Loss of FMR2 further emphasizes the link between deregulation of immediate early response genes FOS and JUN and intellectual disability

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Loss of FMR2 causes Fragile X E (FRAXE) site-associated intellectual disability (ID). FMR2 regulates transcription, promotes alternative splicing with preference for G-quartet structure harbouring exons and is localized to the nuclear speckles. In primary skin fibroblasts from FRAXE patients (n = 8), we found a significant reduction in the number, but a significant increase in the size, of nuclear speckles, when compared with the controls (n = 4). Since nuclear speckles are enriched with factors involved in pre-mRNA processing, we explored the consequence of these defects and the loss of FMR2 on the transcriptome. We performed whole genome expression profiling using total RNA extracted from these cell lines and found 27 genes significantly deregulated by at least 2-fold at P < 0.05 in the patients. Among these genes, FOS was significantly upregulated and was further investigated due to its established role in neuronal cell function. We showed that (i) 30% depletion of Fmr2 in mouse primary cortical neurons led to a 2-fold increase in Fos expression, (ii) overexpression of FMR2 significantly decreased FOS promoter activity in luciferase assays, and (iii) as FOS promoter contains a serum response element, we found that not FOS, but JUN, which encodes for a protein that forms a transcriptional activator complex with FOS, was significantly upregulated in the patients’ cell lines upon mitogen stimulation. These results suggest that FMR2 is an upstream regulator of FOS and JUN, and further link deregulation of the immediate early response genes to the pathology of ID- and FRAXE-associated ID in particular.

INTRODUCTION

The Xq28 folate sensitive fragile site, FRAXE, is associated with an X-linked form of non-syndromic intellectual disability (ID; Mendelian Inheritance in Man, MIM 309548), affecting 1/50 000 males. Patients with FRAXE ID, here onwards referred to as FRAXE patients, show variable phenotypes of mild to borderline ID with or without speech delay, attention problems, hyperactivity and autistic behaviour (1,2). FRAXE ID is caused by an expansion of the CCG repeat at the 5′ end of the FMR2 gene (official gene symbol AFF2), which leads to the hypermethylation of the FRAXE CpG island and subsequent loss of FMR2 transcription (3). The normal size of the CCG repeat varies from 6 to 35, while repeat expansions over 200 triplets become methylated (3–6). At least five affected individuals carrying internal deletions of FMR2 have been described (3,6–8), demonstrating that loss of function of the FMR2 protein is the primary cause of FRAXE ID. More recently, an excess of non-synonymous missense variants in FMR2 has been reported in males with autism spectrum disorders (2), further implicating the role of FMR2 in normal brain function. The FMR2 gene is alternatively

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The longest FMR2 transcript is ~13.7 kb long and is abundant in fetal brain and pituitary gland (9).

FMR2/AFF2 belongs to the AFF (AF4/FMR2) family of proteins that also include AFF1/AF4, AFF3/LAF4 and AFF4/AF5q31 (10–12). The AFF proteins share some highly conserved functional domains, especially in their C-terminal regions (13). All four members have been found to localize in the nucleus, and consistently have been described as potent transcription activators (11,14–16). In particular, AFF1 and AFF4 interact with the positive transcription elongation factor B (P-TEFB) to form the super elongation complex (SEC) which facilitates transcription elongation via RNA polymerase II (PolII) (16–19). AFF2 and AFF3 can also interact with P-TEFB to form the SEC-like 2 and SEC-like 3 complexes, respectively, which can control PolII activity via phosphorylation of its subunits in vitro but not in vivo (19).

In addition, FMR2, AFF3 and AFF4 (but not AFF1) are localized to the nuclear speckles (13), which are sub-nuclear regions where pre-mRNA splicing factors are stored, assembled, modified and recycled (20). Overexpression of FMR2, AFF3 or AFF4 affected the recycling and organization of these structures in HeLa cells. In skin fibroblast cell lines from four FRAXE patients, the nuclear speckles were significantly enlarged, suggesting that FMR2, AFF3 and AFF4 had direct roles in the biogenesis of these structures (13). FMR2, AFF3 and AFF4 also act as splicing enhancers via the interaction with the G-quartet RNA forming structure (13,21).

