SUPPLEMENTARY MATERIALS (MONACO *et al.*, 2009)

Histological Staining for Osteogenic Differentiation

Alizarin Red S Staining

Cells in 25 cm^2 flasks were rinsed with DPBS and fixed in 10% neutral buffered formalin for 30 min. Cells were rinsed with deionized water and incubated with 2% Alizarin Red S (Sigma, A5533) (pH 4.2) for 30 min. Cells were rinsed again with deionized water. Alizarin Red S chelates with the calcium cations present in the tissue to form an Alizarin Red S-calcium complex and positive cells containing calcium deposits appear red [1].

Alkaline Phosphatase Staining

Staining was performed with Sigma Leukocyte Alkaline Phosphatase Kit (Sigma, 85L3R). Briefly, cells in 25 cm^2 flasks were rinsed with DPBS, fixed in citrate buffered acetone for 30 sec and then rinsed with deionized water. Cells were stained with a dye mixture of Fast Violet B and Naphthol AS-MX Phosphate Alkaline solution for 30 min in the dark. After 30 min, cells were rinsed in deionized water and stained in Mayer's Hematoxylin solution for 10 min. Cells were then rinsed with tap water. Cells with alkaline phosphatase activity stain red.

Von Kossa Staining

Cells in 25 cm^2 flasks were rinsed with DPBS and fixed in 10% neutral buffered formalin (Fisher Diagnosis®, Kalamazoo, MI) for 30 min. The cells were washed 3 times with deionized water and stained with 5% silver nitrate (Sigma, 209139) for 30 min. The stain was removed and cells washed with deionized water and exposed to ultraviolet light for 1 h. The presence of black nodules indicates area of calcium deposition.

Histological Staining for Adipogenic Differentiation

Oil Red O Staining

Cells in 25 cm^2 flasks were rinsed with DPBS and fixed in 10% neutral buffered formalin for 30 min. Cells were rinsed with deionized water and then incubated with 60% isopropanol for 5 min. A concentrated stock solution of 2% Oil Red O (Sigma 0-0625) in 99% isopropanol (Sigma I-0398) was prepared. This stock solution was diluted with water for the staining assay. A staining solution of 3:2 (Oil Red O 2% stock: water) was prepared and filtered with common cotton filter 20 min before staining cells. The cells were exposed to this staining solution for 5 min, and then rinsed with tap water followed by counterstaining with Harris Hematoxylin (Sigma HHS-16) for 1 min. Positive cells show bright red intracellular lipid vesicle staining with a blue cell nucleus.

Quantitative Real-Time RT-PCR (qPCR) Analysis

RNA for analysis was diluted to 100 ng/μL using DNase-RNase free water and reverse transcribed into cDNA using 100 ng RNA, 1 μg dT18 (Operon Biotechnologies, Huntsville), 1 μL 10 mM dNTP mix (Invitrogen, Carlsbad, CA), 1 μL Random Primers (Invitrogen), and 10 μL DNase-RNase free water. The reaction was carried out in a Peltier Thermal Cycler PTC-200 (MJ Research, Watertown, MA, http://www.mjr.com) at 65ºC for 5 min and 4ºC for 3 min. A total of 6 μL of Master Mix composed of 4 μL 5X First-Strand Buffer, 1 μL M DTT, 0.25 μL (100 U) Superscript III RT (Invitrogen), 0.25 μL (100 U) RNase Inhibitor (Promega, Madison, WI), and 0.5 μL DNase-RNase free water was added. The mixture was incubated at 25ºC for 5 min, 50ºC for 60 min and 70ºC for 15 min. The cDNA was diluted 1:4 (v/v) with DNase-RNase free water prior qPCR reaction. The real-time RT PCR was performed using 4 μL of diluted cDNA in combination with 6 μL of a mixture composed of 5 μL 1x SYBR Green master mix (Applied Biosystems, Foster City, CA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase-RNase free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate along with a seven-point relative standard curve plus the non-template control. The 5-fold dilution standard curve was made using cDNA from an RNA pool of 4 swine tissues: liver, mammary gland, jejunum, and kidney. The amplification reactions were conducted in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) as follows: 2 min at 50ºC, 10 min at 95ºC, 40 cycles of 15 sec at 95ºC, and 1 min at 60ºC. The presence of a single PCR product was ensured by dissociation protocol using incremental temperatures to 95ºC for 15 sec plus 65ºC for 15 sec. Data were analyzed with the 7900 HT Sequence Detection Systems software (version 2.2.3, Applied Biosystems) using the 7-points standard curve.

