6.06	36.70
Vcsa1*	Variable coding sequence A1	5.56	30.86
Not available*	Not available	3.55	12.61
Oit1_predicted*	Oncoprotein induced transcript 1 (predicted)	2.99	8.92
Igha*	Ig heavy chain (α polypeptide)	2.84	8.06
Cldn10_predicted*	Claudin 10 (predicted)	2.77	7.69
Tacstd1*	Tumor-associated calcium signal transducer 1	2.75	7.57
Alas2†	Aminolevulinic acid synthase 2	2.70	7.29
S100a9†	S100 calcium binding protein A9 (calgranulin B)	2.50	6.25
RGD1310209_predicted†	Similar to KIAA1324 protein (predicted)	2.35	5.54
LOC498228†	Not available	2.27	5.16
Hemgn	Hemogen	2.21	4.87
Krt1-18†	Keratin complex 1, acidic, gene 18	2.18	4.75
Not available†	Not available	2.17	4.73
Not available	Not available	2.10	4.39
Hbb†	Hemoglobin β chain complex	2.08	4.33
Myo5c_predicted	Myosin VC (predicted)	2.05	4.20
RGD:1359209	Globin, α	2.03	4.12
Acdc	Adipocyte complement related protein of 30 kDa	2.03	4.11
pSMAA-hSlo:			
Muc10*	Mucin 10, submandibular gland salivary mucin	7.07	50.05
RGD:708577*	Common salivary protein 1	6.70	44.90
LOC362442*	Not available	6.34	40.16
Vcsa1*	Variable coding sequence A1	6.06	36.70
Pip†	Prolactin induced protein	4.04	16.28
Stfa3_predicted	Stefin A3 (predicted)	3.96	15.66
Not available*	Not available	3.81	14.51
Krtdap	Keratinocyte differentiation associated protein	3.69	13.59
Vcsa2	Variable coding sequence A2	3.60	12.98
Not available†	Not available	3.44	11.83
Oit1_predicted*	Oncoprotein induced transcript 1 (predicted)	3.20	10.22

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		Gene Expression Change	
Gene Symbol	**Gene**	**Log-Fold**	**Fold**
Tacstd1*	Tumor-associated calcium signal transducer 1	3.19	10.18
RGD:1359664	Type II keratin Kb1	3.18	10.13
Not available	Not available	3.16	10.01
Not available	Not available	3.11	9.65
Expi†	Extracellular peptidase inhibitor	3.03	9.18
Not available	Not available	3.00	9.03
Igha*	Ig heavy chain (a polypeptide)	2.93	8.61
Cldn10_predicted*	Claudin 10 (predicted)	2.82	7.97
Not available	Not available	2.80	7.86
RGD:1303044†	Type I keratin KA15	2.79	7.77

*Top 20 changed genes per treatment group.

†Genes changed in 2 treatment groups.

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Table 2

After pVAX-hSlo and pSMAA-hSlo treatment in rats 20 most down-regulated genes, respectively