The role of FMR2 in brain development and function is not well understood. *lilliputian*, the only ortholog of the AFF gene family in *Drosophila*, is indispensible for proper embryogenesis, as most of the *lilliputian* knockout larvae fail to hatch and display multiple defects in cellularization, segmentation and gastrulation (22). On the other hand, the *Fmr2* knockout mouse model does not exhibit any defects in embryogenesis. While no brain malformation was observed, the *Fmr2* knockout mice perform significantly worse in conditioned fear, memory and learning tests than normal littermates (23), which is similar to the clinical features of FRAXE patients. It has been suggested that other members of the AFF protein family could compensate for the loss of FMR2, thus reducing the severity of the neurological phenotype of the knockout mouse and FRAXE patients (24).

In this study, we further investigated the role of FMR2 in the pathology of FRAXE ID. We show that FMR2 is required for proper formation of the nuclear speckle structures. We also find that the expression of *Fos* and *Jun* is perturbed in FRAXE patients, strengthening the hypothesis that deregulation of early response genes is one of the molecular signatures for selected forms of ID.

**RESULTS**

FMR2 is required for proper biogenesis of the nuclear speckles

We had previously reported an increase in the size of nuclear speckles in skin fibroblasts from four FRAXE patients (13). In this study, we extended our analysis using primary skin fibroblasts from eight patients (Supplementary Material, Table S1), four of whom were used in previous study, and four unrelated controls. The patients studied do not have a functional FMR2 protein due to either transcription silencing of FMR2 (Patients 1–7 in Supplementary Material, Fig. S1) or deletion of exons 2 and 3 which causes frameshift (Patient 8 in Supplementary Material, Fig. S1). We studied the sub-nuclear distribution of nuclear speckle marker SC35 by immunofluorescence (Fig. 1A). The average size and number of nuclear speckles were analysed in 916 and 393 cells from the patients and controls, respectively. There was a significant increase in size of nuclear speckles (patients: 1.169 ± 0.014 μm² vs. controls: 1.016 ± 0.02 μm², Fig. 1B), but a significant reduction in their number per cell (patients: 43.41 ± 0.79 structures vs. controls: 61.98 ± 1.77 structures, Fig. 1C) in the patients’ cell lines. To test whether these changes were introduced by variable growth, we serum starved the cells for 20 h in order to synchronize them. After releasing the cells from serum starvation, we repeated the analyses described above and observed similar changes in the size and number of nuclear speckles in the patients’ cell lines (Supplementary Material, Fig. S2).

Since FMR2 has been found in the nucleolus, we also examined nucleoli number and morphology. We employed similar strategy using an antibody against nucleolar marker protein Fibrillarin. However, we did not observe any changes in the distribution and the appearance of nucleoli in the patients’ cell lines (data not shown).

**Genome-wide gene expression analysis of skin fibroblasts of FRAXE patients**

Given the role of FMR2 in gene regulation and alternative splicing together with the defects in the nuclear speckle size and number, we were interested to assess genome-wide consequences of loss of FMR2 expression. We tested total RNA extracted from the patients (*n* = 8, Supplementary Material, Table S1) and controls (*n* = 4) using Affymetrix Human Exon 1.0 ST array (exon array). Compared with the controls at *P* < 0.05 and mean expression difference threshold ≥2-fold, we identified 27 differently expressed genes (DEGs) in the patients (Fig. 2A and Table 1). Sixteen genes were upregulated, whereas eleven genes were down regulated. Twelve of these DEGs were associated with known morbid phenotypes (Table 1). We selected five genes, which were expressed in the brain and had known neuronal functions (Table 1), for further validation using reverse transcribed quantitative PCR (RT–qPCR). We were able to validate the gene expression changes in all five selected genes (Fig. 2B).