Primers Design and Evaluation

Primer pairs were designed with Primer Express, version 3.0 (Applied Biosystems) using default features except for the amplicon length which was set ≥100 bp when possible with the purpose to facilitate amplicon sequencing. Major parts of the primers sets were designed to span exon–exon junctions. Due to the unavailability of pig genome for alignment of transcripts to find exons' junctions, the aligning of porcine annotated mRNA sequences was performed against the human genome [2]. Goodness of fit of primer pairs was assessed by alignment against public sequences database [3]. Features of the primer pairs for the14 selected differentiation gene markers and for the ICG are shown in Table **S1**.

Primers were tested in a 20 μL reaction using the same protocol as qPCR. Five μL of PCR product was analyzed by electrophoresis using a 2% agarose gel stained with ethidium bromide to check the presence of bands at the expected size and the absence of primer-dimer. Fifteen μL of PCR product were purified using PCR-purification kit (Qiagen). Products were then sequenced at the Core DNA sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana Champaign. (Table **S2** and **S3**). Only primers with absence of primer-dimer, unique amplicon and with a correct amplification product, tested by sequencing, were used.

Selection and Evaluation of the Internal Control Genes (ICG)

The assessment of appropriate internal control genes (ICG) to normalize qPCR data is essential to obtain reliable qPCR results [4]. The choice of the ICG for qPCR normalization to be used in this study was carried out following a protocol previously suggested [5]. A minimum of three genes should be used for a reliable normalization of qPCR data [4]. In this manuscript, the selection of the ICG for qPCR normalization was carried out considering several candidates among previously reported ICG used in stem cell differentiation or cell culture [6]. Thirteen potential ICG were selected as suitable candidates (Fig. **S1**). Ingenuity Pathway Analysis® (IPA, Redwood City, CA, www.ingenuity.com) was used to uncover potential co-regulation (i.e., common upstream regulators) among selected genes and geNorm (http:medgen.ugent.be/~jvdesomp/genorm/) was used to assess reliability among ICG tested [4].

Ingenuity Pathway Analysis®, a web-based software that enable to find all known relations and networks among genes, uncovered 8 genes without a known co-regulation (Fig. **S1**). In the name of practicality only 4 genes i.e. *GAPDH*, *GTF2H3*, *NUBP*, and *PPP2CB* were selected. *GAPDH* was chosen because commonly used as ICG. *GTF2H3* and *NUBP* were found to be stably expressed in undifferentiated and early differentiating human ESCs (hESCs) [6]. *PPP2CB* was observed to be stable in differentiating hESCs and also in different adult tissues and cell lines [6]. We ran the qPCR as described above and the 4 ICG values obtained were used for the pair-wise analysis by geNorm. The basic principle of geNorm to uncover reliable ICG has been described in details [4]. Briefly, gene-stability measure (M) refers to the constancy of the expression ratio between two non-co-regulated genes among all samples tested. The lower the M value, the higher the stability. The more stable the expression ratio among two genes, the more likely that the genes are appropriate internal controls. Two ideal control genes should have an identical expression ratio in all samples regardless of experimental conditions, cell, and/or tissue type. geNOrm provides also the optimal number of genes for normalization by calculating the pairwise variation (V) between normalization factor (NF) obtained using n genes (best references) (NFn) and NF obtained using n+1 genes (addition of an extra less stable reference gene) (NFn_{+1}). A large decrease in the pairwaise variation indicates that addition of a subsequent less stable genes (i.e., with higher M value) has a significant effect and should be included for calculation of NF [4]. Once the more stable genes are selected, the normalization factor (NF) is calculated using a geometric mean.

