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Microarray analysis of gene expression changes in mouse liver induced by hydrodynamic DNA injection

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ABSTRACT

The hydrodynamic injection in mice tail vein of a plasmid (40 µg DNA) bearing the human α1-antitrypsin gene mediates: a) good liver gene transfer resulting in therapeutic plasma levels of human protein (>0.9 mg/ml); b) low liver injury as evaluated by a small and transient increase in AST and ALT in mouse plasma; and c) limited expression changes in host liver genes as evaluated by microarray analysis on days 2 and 10 after injection. For microarray analysis, groups of three mice were uninjected (control) or hydrodynamically injected with saline or plasmid DNA and then sacrificed on days 2 and 10 after injection. The results indicate that approximately 20 genes were up-regulated, whereas more than 50 genes were down-regulated. The most important change in up-regulated gene expression corresponded to the serum amyloid A group and the orosomucoid gene. Also, increased expression was observed of genes involved in contractile functions (actin, myosin, troponin) and lipid metabolism (lipin 1 and apolipoprotein A-IV). Relatively more genes were down-regulated, though only a few such as CYP4A10, ATP-binding cassette ABC1, peroxisomal acyl-CoA thioesterase 2A, phospholipids and phosphatidylcholine transfer protein, appear to be mediated by the presence of DNA in the solution. Although hydrodynamic injection appears to mediate only limited changes in host gene expression in the liver, some of the down-regulated genes are associated to atherosclerosis or hypertension diseases. Thus, additional studies will be necessary to clarify the long-term effect of the hydrodynamic gene transfer procedure.

KEYWORDS: microarray, gene delivery, liver, alpha-1 antitrypsin, genomic DNA, DNA injection, hydrodynamic injection

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INTRODUCTION

Hydrodynamic gene transfer is an easy and very efficient procedure for introducing naked DNA into the liver [1] via a rapid i.v. injection of plasmid in a large volume solution. By employing this procedure, high efficiencies of human alpha-1 antitrypsin gene transfer have been described [2, 3], and we have been able to demonstrate [4] that long-term therapeutic levels of human alpha-1-antitrypsin in mouse plasma can be achieved by this procedure. Since hydrodynamic injection opens new perspectives for future safe applications in liver gene therapy, several papers have been directed to determine the safety and efficacy of the procedure as well as the mechanism of gene delivery [4-7]. However, no previous works have addressed the effect of hydrodynamic injection on the change in gene expression pattern in the host liver. Since microarray technology provides a rapid and efficient method for large-scale profiling of gene expression changes [8-13], we have employed this procedure in the present work to investigate the

gene expression changes in livers of mice hydrodynamically injected with a saline solution containing or not 20 µg DNA plasmid bearing the human alpha-1 antitrypsin gene. The aim was to examine whether there are any differences in gene-regulatory activities between treatments, and to identify the genes mainly involved. We now report evidence of the hydrodynamic DNA injection limited effect on host liver gene expression, involving the acute reactant phase and contractile component genes as well as others involved in lipid metabolism and mitochondrial activity.

MATERIALS AND METHODS

Mice

C57BL/6 mice (Harlan, Barcelona, Spain) were used for all experiments. The animals were kept under standard laboratory conditions and housed 3-4 per cage. The experiments were approved by the Biological Research Committee of the Faculty of Medicine of the University of Valencia (Spain).

Hydrodynamic gene transfer

pTG7101 is a plasmid containing the full length of the human AAT. The plasmid was a generous gift from Dr. P. Meulien and Dr. J.P. Lecocq (Transgène S.A., Strasbourg, France). The pTG7101-18.6 kb plasmid employed in the present work contains a 16.5 kb genomic fragment of hAAT gene, cloned in SalI site of Poly III-I plasmid. In addition, it contains a 1.8 kb genome sequence upstream promoter, the promoter and full length of human AAT gene, and 3.2 kb downstream gene segment.

