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Gene expression variation between mouse inbred strains

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Abstract

Background: In this study, we investigated the effect of genetic background on expression profiles. We analysed the transcriptome of mouse hindlimb muscle of five frequently used mouse inbred strains using spotted oligonucleotide microarrays.

Results: Through ANOVA analysis with a false discovery rate of 10%, we show that 1.4% of the analysed genes is significantly differentially expressed between these mouse strains. Differential expression of several of these genes has been confirmed by quantitative RT-PCR. The number of genes affected by genetic background is approximately ten-fold lower than the number of differentially expressed genes caused by a dystrophic genetic defect.

Conclusions: We conclude that evaluation of the effect of background on gene expression profiles in the tissue under study is an effective and sensible approach when comparing expression patterns in animal models with heterogeneous genetic backgrounds. Genes affected by the genetic background can be excluded in subsequent analyses of the disease-related changes in expression profiles. This is often a more effective strategy than backcrossing and inbreeding to obtain isogenic backgrounds.

Background

Due to their isogenicity, inbred mouse strains demonstrate low biological variability within each strain[1,2]. Genetic variation between inbred strains is considerable and has recently been characterized in detail using single nucleotide polymorphisms[3]. Differences in genetic background between strains affect the gene expression levels of a subset of genes, which probably explains phenotypic differences. Indeed, several reports have been published in which gene expression profiles have been used as QTLs in genetic mapping studies to identify complex traits [4-6].

From literature [7-9], it appears that the subset of genes for which expression is significantly affected by genetic background is small. However, this has never been related to the extent of gene expression changes observed due to disease-causing mutations. We are studying differential gene expression between affected and healthy muscle in a range of murine models for neuromuscular disorders with

different genetic backgrounds (Turk *et al.*, manuscript in preparation). We, therefore, determined gene expression levels in hindlimb muscles from five frequently used wildtype mouse inbred strains, and compared these to the differential gene expression levels in affected muscle tissue from a mouse model (*mdx*) for Duchenne muscular dystrophy with healthy muscle tissue. Both the number of differentially expressed genes between strains as well as the fold-change levels are lower when compared to the differences found in affected versus healthy muscle tissue.

Results

Gene expression levels in hindlimb muscle tissue from five different inbred strains (CBA, BALB, BL6, DBA, and BL10) were determined. Total RNA from two individuals per strain was isolated, reversed transcribed, and subsequently labelled according to a recently developed protocol (adapted from Xiang *et al.*, 2002), which requires an input of only 1 μg total RNA. Labelled cDNA was hybridised to murine microarrays containing 7,776 65-mer oligonucleotides spotted in duplicate.

Significance levels (p-values) between the five mouse inbred strains were calculated using analysis of variance[10]. Significance levels among two individual mice within each strain were determined using a hierarchical *t*-test providing higher statistical power than conservative methods for low (2–4) replicate numbers[11]. The higher power is yielded by borrowing information across genes to produce a better expression variance estimator. The gain in power is reported via an increase in the degrees of freedom associated with the t-test. Differentially expressed genes for both computations were selected by controlling the false discovery rate (FDR), as suggested by Benjamini and Hochberg (1995), rather than using pre-defined cut-offs for p-values or corrections for multiple testing. The FDR represents an expectation of the proportion of false positives among the selected differentially expressed genes, which increases dramatically during multiple testing, inherent in microarray experiments[12].

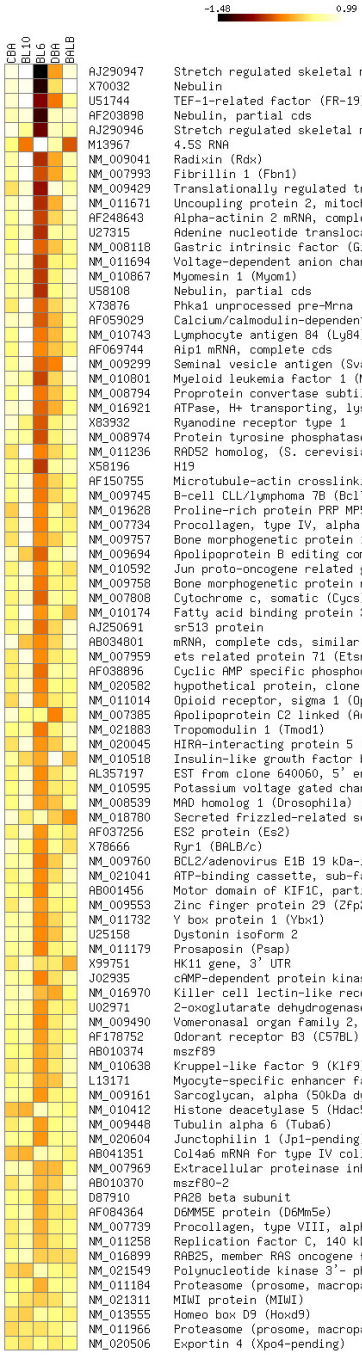
Using an FDR of 10% we selected 88 out of 6144 (1.4%) expressed genes that are differentially expressed between strains (Fig. 1). A lower number of differentially expressed genes was found in the analysis of variation within strains with identical FDRs of 10% (Table 1). Results with other FDR levels are available online as additional file. Correlation between gene expression levels of the two samples from each strain was high (Pearson correlation coefficient ranging from 0.87 to 0.95), also indicating low internal variation (Table 1). A considerable amount of differentially expressed genes (718 genes) were selected when predefined cut-off values ($p < 0.05$) were used to determine the differential gene expression between strains. However, adjusted FDR levels indicated a proportion of false positives equal to 42%. On the other hand, adjusting for multiple testing using Bonferroni correction proved to be too stringent, leaving no or few differentially expressed genes. Controlling the FDR, therefore, appears to be an optimal method for both selecting differential gene expression and simultaneously determining the validity of the experimental outcome.