In our experiment geNORM uncovered PPP2CB and NUBP1 as having the largest stability in ratio expression among samples in osteogenic differentiation (Fig. **S2A**), and GTF2H3 and NUBP1 for the adipogenic differentiation (Fig. **S3A**). However, for the osteogenic differentiation the use of all 4 ICG had the maximal reliability (i.e. minimum pairwise variation, Fig. **S2B**) while for the adipogenic differentiation the NF calculated using 3 ICG resulted more stable than the one calculated using 4 or 2 ICG (M of 0.15 vs. 0.23 Fig. **S3B** and 0.38 Fig. **S3A**, respectively). Therefore, we calculated NF using geometrical mean of 3 genes (*GTF2H3*, *NUBP*, and *PPP2CB*) for the adipogenic differentiation, and 4 ICG for the osteogenic differentiation.

Quantification of Histological Staining

Osteogenic Stains – Alizarin Red, Alkaline Phosphatase and Von Kossa

Stained cell area was determined by threshholding with boundaries being the largest change in contrast. ADSC stained in circular nodules while BMSC stained in sheets. To quantify ADSC nodule radius areas of each were determined assuming perfect circularity.

$$\text{Radius} = (\text{Area} / \pi)^{1/2}$$

The amount of stained area was determined using thresh holding.

Adipogenic Stain - Oil Red O

Stained cells were randomly sampled using 5 properly calibrated light microscope images (Nikon Diaphot microscope) per staining technique at 100x or 200x magnification. Red, green and blue pixels were separated using a color deconvolution algorithm, H& E in ImageJ (National Institutes of Health). Nuclei were counted using blue images and assumed to match the number of cells present. The presence of lipid droplets was determined using the red image and quantified by threshholding. Cells with lipid droplets present were considered differentiated. Derived quantities were calculated as follows:

%differentiated cells = (# differentiated cells)/(total # cells)

Average stained area per cell = (total stained area)/(total # cells)

Table S1. GenBank Accession Number, Gene Symbol, Hybridization Position, Primer Sequence, and Amplicon Size of Primers Used to Analyze Gene Expression by qPCR

Accession no.	Gene	Primer[1]	Primer[2] (5'-3')	Amplicon Size (bp)
AY690660	*ACSL1*	F. 424	AGGCCTGAGTGGGTGATCATT	100
		R. 523	TGATGTAGGTGATGGCCTCAGT	
NM_214200	*ADFP*	F. 737	GGATCCCTGTCCACCAAGCT	123
		R. 859	TTCAATCAGGTTGACAGTGGAATG	
NM_214370	*ADIPOQ*	F. 51	GCAGTCTGTGGCTCTGATTCC	111
		R. 161	AGGCTTCTCGGTGGTTTCCT	
AY145131	*ALP*	F. 409	CCCTTCACTGCCATCCTGTAC	100
		R. 508	GGTAGTTGTCGTGCGCATAGTC	
AY150038	*BGLAP*	F. 34	GAGGGAGGTGTGTGAGCTCAA	105
		R. 138	GGCTGCGAGGTCTAGGCTATG	
NM_001044622	*CD36*	F. 747	CTCTTTCCTACAGCCCAATGGT	100
		R. 846	TACAGCTGCCACAGCCAGAT	
AF103944	*CEBPA*	F. 87	GCAACGTGGAGACTCAACAGAA	101
		R. 187	CCGCAGCGTGTCCAGTTC	
AF201723	*COL1A1*	F. 85	AGAAGAAGACATCCCACCAGTCA	105
		R. 189	CCGTTGTCGCAGACACAGAT	
NM_214119.1	*DBI*	F. 49	CAGGCGGAGTTTGAGAAAGCT	100
		R. 148	TCGCTTGTTTGTAGTGGCTGTAG	
NM_032564.3	*DGAT2*	F. 432	TACTTCACTTGGCTGGCGTTT	120
		R. 551	CACCAGCTGGATGGGAAAGTAG	
AF017079	*GAPDH*	F. 1257	GTCAAGCTCATTTCCTCGTACGA	90
		R. 1346	CTCTTACTCCTTGGAGGCCATGT	
AK240475	*GTF2H3*	F. 1222	CATGCGAGACAGCCTTTAAGATT	110
		R. 1331	CAGCTCTACATGATGGAGAAAAAATT	
AK233504	*NUBP1*	F. 416	ACTGGAGGGAGAACAGGTTCAC	105
		R. 520	TCAGGACTGCTGAGCAAGAAAC	
NM_214379	*PPARG*	F. 1190	GAGCCCAAGTTCGAGTTTGC	100
		R. 1289	GGCGGTCTCCACTGAGAATAAT	
M20193	*PPP2CB*	F. 258	CATTAAAGGTGCGGTATCCAGAA	100
		R. 357	CGCAGACATTCGTCATAAAAGC	
NM_213781	*SCD*	F. 805	TGGTGATGTTCCAGAGGAGGTACTA	120
		R. 924	TGGCGACGAACAGGCTTT	
AY963262	*SPARC*	F. 313	TTTGAGAAGGTATGCAGCAATGA	100
		R. 412	GCTTGTGGCCCTTCTTGGT	
X16575	*SPP1*	F. 128	TAAAGCCTGACCCATCTCAGAAG	100
		R. 327	TTGCTTGGCAGGGTCTCTTG	