C57BL/6 mice (n=3 per group) were injected into the tail vein with a volume of saline solution equivalent to 10% of body weight in 5 sec., containing only saline or 40 µg of pTG7101 plasmid DNA, using a 3 ml latex-free syringe (Beckton Dickinson, Madrid, Spain). The animal groups were sacrified on days 2 and 10 after hydrodynamic injection. Also a non-treated Control group was included, constituted by noninjected animals. After sacrifice, livers were removed and kept at -80ºC until RNA extraction for microarray study of liver gene expression. An additional group was injected with 40 µg of plasmid DNA to evaluate the efficacy of hydrodynamic injection measuring the expression of exogenous hAAT gene as well as the liver injury caused by the hydrodynamic procedure, measuring the AST, ALT, ALP and Transferrine enzyme activity in mouse plasma. Blood samples (200 µl) were taken from the tail vein, using heparinized glass capillaries. After centrifugation, plasma was recovered and a pool was obtained mixing 50 µl/mouse for each group. The pooled plasma samples were inactivated (55°C, 45 min.), centrifuged (20,000 x g, 15 min.) and kept at -20°C until ELISA assay (performed as previously described [4]).

Microarrays

Microarrays for liver gene expression were performed following the manufacturer's instructions (Affymetrix). In brief, after tissue homogeneization in liquid Nitrogen, total RNA was extracted from each liver using Trizol (Invitrogen, Barcelona, Spain) according to the manufacturer's protocol. Biotynilated cRNA and cDNA synthesis were carried out as described in the Expression Analysis Technical Manual. Mouse genome 430 2.0 arrays were hybridized, stained, washed, and screened for quality according to the manufacturer's protocol. The Affymetrix gene chip data was processed, normalized, and statistically analyzed employing the manufacturer's software.

RESULTS AND DISCUSSION

We studied the changes in liver gene expression on days 2 and 10 after hydrodynamic injection, as well as expression efficacy of the exogenous gene and the liver injury caused by the hydrodynamic procedure. The expression of hAAT after hydrodynamic injection of pTG7101 plasmid increased progressively (Table 1) until achievement of the maximal effect on day 5, which remained stable 5 days later (total of 10 days). These kinetics agree with previous studies employing this hAAT gene construct controlled by the natural promoter. Thus, we confirmed the success of the hydrodynamic gene transfer procedure employed in this experiment. In addition, liver injury (Table 1) was very limited and transient, with a maximal increase in plasma AST and ALT two hours after injection. The normal values of liver injury parameters were restored 24-48 hours afterwards. Thus, we expect that days 2 and

Table 1. Gene transfer efficacy and liver injury.

Time	hAAT µg/ml	(SD)	Alb g/l	(SD)	ALT U/l	(SD)	AST U/l	(SD)	GGT U/l	(SD)	ALP U/l	(SD)	Transf. ng/dl	(SD)
15 min	0.7	0.1	15.0	1.4	611.0	14.1	1225.0	14.1	21.0	1.4	166.0	5.7	81.0	1.4
30 min	6.0	0.4	17.5	0.7	770.0	14.1	1685.5	7.8	30.5	2.1	167.5	3.5	104.0	2.8
1h	0.5	0.1	15.5	0.7	1198.0	14.1	2345.5	14.8	35.5	0.7	146.0	4.2	95.0	2.8
2h	16.1	0.8	13.5	0.7	1506.0	7.1	2654.0	5.7	45.0	0.0	156.5	2.1	89.5	0.7
4h	128.1	4.7	17.0	0.0	1008.0	28.3	2378.0	9.9	31.0	1.4	121.5	3.5	112.0	2.8
1d	925.9	5.9	19.5	0.7	255.0	4.2	332.5	6.4	30.0	1.4	152.5	7.8	139.0	1.4
2d	1565.1	20.6	23.5	0.7	55.0	9.9	83.5	3.5	8.5	0.7	132.0	5.7	162.5	7.8
5d	2823.7	100.2	23.0	0.0	47.5	2.1	78.5	2.1	5.5	0.7	153.5	4.9	179.0	9.9
10d	1673.7	17.1	22.0	1.4	42.5	3.5	353.5	9.2	14.0	1.4	151.5	4.9	165.0	2.8
Normal values	>900		25-48		26-77		54-269		< 6		45-222		85-170	

C57BL/6 mice (n=3 per group) were hydrodynamically injected in the tail vein with a volume of saline solution equivalent to 10% of body weight in 5 sec., containing 40 µg of pTG7101 plasmid DNA, using a 3-ml latex-free syringe (Beckton Dickinson). Blood samples (200 µl) were taken from the tail vein, using heparinized glass capillaries. After centrifugation, plasma was recovered and a pool was obtained mixing 50 µl/mouse for each group. The pooled plasma samples were inactivated (55°C, 45 min.), centrifuged (20.000 x g, 15 min.) and kept at -20°C until ELISA assay of hAAT or analysis of liver injury parameters. hAAT, human alpha-1 antitrypsin; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGP, γ-glutamic aminotransferase; ALP, alkaline phosphatase; Transf, transferrin.