To put the influence of differential gene expression due to genetic background in perspective, we studied gene expression between affected and healthy tissue from hindlimb muscle derived from *mdx* mice, and from control mice with identical genetic backgrounds. Selection with an FDR of 10% resulted in 1298 differentially expressed genes. Differential gene expression between the two most divergent mouse inbred strains (BL6 and CBA, data not shown) was determined to allow a direct comparison with identical statistical methods. Selection with an FDR of 10% showed an approximately ten-fold decrease in the number of differentially expressed genes (126). Absolute fold changes were calculated and subsequently a comparison of the distribution was made (Fig. 2). Median gene expression levels are equal between affected/control and inbred/inbred. However, the number of large fold changes (>3) between affected/healthy (221) is much higher than between inbred/inbred (7), consistent with low contribution of differential expression due to genetic background.

Although overall expression levels are similar between strains, a relatively high number of differentially expressed genes was due to deviating gene expression levels in BL6. We performed quantitative real-time RT-PCR (qPCR) on five genes to verify our microarray data. Two genes myomesin 1 and tropomodulin 1, which were 2.2-fold and 1.8-fold lower expressed in BL6 compared to the other strains on our microarrays, were also found to be lower expressed (2.0-fold and 2.2-fold respectively) in our qPCR assay (Fig. 3). Three other genes (dysferlin, cystatin B, and thrombospondin 4) showed no differential expression between any strains.

Discussion

This study shows that variation in overall gene expression levels between mouse inbred strains is relatively low in hindlimb muscle tissue. This is particularly evident when the number of differentially expressed genes between two mouse inbred strains (C57 vs. Bl6, 126 genes with 7 genes having a fold-change > 3) is compared to that between diseased and healthy muscle tissue (*mdx* vs. wild-type, 1298 genes with 221 genes having a fold-change >3). Therefore, the use of mice with deviating genetic background may be justified in disease-related studies. Alternatively, strain-dependent gene expression differences