We also attempted to identify differentially spliced genes in FRAXE patients. However, we were not able to identify any reliable or significant alternative splicing events (data not shown). We also looked at the expression of known nuclear speckle genes, e.g. *CDKL5* (25), *SRSF1*, *SRSF2* and *MALAT1* (20), or other members of the splicing machinery, but did not see any significant difference. As inhibition of transcription has been shown to yield similar defects in the nuclear speckle size and number (21,26), we examined the overall correlation in gene expression among all samples studied. There was minimal impact on the transcriptome upon the loss of FMR2 as indicated by the principal component analysis (Supplementary Material, Fig. S3A) and absolute gene expression...
correlation calculated by Pearson’s correlation ($R > 0.9$, Supplementary Material, Fig. S3B). Therefore, it appears that the observed defects in nuclear speckle size and number were not consequences of significant transcription alteration, but were rather due to the loss of FMR2.

**FMR2 is an upstream regulator of FOS and JUN**

We sought to identify DEGs most relevant to the FRAXE ID phenotype. Two genes stood out as strong candidates for further analysis: GRIK2 (3.3-fold downregulated) for being implicated as the cause of an autosomal recessive ID (MIM 611092) (27); and FOS (5.2-fold upregulated) for its well established role in the regulation of neuronal cell survival, growth and function (28–30). More recently, an impaired response of FOS to serum mitogens has been detected in a non-syndromic autosomal recessive ID (MIM 614249) (31). In these neurons, we saw a 2-fold increase in the expression of Fos, but not Grik2 (Fig. 3A). Since FOS dimerizes with JUN to form the Activator Protein-1 (AP-1) transcription factor complex which regulates gene expression in response to a variety of stimuli, including growth factors (32), we questioned whether Jun expression level has also been affected in Fmr2-depleted cortical neurons. Although expression of JUN was not significantly deregulated in FRAXE patients’ cell lines by exon array analysis, we detected a 20% increase in Jun expression in mouse cortical neurons depleted of Fmr2 (Fig. 3A).

In order to understand how FMR2 regulates FOS expression, we used a luciferase gene reporter construct wherein the expression of luciferase is controlled by the FOS promoter. COS1 cells were co-transfected with the FOS reporter and either pTL1-empty vector or pTL1-FMR2 construct (21). A significant decrease in FOS promoter activity was observed in cells overexpressing FMR2 (Fig. 3B). The FOS promoter includes a serum response element. To check whether the
transcription activation of FOS in FRAXE patients’ fibroblasts could be induced by serum mitogens, we analysed FOS expression after serum starvation in two FRAXE patients’ and two controls’ skin fibroblast cell lines. FOS was significantly upregulated in the patients’ cell lines at 0 h time point, i.e. immediately before serum was added back into the culture; however, this difference was not maintained at the 30 min or subsequent time points (Fig. 3C). On the other hand, JUN was significantly upregulated at 0 h time point and remained significantly upregulated in the patients’ cell lines after 30 min of serum addition (Fig. 3D).

DISCUSSION

We demonstrate that the loss of FMR2 reduces the size and increases the number of nuclear speckles in cell lines of FRAXE patients (Fig. 1). In our previous study, we showed that overexpression of FMR2 caused the nuclear speckles to disassemble (13). Altogether, these findings suggest a role for FMR2 in biogenesis and/or remodelling of nuclear speckles, which are known to be very dynamic. It is interesting to underline that the protein SON, a transcription factor that is also a component of nuclear speckles, has a similar effect on nuclear speckle morphology when it is depleted (33). Due to the association of splicing and transcription during mRNA synthesis, we can also propose that FMR2 is a link between these two important events of mRNA metabolism; for instance, FMR2 may facilitate the interaction between serine/arginine-rich proteins and RNA PolII or participate in the recruitment of splicing factors to the RNA synthesis loci.