10 could be good timepoints for microarray analysis of liver changes in mouse gene expression induced by the hydrodynamic procedure. The information from day 2 should circumvent the background due to direct injury and/or cell death, and therefore could offer more specific information on the early gene expression changes mediated by hydrodynamic injection and gene transfer. Day 10 represents a time period >5 days of stabilization of the exogenous gene, and could represent a second step of later changes in liver gene expression.

The preliminary microarray analysis with clustering method, based on the measure of similarity on Euclidean distances, shows a difference between the control group (CON) and the groups injected 2 or 10 days before with saline solution (Hyd-2d and Hyd-10d, respectively), but close similarity was observed between the last groups (data not shown). Differences were also observed between the hydrodynamic DNA injected groups (HydDNA-2d and HydDNA10d) and the respective mice groups injected with saline (Hyd-2d and Hyd-10d) - suggesting that nonspecific and DNA specific changes in gene expression are induced.

Tables 2 and 3 show up- and down-regulation, respectively, of representative genes whose hepatic mRNA levels were judged to be increased or decreased by Affymetryx Analysis Software, with changes of >1.9 or <-1.9. In all cases, the number of up-regulated genes was lower than the number of down-regulated genes. The up-regulated

Table 2. Up-regulated genes.

UP REGULATION **Gene**	**Accession**	**CON**	**Hyd 2d**	**Fold Change**	**HydDNA 2d**	**Fold Change**	**Hyd 10d**	**Fold Change**	**HydDNA 10d**	**Fold Change**
Acute phase response										
serum amyloid A 1	NM_009117	1040.84	4094.72	3.9	6807.18	**6.5**	1006.3	-1.0	5288.1	5.1
serum amyloid A 2	NM_011314	1761.97	4638.6	2.6	8128.66	**4.6**	1172.07	-1.5	5702.2	3.2
serum amyloid A 3	NM_011315	349.88	720.89	2.1	1114.06	**3.2**	308.57	-1.1	429.44	1.2
orosomucoid 2	NM_011016	191.87	678.51	3.5	2592.25	**13.5**	110.22	-1.7	915.31	4.8
Actin/Myosin/Troponin										
actin, alpha 1, skeletal muscle	M12233	105.85	69.45	-1.5	129.36	1.2	54.05	-2.0	597.82	**5.6**
myosin light chain, phosphorylatable, fast skeletal muscle	NM_016754	45.13	52.56	1.2	95.12	2.1	39.52	-1.1	428.85	**9.5**
myosin, heavy polypeptide 1, skeletal muscle, adult	AJ293626	16.44	28.78	1.8	90.48	5.5	14.29	-1.2	484.72	**29.5**
myosin, heavy polypeptide 2, skeletal muscle, adult	BC008538	38.77	50.06	1.3	77.74	2.0	37.05	-1.0	457.31	**11.8**
troponin C, fast skeletal	NM_009394	36.15	56.21	1.6	140.37	3.9	31.94	-1.1	672.81	**18.6**
troponin I, skeletal, fast 2	AV007148	104.78	128.18	1.2	148.22	1.4	137.05	1.3	590.98	**5.6**
Lipid Metabolism & Transport										
lipin 1	NM_015763	565.73	404.4	-1.4	778.21	1.4	1848.67	3.3	2373.5	**4.2**
lipin 1	AK014526	530.95	355.68	-1.5	674.7	1.3	1653.94	3.1	2166.3	**4.1**
sterol carrier protein 2, liver	C76618	234.51	350.94	1.5	362.56	1.5	545.77	2.3	534.32	**2.3**
fatty acid binding protein 4, adipocyte	BC002148	102.1	277.36	2.7	257.81	2.5	688.36	**6.7**	203.36	2.0
fatty acid binding protein 4, adipocyte	NM_024406	134.11	320.59	2.4	283.65	2.1	763.68	**5.7**	220.43	1.6
apolipoprotein A-IV	AV027367	589.8	2810.2	4.8	3574.59	**6.1**	921.22	1.6	933.72	1.6
apolipoprotein A-IV	BC010769	547.37	2034.71	3.7	2611.13	**4.8**	661.18	1.2	562.12	1.0

Table 2 continued..