AJ290947	Stretch regulated skeletal muscle protein (Usmg5)	signal transducer	Mn.29722
X70032	Nebulin	muscle development	
U51744	TEF-1-related factor (FR-19)	biological_process unknown	
AF203898	Nebulin, partial cds	muscle development	
AJ290946	Stretch regulated skeletal muscle protein (Usmg4)	biological_process unknown	Mn.27881
M13967	4.5S RNA	biological_process unknown	
NM_009041	Radixin (Rdx)	cytoskeletal anchoring	Mn.7051
NM_007993	Fibrillin 1 (Fbn1)	skeletal development	Mn.735
NM_009429	Translationally regulated transcript (21 kDa) (Trt)	protein folding	Mn.254
NM_011671	Uncoupling protein 2, mitochondrial (Ucp2)	ATP/ADP exchange	Mn.144413
AF248643	Alpha-actinin 2 mRNA, complete cds	invasive growth	Mn.10711
U27315	Adenine nucleotide translocase-1 (Ant1)	ATP/ADP exchange	Mn.16228
NM_008118	Gastric intrinsic factor (Gif)	cobalt ion transport	Mn.456
NM_011694	Voltage-dependent anion channel 1 (Vdac1)	apoptotic program	Mn.3555
NM_010867	Myomesin 1 (Myom1)	muscle development	Mn.4103
U58108	Nebulin, partial cds	muscle development	
X73876	Phka1 unprocessed pre-Mrna	biological_process unknown	
AF059029	Calcium/calmodulin-dependent protein kinase II delta	learning and memory	Mn.34377
NM_010743	Lymphocyte antigen 84 (Ly84)	cell surface receptor linked signal transduction	Mn.35692
AF069744	Aip1 mRNA, complete cds	biological_process unknown	Mn.42086
NM_009299	Seminal vesicle antigen (Sva)	transmembrane receptor protein tyrosine phosphatase signaling pathway	Mn.4119
NM_010801	Myeloid leukemia factor 1 (Mlf1)	GTPase activator	Mn.10414
NM_008794	Proprotein convertase subtilisin/kexin type 7 (Pcsk7)	proteolysis and peptidolysis	Mn.3255
NM_016921	ATPase, H+ transporting, lysosomal I (Atp6i)	positive control of cell proliferation	Mn.19185
X83932	Ryanodine receptor type 1	calcium ion transport	Mn.4519
NM_008974	Protein tyrosine phosphatase 4a2 (Ptp4a2)	DNA replication	Mn.6355
NM_011236	RAD52 homolog, (S. cerevisiae) (Rad52)	DNA repair	Mn.149
X58196	H19	biological_process unknown	
AF150755	Microtubule-actin crosslinking factor (Macf)	cytoskeletal anchoring	Mn.3350
NM_009745	B-cell CLL/lymphoma 7B (Bcl7b)	biological_process unknown	Mn.31
NM_019628	Proline-rich protein PRP MP5 (PRPMP5)	cell growth and/or maintenance	Mn.4491
NM_007734	Procollagen, type IV, alpha 3 (Col4a3)	cell adhesion	Mn.8069
NM_009757	Bone morphogenetic protein 15 (Bmp15)	oogenesis	Mn.42160
NM_009694	Apolipoprotein B editing complex 2 (Apobec2)	mRNA editing	Mn.27822
NM_010592	Jun proto-oncogene related gene d1 (Jund1)	transcription regulation	Mn.1175
NM_009758	Bone morphogenetic protein receptor, type 1A (Bmpr1a)	TGFbeta receptor signaling pathway	
NM_007808	Cytochrome c, somatic (Cycs)	electron transport	Mn.35389
NM_010174	Fatty acid binding protein 3, muscle and heart (Fabp3)	negative control of cell proliferation	Mn.22220
AJ250691	sr513 protein	biological_process unknown	Mn.52047
AB034801	mRNA, complete cds, similar to L-amino acid oxidase	apoptosis	Mn.26698
NM_007959	ets related protein 71 (Etsrp71)	transcription regulation	Mn.4829
AF038896	Cyclic AMP specific phosphodiesterase	olfaction	
NM_020582	hypothetical protein, clone:2-31 (LOC57423)	hydrogen transport	Mn.1674
NM_011014	Opioid receptor, sigma 1 (Oprs1)	steroid biosynthesis	Mn.22745
NM_007385	Apolipoprotein C2 linked (Acl)	biological_process unknown	Mn.8239
NM_021883	Tropomodulin 1 (Tmod1)	actin filament organization	Mn.139690
NM_020045	HIRA-interacting protein 5 (HIRIP5)	iron homeostasis	Mn.23809
NM_010518	Insulin-like growth factor binding protein 5 (Igfbp5)	receptor	Mn.578
AL357197	EST from clone 640060, 5' end	biological_process unknown	
NM_010595	Potassium voltage gated channel, shaker related subfamily, member 1 (Kcna1)	potassium transport	Mn.2493
NM_008539	MAD homolog 1 (Drosophila) (Madh1)	common-partner SMAD protein phosphorylation	Mn.15185
NM_018780	Secreted frizzled-related sequence protein 5 (Sfrp5)	establishment and/or maintenance of cell polarity	Mn.103731
AF037256	ES2 protein (Es2)	leading strand elongation	Mn.10094
X78666	Ryr1 (BALB/c)	calcium ion transport	
NM_009760	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3 (Bnip3)	apoptosis	Mn.2159
NM_021041	ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (Abcc9), transcript variant 2	potassium transport	Mn.35670
AB001456	Motor domain of KIF1C, partial cds	microtubule-based process	MM.90223
NM_009553	Zinc finger protein 29 (Zfp29)	transcription regulation	Mn.5010
NM_011732	Y box protein 1 (Ybx1)	response to pest/pathogen/parasite	Mn.21054
U25158	Dystonin isoform 2	developmental processes	
NM_011179	Prosaposin (Psap)	respiration	Mn.3363
X99751	HK11 gene, 3' UTR	biological_process unknown	
J02935	cAMP-dependent protein kinase type II regulatory subunit mRNA, 3' end	intracellular signaling cascade	Mn.3176
NM_016970	Killer cell lectin-like receptor subfamily G, member 1 (Klrg1)	cell surface receptor linked signal transduction	Mn.20434
U02971	2-oxoglutarate dehydrogenase E1 component (BALB/c)	glycolysis	
NM_009490	Vomeronasal organ family 2, receptor, 15 (V2r15)	ossification	Mn.34447
AF178752	Odorant receptor B3 (C57BL)	olfaction	
AB010374	mszf89	transcription regulation	
NM_010638	Kruppel-like factor 9 (Klf9)	transcription regulation	Mn.19788
L13171	Myocyte-specific enhancer factor 2 (MEF-2C) mRNA sequence	transcription regulation	Mn.24001
NM_009161	Sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein) (Sgca)	muscle development	Mn.18709
NM_010412	Histone deacetylase 5 (Hdac5)	repression of transcription from Pol II promoter	Mn.22665
NM_009448	Tubulin alpha 6 (Tuba6)	nuclear congression	Mn.88212
NM_020604	Junctophilin 1 (Jp1-pending)	protein folding	Mn.143761
AB041351	Col4a6 mRNA for type IV collagen alpha 6 chain	cell adhesion	Mn.155586
NM_007969	Extracellular proteinase inhibitor (Expi)	developmental processes	Mn.1650
AB010370	mszf80-2	transcription regulation	
D87910	PA28 beta subunit	cell adhesion	Mn.626
AF084364	D6MM5E protein (D6Mm5e)	mitochondrial processing	Mn.100652
NM_007739	Procollagen, type VIII, alpha 1 (Col8a1)	cell adhesion	Mn.86813
NM_011258	Replication factor C, 140 kDa (Recc1)	DNA replication	Mn.148877
NM_016899	RAB25, member RAS oncogene family (Rab25)	intracellular protein traffic	Mn.26994
NM_021549	Polynucleotide kinase 3'- phosphatase (Pnkp)	histidine metabolism	Mn.29545
NM_011184	Proteasome (prosome, macropain) subunit, alpha type 3 (Psma3)	ubiquitin-dependent protein degradation	Mn.1007
NM_021311	MIWI protein (MIWI)	cell growth and/or maintenance	Mn.87282
NM_013555	Homeo box D9 (Hoxd9)	transcription regulation	Mn.57122
NM_011966	Proteasome (prosome, macropain) subunit, alpha type 4 (Psma4)	ubiquitin-dependent protein degradation	Mn.30270
NM_020506	Exportin 4 (Xpo4-pending)	protein modification	Mn.143753