Our results suggest that FMR2 regulates the basal expression of both FOS and JUN, while it only regulates JUN expression upon mitogen induction (Fig. 3). The deregulation of both of these factors upon mitogen induction was recently shown in skin fibroblasts derived from an ID patient carrying a missense mutation in MED23. This gene encodes a protein member of the multiprotein mediator complex, which functions as a transcriptional coactivator and is composed of at least 31 subunits. In this instance, the mutated MED23 protein disrupted histone acetylation and methylation markers which regulated the activity of FOS and JUN promoters, subsequently altering their expression upon mitogen activation (31). It is then easy to hypothesize that a similar mechanism causes abnormal levels of FOS and JUN observed in our study (Fig. 3). Under this scenario, FMR2 should interact with the mediator complex. Interestingly, AFF1 was found to directly interact with 14 of the mediator complex subunits, for examples, MED1, MED12, MED14, MED17 as well as MED23 (34). Moreover, AFF1 and AFF4 are part of SEC which can regulate the transcription level of FOS and JUN (19). By homology and analogy, it is plausible that FMR2 can regulate FOS and JUN expression by interacting with either some members of the mediator complex or the SEC-like 2 complex and PolII.

FOS and JUN are members of the AP-1 complex, which is a transcription complex known to play a role in cell proliferation and neuronal activation. Consequently, failure to properly activate a neuronal response during learning and memory forming processes may be the underlying aetiology of this non-syndromic form of ID. Indeed, we were able to provide evidence to link the deregulation of FOS, and by inference AP-1 complex, to the deregulation of eight other DEGs identified in the patients’ cell lines (Table 1). First, Fos directly regulates the expression of Grik2 in mouse hippocampal neurons (28). Second, SERPINB2 and PTGS2 are direct targets activated by the AP-1 complex (35), while PPARG and LYPD6 directly regulate FOS/AP-1 expression (36,37). Finally, DAPK1, DUSP1 and IFIT1 expression were shown to positively correlate with FOS expression upon stimulations (38,39). As such, it is tempting to speculate that the deregulation of a large proportion of DEGs (~30%) in FRAXE patients’ cell lines was mediated through FOS and genes participating in the same pathways (Supplementary Material, Fig. S5). We attempted to validate this hypothesis by examining the expression of Grik2 in mouse cortical neurons depleted of Fmr2 and showing upregulation of Fos. However, we could not detect any change in the expression of Grik2. This was most likely due to either the low knockdown efficiency that we could achieve with this assay, the level of Fos upregulation which was lower in mouse neurons than in human skin.

![Figure 2.](http://example.com/fig2.png) Identification of DEGs as a consequence of loss of FMR2. (A) DEGs identified in FRAXE patients at the conservative threshold of P < 0.05, mean expression difference ≥2-fold. (B) RT–qPCR of five genes chosen for validation of the exon array results. Mean expression (±SD) from technical triplicates of FOS, SCIN, GRIK2, PNMA2 and LYPD6 of controls (n = 4) and FRAXE patients (n = 7) were calculated by the comparative Ct method using ACTB measured in the same sample as reference gene. Expression difference was obtained from ratio between the patient and the control groups, and converted to fold change.
## Table 1. DEGs identified in FRAXE patients

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Associated morbid phenotypes</th>
<th>Brain expression</th>
<th>P-value</th>
<th>Fold change</th>
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<tr>
<td>GRIK2</td>
<td>Glutamate Receptor, Ionotropic, Kainate 2</td>
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<td>DAPK1</td>
<td>Death-Associated Protein Kinase 1</td>
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<td>GPR37</td>
<td>G Protein-Coupled Receptor 37</td>
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<td>PNN1A</td>
<td>Paraneoplastic Antigen Ma2</td>
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<td>COMP</td>
<td>Cartilage Oligomeric Matrix Protein</td>
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<td>RAS4A</td>
<td>Ras P21 Protein Activator 4</td>
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<td>APOL1</td>
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<td>PDE4B</td>
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<td>Transmembrane Protein 51</td>
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<td>SERPINB2</td>
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<td>ASPN</td>
<td>Asporin</td>
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<tr>
<td>IFIT1</td>
<td>Interferon-Induced Protein With Tetracic peptide Repeats</td>
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<td>CDH10</td>
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<td>PTG2</td>
<td>Prostaglandin-Endoperoxide Synthase 2</td>
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<td>FOS</td>
<td>Fos Fbj Murine Osteosarcoma Viral Oncogene Homolog</td>
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<td>FBN2</td>
<td>Fibrillin 2</td>
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<tr>
<td>ABC9</td>
<td>Atp-Binding Cassette, Sub-Family C</td>
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Associated morbid phenotypes were extracted from the Online Medelian Inheritance in Man (OMIM). The level of gene expression (measured by RNA sequencing) in the brain was extracted from public database BrainSpan: http://www.brainspan.org.