Mitochondrial										
aldehyde dehydrogenase 2, mitochondrial	AI462635	88.2	187.27	2.1	172.91	2.0	288.78	**3.3**	257.23	2.9
aldehyde dehydrogenase 2, mitochondrial	AI462635	83.64	165	2.0	145.59	1.7	247.38	**3.0**	198.75	2.4
solute carrier family 25 (mit. carrier; aden. nucl. transl), member 4	BC026925	114.87	177.38	1.5	187.14	1.6	170.5	1.5	498.29	**4.3**
solute carrier family 25 (mit. carrier; aden. nucl. transl), member 4	BF225398	133.6	184.19	1.4	214.92	1.6	16.89	-7.9	498.46	**3.7**
Others										
glucocorticoid-induced leucine zipper	NM_010286	300.75	640.98	2.1	921.07	**3.1**	292.4	1.0	435.29	1.4
glucocorticoid-induced leucine zipper	AF201289	198.78	521.5	2.6	722.69	**3.6**	191.76	1.0	233.39	1.2
glucocorticoid-induced leucine zipper	AF201288	571.45	1228.68	2.2	1679.7	**2.9**	654.18	1.1	1011.8	1.8
insulin-like growth factor binding protein 1	NM_008341	617.42	1991.55	3.2	3675.82	**6.0**	1044.62	1.7	1344.3	2.2
cysteine-rich motor neuron 1	AV227314	153.81	237.47	1.5	240.83	1.6	305.95	2.0	330.46	**2.1**
erythrocyte protein band 4.1-like 2	AJ245854	138.06	243.94	1.8	313.31	2.3	354.24	2.6	335.86	**2.4**
5'-3' exoribonuclease 1	NM_011916	23.11	40.24	1.7	49.12	**2.1**	44.45	1.9	46.97	2.0

For microarray experiments, the groups of animals (n=3) were sacrificed on days 2 and 10 after hydrodynamic injection of saline (Hyd-2d and Hyd-10, respectively) or saline containing DNA (HydDNA-2d and HydDNA-10d, respectively). The control group (CON) was composed of noninjected animals. After sacrifice, livers were removed and kept at -80ºC until RNA extraction for microarray analysis of gene expression changes induced by hydrodynamic injection. For microarray analysis, total RNA was extracted from each liver using TRIzol (Invitrogen) according to the manufacturer's protocol. Biotinylated cRNA and cDNA synthesis was carried out as described in the Expression Analysis Technical Manual (Affymetrix). Mouse genome 430 2.0 arrays were hybridized, stained, washed, and screened for quality according to the manufacturer's protocol. The Affymetrix gene chip data were processed, normalized, and statistically analyzed using GCOS 1.4 software. We filtered and sorted the list of genes using the change p value change call, and signal log ratio. Subsequent agglomerative hierarchical clustering analysis was performed using the SpotFire DecisionSite for Functional Genomics software (Somerville, MA). We used the UPGMA (unweighted average) clustering method, and based the measure of similarity on Euclidean distances. Function annotations were generated using the gene ontology browser tool included in the SpotFire software and the ontology information available in the NetAffx analysis center. In bold we underscore the higher value of fold change with respect to the CON group, in each file.

Table 3. Down-regulated genes.