Figure 1

Differentially expressed genes between mouse inbred strains Relative expression levels of differentially expressed genes between mouse inbred strains are depicted in colour as relative intensity levels. Shown for each gene are GenBank accession number, description, functional annotation according to Gene Ontology, and UniGene cluster IDs. Relative expression levels are calculated by subtracting the average intensity value per gene from the strain-dependent intensity values. Differential expression was determined by selecting p-values from analysis of variance based on a false discovery rate of 10%.

Table 1: Number of differentially expressed genes using several cut-off strategies

	Between strains MA-ANOVA	**Within strains** Hierarchical *t*-test				
		CBA	**BL10**	**BL6**	**DBA**	**BALB**
Correlation		0.95	0.95	0.87	0.87	0.92
Naive ($p < 0.05$)	718	737	610	963	1043	483
Bonferroni	0	2	4	1	0	3
FDR 10%	88	2	4	14	0	16

Correlation between two individuals per strain was calculated using Pearson's correlation coefficient. Significance levels (p-values) between strains were calculated with MA-ANOVA, and within strains using the hierarchical *t*-test. Differential gene expression was determined by selecting genes with p-values lower than a specified threshold. Thresholds were selected using three different strategies; naive, Bonferroni corrected, and False Discovery Rate (10%), and resulted in different numbers of significantly differentially expressed genes.