The deregulation of FOS and JUN provides plausible explanation for the neuronal phenotype seen in FRAXE patients. In the normal situation, these two genes are expressed at low levels in neuronal cells; however, their expression is markedly elevated upon neuronal activation (40). The constitutive high levels in neuronal cells; however, their expression is markedly elevated upon neuronal activation (40). The constitutive high basal level of FOS and JUN in FRAXE patients might lead to over stimulation of downstream responses. This is supported by the enhanced long-term potentiation, another marker for neuronal activation, in the hippocampal slices of the Fmr2 knockout mice (23). As Fos has been shown to regulate neuronal excitability and survival via kainic acid receptor, which is encoded by Grik2 (28), it would also be interesting to assess the response of these mice to kainic acid induction. Finally, as non-synonymous missense mutations in FMR2 have recently been linked to autism spectrum disorders (2), our results provide a testable hypothesis to assess the possible functional consequences of these variants.

Our data reinforce previous findings that deregulation of immediate early response genes might represent a molecular signature for selected forms of ID. Additionally, we show that such deregulation (via FMR2) may be mitogen independent. Testing additional cell lines or induced pluripotent stem cells derived neurons from patients with FRAXE ID, other ID syndromes or autism spectrum disorders for the expression of FOS and JUN should be warranted.

## MATERIALS AND METHODS

### Cell culture, transfection and nucleofection

Primary skin fibroblast cell lines were established from skin punches of patients and controls, and maintained in RPMI (Sigma) supplemented with 10% fetal calf sera, 2 mM L-glutamine and 0.017 mg/ml benzylpenicillin (CLS). For cell synchronization, fibroblasts were serum-starved for 20 h in RPMI, 0.1% fetal bovine serum and 100 mg/ml penicillin/streptomycin and then cultured 6 h in RPMI, supplemented with 10% fetal bovine serum and 100 mg/ml penicillin/streptomycin. For FOS and JUN expression analysis, fibroblasts were serum-starved for 20 h in RPMI, 0.1% fetal bovine serum and 100 mg/ml penicillin/streptomycin and then cultured 30 min, 1 h and 3 h in RPMI, supplemented with 15% fetal bovine serum and 100 mg/ml penicillin/streptomycin.

COS1 cells were co-transfected with FOS-promoter-Firefly-Luciferase construct, a kind gift of Dr Schwartz (41,42), Renilla-reporter vector and pTL1-empty vector or pTL1-FMR2 construct (21) using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol.

Primary cultures of cortical neurons were obtained from wild-type mouse embryos at 18 days of gestation. Amaya mouse neuron Nucleofector kit (Lonza) was used for nucleofection of primary cortical neurons. Four million cortical neurons were resuspended in 100 μl of the nucleofection solution.
reagent. Thirty picomoles of Stealth™ RNAi negative universal control medium (Invitrogen) or three different Stealth™ RNAi siRNAs (Invitrogen, Supplementary Material, Table S2) directed against Fmr2 were mixed with cells and subsequently transferred into nucleofection cuvettes (Lonza). Cortical neurons were nucleofected by using the O-05 program in the nucleofection device (Nucleofector I) and pre-warmed culture media (DMEM supplemented with 10% fetal bovine serum, 10 μg/ml gentamicin) was immediately added into the cells. Nucleofected cells (two millions per plate) were seeded onto 60 mm culture plates (Nunc) pre-coated with l-ornithine (30 ug/ml, Sigma). After 4 h, media were carefully replaced with Neurobasal (Gibco) supplemented with B27 (Gibco) and 0.5 mm L-glutamine and primary cortical neurons cultures are incubated 72 h. All cells were cultured at 37°C, 5% CO2.