		Gene Expression Change	
Gene Symbol	**Gene**	**Log-Fold**	**Fold**
pVAX-hSlo:			
RGD:1303126*	SPARC-related modular calcium binding protein 1	−2.40	5.77
Col6a3_predicted	Procollagen, type VI, α3 (predicted)	−2.07	4.28
Ptrf_predicted*	Polymerase I and transcript release factor (predicted)	−2.06	4.23
Nedd4a†	Neural precursor cell gene 4A	−1.99	3.98
Lcn2	Lipocalin 2	−1.97	3.89
App†	Amyloid β (A4) precursor protein	−1.82	3.30
Cav†	Caveolin	−1.74	3.04
Prnd_predicted*	Prion protein dublet (predicted)	−1.72	2.95
Not available*	Not available	−1.72	2.94
Mfap2_predicted†	Microfibrillar-associated protein 2 (predicted)	−1.71	2.91
RGD:1359529	Similar to hypothetical protein dJ465N24.2.1	−1.68	2.83
Mmp3*	Matrix metallopeptidase 3	−1.66	2.75
Not available	Not available	−1.64	2.70
LOC294435	Not available	−1.59	2.54
Aebp1_predicted	AE binding protein 1 (predicted)	−1.59	2.53
Nisch_predicted	Nischarin (predicted)	−1.57	2.46
Mtmr1_predicted†	Myotubularin related protein 1 (predicted)	−1.51	2.27
Col18a1	Collagen, type XVIII, α 1	−1.48	2.20
Eef1a1	Eukaryotic translation elongation factor 1 α 1	−1.46	2.14
Not available	Not available	−1.46	2.13
RGD1309414_predicted	Similar to KIAA0913 protein (predicted)	−1.46	2.13
pSMAA-hSlo:			
RGD:1303126*	SPARC-related modular calcium binding protein 1	−2.00	4.00
Ptrf_predicted*	Polymerase I and transcript release factor	−1.96	3.86
LOC499533	Not available	−1.77	3.15
Prnd_predicted*	Prion protein dublet (predicted)	−1.66	2.77
Mfap2_predicted	Microfibrillar-associated protein 2 (predicted)	−1.66	2.75
RT1-Bb	RT1 class II locus Bb	−1.65	2.73
Not available*	Not available	−1.61	2.60
Mmp3*	Matrix metallopeptidase 3	−1.53	2.35
Not available	Not available	−1.52	2.31
LOC360840†	Not available	−1.48	2.20
Scgb2a1	Secretoglobin, family 2A, member 1	−1.45	2.11
Not available	Not available	−1.44	2.08
LOC503252	Not available	−1.44	2.08

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		Gene Expression Change	
Gene Symbol	**Gene**	**Log-Fold**	**Fold**
Not available	Not available	−1.44	2.07
LOC498539	Not available	−1.43	2.05
Ap1s1_predicted	Adaptor protein complex AP-1, Σ 1	−1.43	2.05
Lamc1†	Laminin, γ 1	−1.43	2.05
LOC360596	Not available	−1.42	2.03
Gnb2†	Guanine nucleotide binding protein, β polypeptide 2	−1.42	2.00
Fgfr1	Fibroblast growth factor receptor 1	−1.40	1.97
Tm9sf4_predicted	Transmembrane 9 superfamily protein member 4	−1.40	1.97

*Top 20 changed genes per treatment group.

†Genes changed in 2 treatment groups.

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Table 3

Quantitative RT-PCR of gene expression changes after pVAX-hSlo or pSMAA-hSlo intracorporeal injection

	Mean ± SD Fold Change vs pVAX	
	pVAX-hSlo	**pSMAA-hSlo**
Vcsa1	10.56 ± 2.43	320 ± 82
Slo	1.23 ± 0.51	1.33 ± 0.61
Muc10	17.425 ± 2.3	210.5 ± 142.5
Alas2	19 ± 3.23	5.84 ± 1.34
Pbsn	4.7 ± 1.16	2.16 ± 1.05
S100a9	6.46 ± 1.62	4.6 ± 1.875
Krt1-18	1.56 ± 0.06	2.03 ± 0.23
Expi	11.16 ± 2.62	42.53 ± 11.23