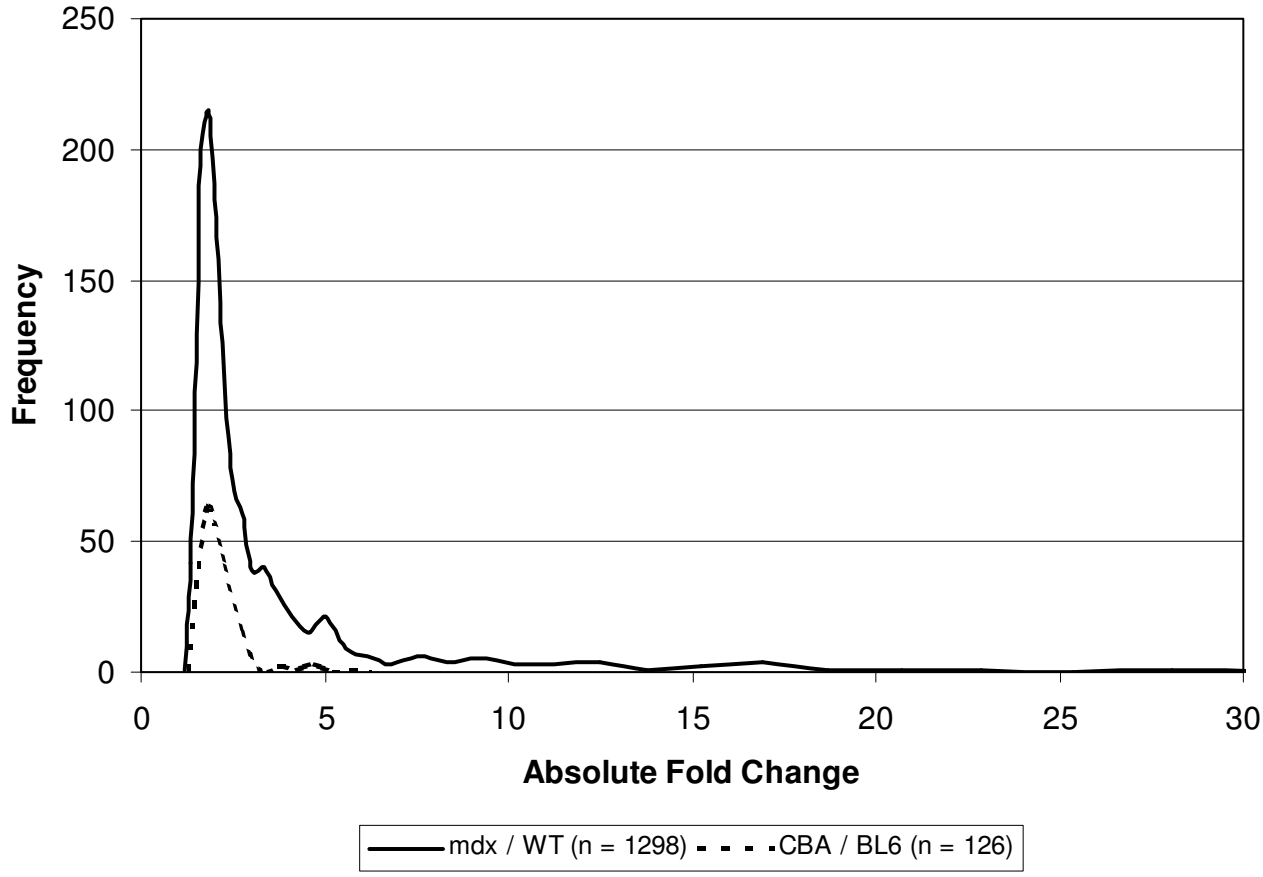


Figure 2
Effect of different genetic background on differential gene expression The distribution of absolute fold changes of differentially expressed genes (n = 1298) between affected (*mdx*) and healthy (WT) muscle were compared to the distribution of absolute fold changes of differentially expressed genes (n = 126) between two mouse inbred strains (CBA and BL6). Selections were based on a FDR of 10%.