MAINLY DOWN REGULATION	Accession	CON	Hyd 2d	Fold Change	HydDNA 2d	Fold Change	Hyd 10d	Fold Change	HydDNA 10d	Fold Change
Aldehyde dehydrogenase										
aldehyde dehydrogenase family 3, subfamily A2	NM_007437	2824.94	1842.47	-1.5	1272.67	**-2.2**	1485.77	-1.9	1322.5	-2.1
aldehyde dehydrogenase 9, subfamily A1	BB703752	2198.29	1334.17	-1.6	1328.62	-1.7	1007.22	**-2.2**	1195	-1.8
Oxidoreductase										
NADPH oxidase 4	AB041034	463.75	263.4	-1.8	226.96	-2.0	169.57	**-2.7**	203.66	-2.3
interleukin 4 induced 1	BC017607	246.87	141.97	-1.7	151.68	-1.6	110.31	**-2.2**	120.88	-2.0
retinol dehydrogenase 6	NM_009040	1066.57	516.91	-2.1	463.36	**-2.3**	605.79	-1.8	515.41	-2.1
hypothetical protein LOC269575	AI327006	3625.5	1057.97	-3.4	706.12	-5.1	618.19	-5.9	219.41	**-16.5**
diaphorase 1 (NADH)	AF332060	2046.75	1375.81	-1.5	1355.45	-1.5	1011.69	-2.0	661.75	**-3.1**
RIKEN cDNA 4632417N05 gene	AK014586	1930.02	1306.38	-1.5	1225.76	-1.6	740.34	**-2.6**	884.14	-2.2
fatty acid desaturase 2	NM_019699	1847.86	970.81	-1.9	1053.46	-1.8	943.74	**-2.0**	943.34	-2.0
hydroxysteroid (17-beta) dehydrogenase 9	BC021836	3000.69	1076.6	**-2.8**	1574.44	-1.9	1350.04	-2.2	1478.9	-2.0
Cytochrome P450										
cytochrome P450, 2b9, phenobarbitol inducible, type a	NM_010000	224.73	112.8	-2.0	63.62	-3.5	6.12	**-36.7**	10.52	-21.4
cytochrome P450, 4a10	BC010747	7294.01	3061.38	-2.4	1614.96	**-4.5**	2160.38	-3.4	1655.5	-4.4
solute carrier family 27 (fatty acid transporter), member 2	BC013442	7015.1	5634.27	-1.2	5309.63	-1.3	5189.98	**-1.4**	6564.7	-1.1
Carboxylic Acid Metabolism										
ATP citrate lyase	AF332052	908.02	430.51	-2.1	658.35	-1.4	387.1	-2.3	333.95	**-2.7**
glutamate oxaloacetate transaminase 2, mitochondrial	U82470	1822.6	1047.71	-1.7	1191.17	-1.5	551.27	**-3.3**	575.72	-3.2
expressed sequence AA415817	BB026304	215.99	147	-1.5	159.18	-1.4	64.18	**-3.4**	70.77	-3.1
malic enzyme, supernatant	AK006387	3396.37	1594.41	-2.1	1314.06	**-2.6**	2571.17	-1.3	1625.2	-2.1
RIKEN cDNA 1300002P22 gene	NM_023737	3962.67	1663.69	0.4	1415.63	-2.8	1359.06	**-2.9**	1314.8	0.3

Table 3 continued..