Luciferase assay
Assays were performed 16 h after transfection. Starting from cell monolayer in 96-well dishes, Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) and following the manufacturer’s instructions. Luciferase activities were measured using a luminometer. In each transfection, firefly luciferase values were normalized with Renilla luciferase values.

RNA extraction
Total RNA from cell pellets were extracted using a combination of Trizol Reagent (Invitrogen), RNeasy Mini Kit (QIAGEN) and RNase-Free DNase set (QIAGEN) according to the manufacturers’ protocols. RNA concentration was determined using the Spectrophotometer (Nanodrop).

Immunofluorescence and nuclear speckles analysis
For immunofluorescence experiments, 5000 cells of each fibroblast cell line were seeded into 24-well culture plate (Nunc) and cultured for 16 h. Cells were fixed in ice cold methanol:acetone (1:1) solution for 10 min at −20°C. Cover slips were air-dried and then cells were treated 45 min at 37°C with DNase I (10 U/cover slip, New England Biolabs) in DNase I buffer (40 mM Tris pH 7.4, 6 mM MgCl2, 150 mM NaCl) to enhance the antibody penetration. Cells were rinsed with PBS and incubated with monoclonal anti-SC35 (Abcam) at 1:2000 dilution factor, rinsed three times with PBS 0.1% Triton and incubated with secondary antibody: Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes). Cells were then rinsed with PBS and mounted in Dako fluorescent mounting medium (Dako). Immunofluorescence was analysed using a Zeiss fluorescence microscope (Axioplan2 Imaging) at ×63 magnification. Images were captured using a charge coupled device camera (CoolSNAP HQ) and Metavue software. Images were analysed by the ImageJ software after programming an automated Macro aiming to find cell nuclei, counting nuclear speckles and measuring their areas.

Immunoblot analysis
Protein extraction and immunoblot analysis were performed as described previously (43). The antibodies used in immunoblot
Microarray hybridization

Seven micrograms of total RNA extracted from fibroblast was sent to the Australian Genome Research Facility (Melbourne, Australia) for labelling and hybridizing to Affymetrix Human Exon 1.0 ST arrays (exon array).

Microarray analysis

The exon array contains ~1.4 million probesets targeting all known and predicted exons. We only utilized the core meta probesets (~250 000 probesets), which targets the most confident exons and have high specificity, for analysis. In short, intensity values were normalized using Robust Multi Array (44) analysis and log 2 transformed. Gene expression was calculated as median of all probesets targeting that gene. Significant difference in mean expression between groups was calculated using ANOVA-one-way assuming equal variance. Alternatively, spliced exon was determined using the Alternative Splicing ANOVA algorithm. All analysis was performed using Partek Genomics Suite V6.5.

Reverse transcription reaction

cDNA was synthesized from RNA using Reverse Transcription kit with Super Script RT III (Invitrogen) following the manufacturer’s protocol. The efficiency of the RT–PCR was determined by PCR using primers specific to the ubiquitously expressed ESD gene.

Polymerase chain reaction

RT–PCR was performed to check for FMR2 expression as previously described (45).

Real-time PCR validation

Expression of gene of interest was measured using gene specific primers (Supplementary Material, Table S2), and normalized against reference gene in the same sample using the comparative ct method. Reaction was set up as followed: 2 μl cDNA template, 2 μl of 10 mM primer pair, 10 μl of SYBR Green PCR Master Mix (Applied Biosystems) and 6 μl of H2O. RT–qPCR was carried out using a StepOne Plus Real Time PCR System (Applied Biosystems). Expression (± SD) was averaged from technical triplicates.

Statistical analysis

Student’s t-test was employed to assess difference in mean value between groups unless otherwise stated.