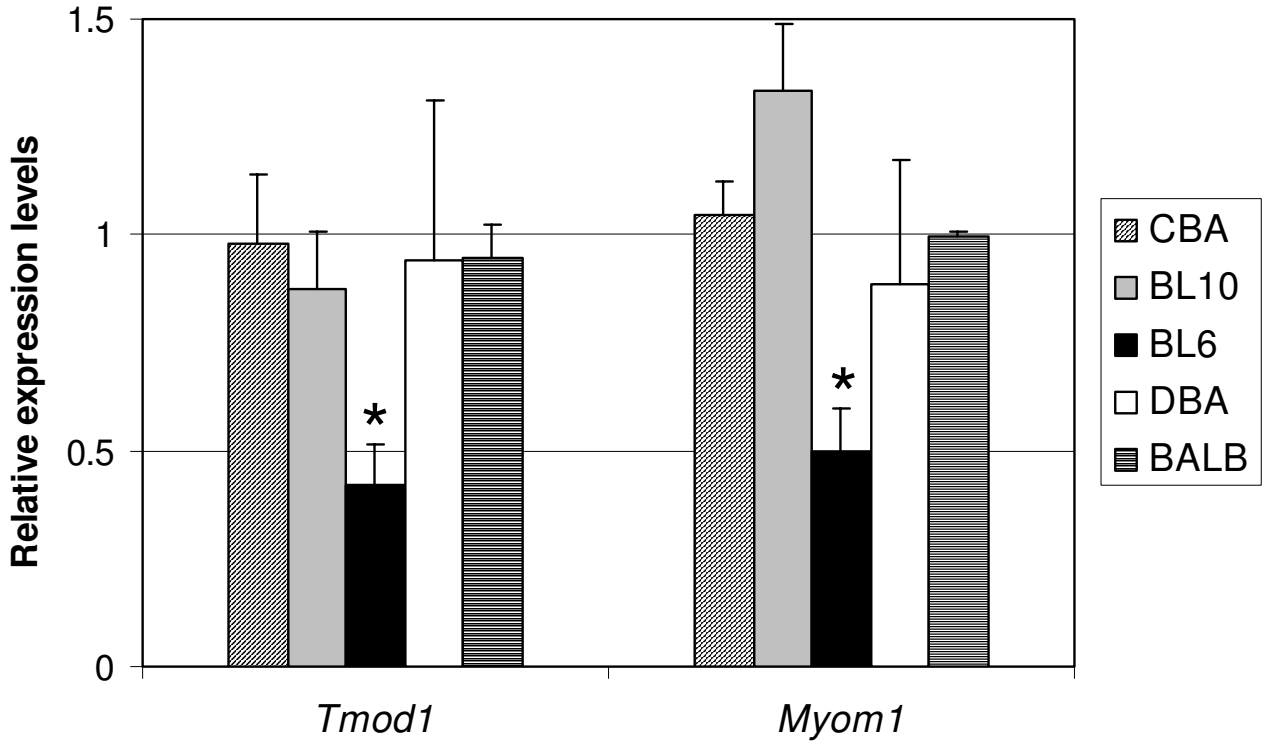


Figure 3
Validation of BL6-dependent gene expression with qPCR Relative gene expression levels between mouse inbred strains of tropomodulin 1 (*Tmod1*) and myomesin 1 (*Myom1*) as determined by quantitative RT-PCR. Significantly lower expression ($p < 0.01$, marked by *) for both genes was shown in BL6 compared to other strains.

may be evaluated in the initial study phase of gene targeting experiments, although the effect of hybrid backgrounds is difficult to assess.

Gene expression studies in the brain revealed that approximately 1% of expressed genes differ between two mouse strains[8]. Application of alternative statistical methods, similar to those used in our study, on this dataset resulted in an increase in the number of differentially expressed genes (approx. 3%) between the two mouse strains[7], demonstrating that the number of differentially expressed genes is highly dependent on the statistical criteria used. A similar number of differentially expressed genes was found in a comparison of hippocampal gene expression between 8 different mouse strains[9]. The results of our study in muscle tissue demonstrated that approximately 1.4% of the expressed genes show differential expression between mouse strains. Based on these results, strain differences in gene expression seem to have a similar magnitude across different tissues.

Genomic variability could be correlated with high levels of single nucleotide polymorphisms (SNPs) occurring in specific blocks between mouse inbred strains. The presence of cis-acting (single nucleotide) polymorphisms may be associated with regulatory variation affecting gene expression levels. It was estimated that probably a consistent amount (up to 6%) of the roughly estimated 35,000 mouse genes contain such functional regulatory variants[13]. We investigated if differentially expressed genes were localized in blocks with high genomic variability, but our number of differentially expressed genes was too low to obtain statistically significant answers (data not shown).

This study suggests an additional method for phenotyping mouse inbred strains and provides a list of genes with significant differential expression based upon false discovery rate selection. Although overall gene expression profiles are highly similar, most significant differences are determined by low gene expression levels of BL6 compared to the other strains. A large proportion of these BL6-specific genes function as structural muscle proteins (i.e. nebulin, alpha-actinin 2, myomesin 1 and radixin). To date, however, no major differences in muscle physiology in BL6-mice have been described which can be attributed to these reduced gene expression levels.

Perfectly isogenic backgrounds are sometimes difficult to obtain. This explorative study demonstrates that the effect of genetic background on muscle expression profiles is significant but rather limited compared to other effects, e.g. the dystrophic genetic defect (*mdx*) we study. As such, the genetic background will only marginally interfere with data analysis. Determination of gene expression profiles between mouse strains enables flagging a modest number of differentially expressed genes, and is an efficient and sensible approach to circumvent tedious backcrossings, necessary to obtain isogenic animals.