mitochondrial acyl-CoA thioesterase 1	NM_134188	519.42	316.72	-1.6	168.25	**-3.1**	362.15	-1.4	247.26	-2.1
acetyl-Coenzyme A acyltransferase 1	NM_130864	6520.24	4054.96	-1.6	2429.32	**-2.7**	3634.92	-1.8	2894.1	-2.3
fatty acid Coenzyme A ligase, long chain 2	NM_007981	4266.79	2919.53	-1.5	2848.44	-1.5	1262.04	**-3.4**	1269.9	-3.4
peroxisomal acyl-CoA thioesterase 2A	NM_134246	498.71	116.86	-4.3	87.65	**-5.7**	483.92	-1.0	303.81	-1.6
stearoyl-Coenzyme A desaturase 1	NM_009127	2895.21	1266.68	-2.3	1134	**-2.6**	1965.36	-1.5	1436.7	-2.0
Lipid Metabolism & Transport										
phosphate cytidylyltransferase 1, choline, alpha isoform	BF714935	302	183.8	-1.6	190.48	-1.6	127.79	-2.4	112.61	**-2.7**
steroid sulfatase	NM_009293	776.83	372.05	-2.1	332.97	**-2.3**	356.71	**-2.2**	526.67	-1.5
diacylglycerol O-acyltransferase 2	NM_026384	2925.8	1350.39	-2.2	1656.97	-1.8	1162.03	**-2.5**	1182.4	-2.5
phosphotidylinositol transfer protein, beta	BG975479	308.29	176.45	-1.7	253.5	-1.2	113.91	-2.7	136.79	-2.3
phosphotidylinositol transfer protein, beta	NM_019640	440.38	318.65	-1.4	382.2	-1.2	124.21	-3.5	105.64	**-4.2**
phospholipid transfer protein	NM_011125	958.26	420.1	-2.3	222.87	**-4.3**	479.12	-2.0	243.98	-3.9
phosphatidylcholine transfer protein	NM_008796	837.26	516.76	-1.6	446.58	-1.9	234.47	-3.6	178.13	**-4.7**
phospholipid transfer protein	AI591480	2215.02	1424.75	-1.6	710.91	**-3.1**	2004.58	-1.1	1348	-1.6
solute carrier family 27 (fatty acid transporter), member 2	BC013442	7015.1	5634.27	-1.2	5309.63	-1.3	5189.98	**-1.4**	6564.7	-1.1
LXR and SRBP										
nuclear receptor subfamily 1, group H, member 3	NM_013839	444.83	407.73	-1.1	434	-1.0	292.05	**-1.5**	300.51	-1.5
nuclear receptor subfamily 1, group H, member 2	NM_009473	408.04	305.47	-1.3	282.95	-1.4	237.87	**-1.7**	290.74	-1.4
sterol regulatory element binding factor 2	BM123132	268.67	172.99	-1.6	204.67	-1.3	135.36	**-2.0**	132.63	-2.0
Adenine transloc. & mit subs carrier-domain										
RIKEN cDNA 4933433D23 gene	NM_026232	399.62	270.13	-1.5	188.15	-2.1	218.93	-1.8	120.08	**-3.3**
RIKEN cDNA 4933433D23 gene	BB032012	675.78	508.18	-1.3	266.91	-2.5	248.03	-2.7	212.63	**-3.2**

Table 3 continued..

RIKEN cDNA 4933433D23 gene	BC022676	160.57	122.09	-1.3	47.57	-3.4	14.97	**-10.7**	21.02	-7.6
solute carrier family 25 (mit. carrier; aden. nucl.transl), member 10	BC003222	717	471.44	-1.5	371.49	-1.9	263.51	-2.7	217.96	**-3.3**
selenocysteine lyase	NM_016717	682.36	372.21	-1.8	356.63	-1.9	229.23	**-3.0**	284.69	-2.4
solute carrier family 39 (iron-regulated transporter), member 1	AF226613	857.6	475.91	-1.8	530.29	-1.6	284.6	**-3.0**	390.54	-2.2
IGFBP-Domain										
insulin-like growth factor binding protein 4	NM_010517	2838.34	1107.84	-2.6	1396.69	-2.0	708.11	**-4.0**	938.82	-3.0
insulin-like growth factor binding protein 4	AA119124	3989.25	2048.54	-1.9	2433.04	-1.6	1581.42	**-2.5**	2080.7	-1.9
insulin-like growth factor binding protein 2	AK011784	4677.86	1506.48	**-3.1**	1716.38	-2.7	2126.53	-2.2	2361.4	-2.0
Regulation of Cell Shape										
ras homolog gene family, member U	NM_133955	1354.35	753.55	-1.8	838.55	-1.6	570.75	**-2.4**	562.05	-2.4
guanine nucleotide binding protein, alpha 12	NM_010302	188.24	87.75	-2.1	88.92	-2.1	44.03	**-4.3**	54.99	-3.4
guanine nucleotide binding protein, alpha 12	BF302166	212.59	124.87	-1.7	98.42	-2.2	37.36	**-5.7**	57.53	-3.7
ATP-Binding Cassette Transporters										
ATP-binding cassette, sub-family A (ABC1), member 1	NM_013454	470.66	264.51	-1.8	342.7	-1.4	168.26	**-2.8**	118.26	-4.0
ATP-binding cassette, sub-family A (ABC1), member 1	BB144704	194.94	121.33	-1.6	169.45	-1.2	26.18	**-7.4**	32.97	-5.9
ATP-binding cassette, sub-family A (ABC1), member 3	AY083616	286.36	222.04	-1.3	253.74	-1.1	111.46	**-2.6**	137.04	-2.1
Nucleases										
deoxyribonuclease I	BC014718	40.28	33.86	-1.2	19.71	-2.0	12.99	**-3.1**	19.69	-2.0