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APPENDIX 1

Primers used to confirm microarray gene expression

Gene	Primer
Vcsa1:	
Forward	5′-GAGGGTGTCAGAGGCCC-3′
Reverse	5′-GAGCAGTTAGCTGCCACTGATA-3′
Slo:	
Forward	5′-TACTTCAATGACAATATCCTCACCCT-3′
Reverse	5′-ACCATAACAACCACCATCCCCTAAG-3′
Expi:	
Forward	5′-TGTTCCAATGGCTGTGGTCA-3′
Reverse	5′-GGCCATCAGTCGTGCTTATGA-3′;
Krt1-18:	
Forward	5′-CAGACCTTGGAGATTGACCTGG-3′;
Reverse	5′-TTGCTCCATCTGCACCCTGTA-3′;
Cav:	
Forward	5′-ACCATCTTCGGCATCCCTATG-3′
Reverse	5′-AGGAAGCTCTTGATGCACGGT-3′;
Eef1a1:	
Forward	5′-GTCAGAACGCAGGTGTTGTGAA-3′
Reverse	5′-GCCGGAATCTACGTGTCCAAT-3′;
Emp1:	
Forward	5′-TCAAAGTGCATGCCCACCA-3′
Reverse	5′-GCGATGGAACATGTGCATCTC-3′ ‘;
RGD:1303126:	
Forward	5′-TCTGACGGCAGGTCCTATGAGT-3′
Reverse	5′-TGGCCAGCATCTTTGCATC-3′.
Muc10:	
Forward	5′-TCCCACCAAGGAGCAACATTAA-3′
Reverse	5′-GGATGTGGTTTTGGCTGGAAG-3′.
Alas2:	
Forward	5′-ACCTCCCCTGCTGATTCAGAAT-3′
Reverse	5′-ACGGTATGTGTGGTCCTGCTTC-3′.
S100a9:	
Forward	5′-ACCCTGAACAAGGCGGAATT-3′
Reverse	5′-TTTGTGTCCAGGTCCTCCATG-3′.
Pbsn:	
Forward	5′-TGCTCACACTGGATGTGCTAGG-3′
Reverse	5′-TCCACGCTACTGGCAGCTAAGT-3′.
Rpl24:	
Forward	5′-TCGAGCTGTGCAGTTTTAGTGG-3′
Reverse	5′-GCGGACTCACATTTGGCATTA-3′
Glyceraldehyde-3-phosphate	

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Gene	Primer
dehydrogenase	
Forward	5′-GCCGCCTGCTTCACCACCTTCT-3′
Reverse	5′-GCATGGCCTTCCGTGTTCCTACC-3′

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APPENDIX 2

Intermediate filament genes up-regulated after pVAX-hSlo or pSMAA-hSlo treatment

Affymetrix Identification No.	Gene
1371530_at	Keratin complex 2, basic, gene 8
1370868_at	Type II keratin kb1
1370863_at	Keratin complex 2, basic, gene 5
1388433_at	Keratin complex 1, acidic, gene 19
1373900_at	Keratin complex 2, basic, gene 7 (predicted)
1372153_at	Type I keratin ka15
1388155_at	Keratin complex 1, acidic, gene 18
1373254_at	Keratin 10

Keratins belonging to the intermediate filament group of genes were up-regulated in corporeal tissue after treatment with pVAX-hSlo and pSMAA-hSlo (enrichment score 3.5).

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APPENDIX 3

Genes enriched in transcription regulation

pVAX-hSlo		pSMAA-hSlo	
Affymetrix Identification No.	**Gene**	**Affymetrix Identification No.**	**Gene**
1368813_at	ccaat/Enhancer binding protein (c/ebp), Δ	1368813_at	ccaat/Enhancer binding protein (c/ebp), Δ
1388088_a_at	Upstream transcription factor 2	1388088_a_at	Upstream transcription factor 2
1380552_at	Lymphoblastic leukemia derived sequence 1	1390399_at	Camp responsive element binding protein-like 2
1395695_at	ae Binding protein 1 (predicted)	1380552_at	Lymphoblastic leukemia derived sequence 1
1367468_at	Scan domain-containing 1 (predicted)	1367468_at	Scan domain-containing 1 (predicted)
1372097_at	Interferon consensus sequence binding protein 1	1374974_at	Cell division cycle associated 7
1374974_at	Cell division cycle associated 7	1374085_at	Max dimerization protein 4 (predicted)

Genes related to transcription regulation were identified as down-regulated in corporeal tissues after treatment with pVAX-hSlo (enrichment score 0.63) or pSMAA-hSlo (enrichment score 1.01)