Methods

Mouse breeding, tissue preparation and total RNA isolation

We obtained CBA/CaOlaHsd (CBA), BALB/cOlaHsd (BALB), C57Bl/6JOlaHsd (BL6), DBA/2OlaHsd (DBA), and C57Bl/10ScSnOlaHsd (BL10) mice from Harland Laboratories, and C57Bl/10ScSn-Dmd$^{mdx/J}$ (*mdx*) mice from Jackson Laboratory at the age of 6 weeks. Mice were kept under standard conditions and were sacrificed by cervical dislocation when 8 weeks old. Hindlimb muscles (m. quadriceps femoris) were dissected and promptly snap-frozen in isopentane at -80°C. Total RNA was prepared by disrupting tissue using mortar and pestle and subsequent homogenisation by a rotor-stator homogenizor (Ultra-Turrax T25, Janke & Kunkel IKA-Labortechnik) in RNA-Bee (Campro Scientific) until uniformly homogenous (15–45 sec). Total RNA was isolated according to manufacturer's instructions followed by purification using RN-easy columns (Qiagen). Quality and yield was determined using Lab-on-a-chip (BioAnalyzer, Agilent).

Target preparation and hybridisation

Aminoallyl labelled cDNA (aa-cDNA) was prepared based on a previously described protocol[14]. Aliquots of 1 μg of total RNA in the presence of 2 μg amino-TN_6 primer (5'-NH_2-$(CH_2)_6$-TN_6, Eurogentec) were adjusted to a volume of 21 μl with DEPC-treated H_2O (diethyl pyrocarbonate, Sigma), heated for 10 minutes at 70°C and chilled on ice for 10 minutes. Reverse transcription mastermix (1.8 μl RevertAid RNaseH-M-MuLV reverse transcriptase (200 U/μl, MBI Fermentas), 6 μl 5x first-strand buffer (MBI Fermentas), and 1.2 μl 25x aa-dUTP / dNTP solution (2 μl 50 mM dATP, 2 μl 50 mM dCTP, 2 μl 50 mM dGTP, 1.2 μl 50 mM dTTP, 0.8 μl 50 mM aminoallyl-dUTP (Ambion)) was added per reaction and incubated at room temperature for 10 minutes followed by 2 hours at 42°C. RNA was hydrolysed by addition of 10 μl 0.5 M EDTA and 10 μl 1 M NaOH and incubation at 65°C for 30 minutes followed by neutralization by addition of 10 μl 1 M HCl. Aminoallyl labelled cDNA was then purified by combining 300 μl of PB-buffer (Qiagen) to 60 μl of the neutralized sample and centrifuged through a Qiaquick column (Qiagen) at 13000 rpm for 1 minute. Two washing steps were performed by spinning 500 μl of 75% EtOH at 13000 rpm for 1 minute while discarding the flow-through. To remove ethanol-traces the columns were centrifuged for an additional minute. cDNA was recovered by eluting three times using 30 μl basic H_2O (3.3 mM $NaHCO_3$ buffer, pH 9.0) and concentrated to a volume of 6.66 μl using a speedvac. Aliquots of Cy3 and Cy5 reactive dyes (PA23001, PA25001, Amersham) were prepared by dissolving each vial of monoreactive dye in 40 μl fresh anhydrous DMSO (Sigma) and dividing into aliquots of 2 μl followed by vaccuumdrying until dry and subsequent storage at 4°C in the presence of silica. Fluorescent dyes were coupled by adding 3.33 μl of bicarbonate buffer (1 M $NaHCO_3$ buffer, pH 9.0) to the aa-cDNA sample and dissolving the dried aliquot of reactive dye, followed by incubation at room temperature for 1 hour in the dark. To the samples 4.5 μl 4 M hydroxylamine (Sigma) was added and incubated at room temperature in the dark for 15 minutes, followed by addition of 186 μl TE^{-3}-buffer. Hybridisation mixtures were prepared by combining a Cy3-labeled cDNA sample with a Cy5-labeled cDNA sample and 10 μl Mouse-Hybloc (1 μg/μl, Applied Genetics Laboratories) followed by removing uncoupled dyes by spinning through a pre-wetted Microcon column (YM30, Amicon) for 8 minutes at 13000 rpm. Hybridisation mixture was washed by spinning 500 μl TE^{-3}-buffer through the column and discarding the flow-through. This step was repeated two times as 2 μl yeast-tRNA (10 μg/μl, Sigma) and 2 μl polyA-RNA (10 μg/μl, Sigma) were added during the last step. Mixture was collected by inverting the column and spinning for 1 minute at 13000 rpm. Hybridisation mixture was finalized by adding TE^{-3}-buffer to 84 μl together with 17 μl 20x SSC and 3 μl 10% SDS followed by denaturing at 100°C for 2 minutes, renaturing at room temperature for 15 minutes and spinning at 13000 rpm for 10 minutes. Labelled target was hybridised overnight on murine oligonucleotide microarrays (65-mer with 5'-hexylaminolinker, Sigma-Genosys mouse 7.5 K oligonucleotide library, spotted in duplicate). Hybridisation occurred in a automatic hybridisation station (GeneTac, Perkin Elmer) and was followed by washing with 5x

2xSSC + 0.1% SDS at 30°C, 5x 1xSSC at 30°C, 3x 0.2xSSC at 30°C, 1x 0.2xSSC at 65°C, 2x 0.2xSSC at 30°C, and subsequently scanned as described previously[15].