With the same methods as in table 2, table 3 shows the downregulated genes.

genes can be grouped into four categories according to their main function: acute phase reactants (APRs), contraction, lipid metabolism and mitochondrial activity. The major APRs in mammals include serum amyloid A (SAA) and either C-reactive protein or serum amyloid P component, depending on the species. They have a wide range of activities that contribute to host defense, since they can directly neutralize inflammatory agents, help minimize the extent of local tissue damage, and participate in tissue damage repair and regeneration. In our experiment, saline hydrodynamic injection mediates early (day 2) up-regulation of serum amyloid A gene (~2.8 fold), and the effect was greater (~4.8 fold) when DNA was injected. However, the effect must be transient, since lower values of SAA were observed 10 day after both saline and DNA injection (~ -1.2 and 3.1 fold, respectively). The data from serum amyloid A and orosomucoid 2 genes suggest that up-regulation was a nonspecific process induced by hydrodynamic injection, and that the presence of DNA contributes to increase this effect. In addition, the AST and ALT activity in plasma (Table 1) indicate that hydrodynamic injection mediates early and transient liver injury, and therefore we think that SAA up-regulation is a good liver response to neutralize the proinflammatory liver damage induced by this procedure. In this respect, SAA is the collective name referring to a polymorphic protein family encoded by multiple genes that represent the archetypal example of plasma proteins that are likely to be beneficial during the transient acute phase reaction response, although they could mediate detrimental effects in chronic inflammation. Thus, the hydrodynamic dose of 40 µg DNA plasmid employed in the present study mediates plasma levels of hAAT very close to the maximal effect, as previously described in dose-response studies, but a higher DNA dose (160 or 320 µg DNA plasmid) results in dose-dependent lower efficiencies - an effect that could be due, at least partially, to high steady-state SAA concentration in plasma.

Functionally, SAAs are small apolipoproteins that associate rapidly during the acute phase response with the third fraction of high-density lipoprotein (HDL3), on which they become the predominant apolipoprotein. We have observed in this study that hydrodynamic injection induces up-regulation of apolipoprotein A-IV, and the increased apolipoprotein expression is an early event since the effect was mainly observed 2 days after both saline and DNA injection (3.7-4.8 to 4.8-6.1 fold change, respectively). The normal values were recovered 10 days later, but it appears to be better induced by DNA injection, and the effect should be independent upon a direct liver X receptor [14, 15], because these genes were down-regulated after hydrodynamic injection. Since SAA enhances the binding of HDL3 to macrophages during inflammation, concomitant to a decrease in binding capacity to hepatocytes, we suggest that SAA acts retargeting the lipoprotein from hepatocytes to macrophages, which then can engulf cholesterol and lipid debris at the site of liver damage. This agrees with our electron microscopy observation that shows hydrodynamic injection to mediate transient formation of massive endocytic vesicles in the cell membrane of hepatocytes, in contact with the Disse space [7]. Thus, redirectioning of lipoproteins to macrophages may offer an advantage to restore hepatocyte normality.

In a way similar to SAA, we found early up-regulated expression of both the glucocorticoid-induced leucine zipper (GILZ) and the insulin-like growth factor binding protein 1 (IGFBP-1) genes. GILZ is a leucine zipper protein that mimics some of the effects of glucocorticoid hormones [16-19], including immunosuppressive and anti-inflammatory activity, and therefore, we could expect that augmented expression will contribute to limit the inflammatory reaction after hydrodynamic injection. On the other hand, IGFBP-1 is a 25 kD protein that is produced predominantly by hepatocytes, and hypoxia strongly induces its expression [20]. Since hydrodynamic injection mediates liver sinusoidal blood stasis for several minutes [7], we think that hydrodynamic mediated liver hypoxia could induce early IGFBP-1 over-expression.