Experimental design, data extraction and analysis

Gene expression profiles from hindlimb muscle derived from 2 male animals of each strain were generated using dye-swap experiments. Subsequent duplicate spots on each array resulted in 8 replicate measurements per gene. Targets were assigned at random to the arrays, while avoiding co-hybridisation of samples from the same strain. GenePix Pro 3.0 (Axon) was used for feature extraction and quantification. Genes were considered as being expressed when the corresponding feature was not flagged by the algorithm provided by GenePix. Local background corrected spot intensities were normalized using Variance Stabilization and Normalization (VSN) in R [16]. Array data has been made available through the GEO data repository of the National Center for Biotechnology Information under series GSE662. Correlation between individuals was calculated using Pearson's correlation coefficient. Significantly differential expression levels were determined using MA-ANOVA (**MAANOVA2.0 The Jackson Laboratory** http://www.jax.org/staff/churchill/labsite/software/anova/), hierarchical *t*-test [11] and the False Discovery Rate [17] selection procedure.

Quantitative Reverse Transcription Polymerase Chain Reaction

qPCR was performed in duplicate for each individual resulting in four measurements per strain per gene. cDNA was prepared by reverse transcription using 1 μg total RNA as template. Random hexamers (40 ng) were used to prime the transcription after heating 10 minutes at 70°C followed by chilling on ice for 10 minutes. cDNA was synthesized by RevertAid RNaseH$^-$ MuLV reverse transcriptase and accompanying buffer (MBI-Fermentas) using 1 mM dNTPs. The mixture was incubated at room temperature for 10 minutes before a 2 hour incubation step at 42°C, followed by 10 minutes at 70°C. Quantitative PCR was performed using the Lightcycler (Roche). PCR mixture was prepared by combining cDNA dilution, 10 pmol forward and reverse primer, $MgCl_2$ (4 mM) with 4x homemade LC mastermix (0.9 mM dNTPs, BSA (1 μl/μl, Pharmacia Biotech), Taq polymerase (0.8 U/μl), 4x SYBR Green I (Molecular Probes), 4x AmpliTaq Reaction Buffer (Perkin Elmer)) to a total volume of 20 μl. Amplicons were generated during 45 cycles with annealing temperature set at 55°C. Optimal cDNA dilutions and relative concentrations were determined using a dilution series per gene. Replicate experiments (n = 4) were normalized to 1 and relative expression values were determined by calculating the ratio per gene over the average relative expression of genes, which show no differential expression on both microarray and qPCR (dysferlin, cystatin B, and thrombospondin 4). Significance levels were calculated with a one-sample *t*-test. PCR primer pairs were designed using the Primer3 search engine, available at: **Primer3 Software Distribution** http://frodo.wi.mit.edu/primer3/primer3_code.html. The screened genes and the oligonucleotide primer pairs used for each of the genes in this study correspond to the following nucleotides: myomesin1, 4761–4780 and 4865–4884 (NM_010867); tropomodulin1, 670–689 and 878–897 (NM_021883); dysferlin, 4218–4237 and 4353–4372 (AF188290); cystatinB, 3–22 and 151–170 (NM_007793); thrombospondin4, 2167–2186 and 2289–2308 (NM_011582).

Authors' contributions

RT carried out the tissue preparation, total RNA isolation, target preparation, hybridisations, experimental design, data extraction, data analysis, rt-PCR, and the drafting of the manuscript. PH participated in the experimental design, analysis, rt-PCR, and study coordination. ES participated in the experimental design and analysis. RM provided statistical support. EM was responsible for mouse breeding and tissue preparation. JM participated in experimental design. GO and JD coordinated the study. All authors read the final manuscript.

Additional material

Additional File 1

Differentially expressed genes between mouse inbred strains are selected with a false discovery rate of 10, 15, and 20%. Selected genes are indicated with 1, genes not selected by the specified criteria are indicated with 0. The mean of the relative gene expression levels of each of the five mouse strains is shown. For each gene the GenBank accession number is shown as well as the UniGene ID, gene description and the gene ontology description. The additional file is formatted as Comma Separated Values (CSV) file, and is named Turketal2004_Additional_File.csv.

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[http://www.biomedcentral.com/content/supplementary/1471-2164-5-57-S1.csv]

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