The increased lipin 1 expression is a late response to hydrodynamic injection, since the maximal effect was observed 10 days after injection, and

both saline and DNA injection mediated 3.1-3.3 and 4.1-4.2 fold changes, respectively. Lipin defines a family of nuclear proteins required for normal adipose tissue development, and provides a candidate gene for human lipodystrophy [21]. Recent studies in mice have demonstrated that variations in lipin expression levels in adipose tissue have marked effects on adipose tissue mass and insulin sensitivity [22, 23], and that mutations in the Lipin 1 gene cause steatosis in a model of lipodystrophy. Although the molecular function of lipin 1 is unclear, recent results identify it as a selective physiological controller of hepatic lipid metabolism [24], since it selectively activates a subset of target pathways, including fatty acid oxidation and mitochondrial oxidative phosphorylation, while suppressing the lipogenic program. In this respect, we observed that hydrodynamic injection also mediates up-regulation of liver sterol carrier protein-2 and aldehyde dehydrogenase-2 genes. Sterol carrier protein-2 is a peroxisomal protein most highly expressed in non-steroidogenic tissues such as the liver and small intestine [25], and that plays a crucial role in the trafficking and metabolism of cholesterol and other lipids in mammalian cells - including the potential translocation of lipid hydroperoxides in oxidatively stressed cells [26]. Aldehyde dehydrogenase comprises a family of several isoenzymes [27, 28] involved in cell defense against both exogenous [27, 29] and endogenous aldehydes such as those derived from lipid peroxidation [30]. Class 2 aldehyde dehydrogenase is expressed in a large number of tissues and mediates an important role in aldehyde metabolism derived from lipid peroxidation in the liver [31].

Late up-regulation response was also induced by hydrodynamic DNA injection in relation to genes involved in contractile/cytoskeleton functions. Thus, no changes were observed after hydrodynamic injection of saline solution, though expression increased two days after hydrodynamic DNA injection - and this effect was greater 10 days later. Although the fold changes between saline versus DNA injected groups were 1.2 and 5.5 and 5.6 and 29.5, on days 2 and 10 respectively, we must consider that very low levels of gene expression were observed in the noninjected control group. It has been established that the liver possesses its own actin and myosin [32-35], and these filaments are distributed through the cytoplasm but are particularly concentrated beneath the plasma membranes in the region of the bile canaliculi [36-38]. Since bile canaliculi are cavities surrounded by the plasma membranes of adjacent liver cells and have no smooth muscle wall, it is reasonable to assume that actomyosin of the liver cells is the functional unit of this contraction, but also of vesicular transport which is energically perturbed by hydrodynamic injection - as has been reported in mice [4, 7], and more recently in a model of hydrodynamic injection in larger animals [39-40]. On the other hand, we have observed up-regulation of troponin C and I genes 10 days after hydrodynamic DNA injection. Troponin is a thin filament-associated protein of the myocyte with three components: troponin C, troponin I and troponin T. The expression of troponins I and C is mainly restricted to the skeletal muscle, but they also can be expressed in the liver [41, 42]. The expression pattern of these genes can be altered in fatty liver dystrophy, and this effect could be mediated by signaling pathways triggered by insulin and/or insulin-like growth factors which are crucial for the regulation of differentiation and metabolism [43], and which was down regulated after hydrodynamic injection, in our study.

Other genes mainly involving the lipid metabolism pathway were down-regulated after hydrodynamic injection. However, in a large number of cases gene expression down-regulation was mediated by unspecific mechanisms, since the effect was similar in the saline and DNA injected groups. The few cases in which down-regulation was more prominent in the DNA injected groups were associated with cholesterol metabolism (peroxisomal acyl-CoA thioesterase) and disorders such as atherosclerosis (ATP-binding cassette, phospholipids and phosphatidylcholine transfer proteins) and hypertension (CYP4A10) [44]. We finally comment that hydrodynamic injection appears to mediate only limited changes in host gene expression in the liver, but some of the down-regulated genes are associated to atherosclerosis or hypertension. Thus, the long-term effects on changes in liver gene expression induced by

hydrodynamic injection should be investigated in future studies.

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