[1] Primer direction (F = forward; R = reverse) and hybridization position for each primer **(5'-3').**
[2] Exon-exon junctions in primer sequences are underlined.

Table S2. Sequencing Results Using BLASTN from NCBI

(http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on) against nucleotide collection (nr/nt)

Gene	Best Hit in NCBI	% Identity
ACSL1	Sus scrofa acyl coenzyme A synthetase long-chain 1 mRNA, partial	100.0
ADFP	Sus scrofa adipose differentiation-related protein (ADFP) mRNA, complete cds	90.0
ADIPOQ	Sus scrofa adiponectin mRNA, complete cds	97.0
ALP	Sus scrofa alkaline phosphatase mRNA, partial cds	95.0
BGLAP	Sus scrofa osteocalcin mRNA, partial cds	96.0
CD36	Sus scrofa CD36 molecule (thrombospondin receptor) (CD36)	98.0
CEBPA	Homo sapiens CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA) gene, complete cds	90.0
COL1A1	Sus scrofa type I collagen alpha1 mRNA, partial cds	95.0
DBI	Sus scrofa endozepine (DBI) mRNA, complete cds	100.0
DGAT2	PREDICTED: Canis familiaris similar to diacylglycerol O-acyltransferase 2 (LOC485185), mRNA	86.0
GAPDH	Sus scrofa glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, complete cds	100.0
GTF2H3	PREDICTED: Sus scrofa similar to general transcription factor IIH, polypeptide 3, 34kDa (LOC100152121)	93.0
NUBP1	Sus scrofa mRNA, clone:LVRM10161D05, expressed in liver	95.0
PPARG	Sus scrofa peroxisome proliferator-activated receptor gamma 2 mRNA, complete cds	92.0
PPP2CB	Porcine protein phosphatase 2A beta subunit mRNA, complete cds	91.0
SCD	Sus scrofa stearoyl-CoA desaturase (SCD) gene, exons 1 through 6	97.0
SPARC	Sus scrofa partial mRNA for secreted protein, acidic, cysteine-rich (SPARC gene)	97.0
SPP1	Sus scrofa secreted phosphoprotein 1 (SPP1) mRNA, complete cds	96.0

Table S3. Sequencing Results Obtained from PCR Products

Gene	Sequence
ACSL1	TTGCTTATTCCATGGTGGTCGTCCCCCTCTACGACACCCTCGGAACTGAGGCCATCACCTACTCAA
ADFP	CAGTCATCACAGCAGCTCACCAGGTTAAGAAGTCAAGCAAAAAAGCCAGGAGACCATTTC-TCAGCTCCACTTCTCACGTGTCAACCTGATTGAAA
ADIPOQ	CGATAGCTAGATGCTGTGTGGAGCTGTTCTACTGCTACTAGCCCTGCCCAGTCTCGGC-CAGGAAACCACCGAGAAGCCTAGTAGCCCTGCCCAGTCTCGGCCAGGAAACCACCGAGAAGCCTA
ALP	GTGCTGGTCAGGTGGTGGGTGGTGAGAGAGAGACGTCTCCTGGTGGACTATGCGCCGACAACTACCTATC
BGLAP	GCGTGCTGATCACATCGGCTTCAGGAGGCCTATCGGCGCTTCTATGGCATAGCCTAGACCTCAGCAGCCA
CD36	GACTGTACTCGTATCAGTTGGACAGAGAACGACACCTTCACTGTTCTCAATCTGGCTGTGGCAGCTGTAAC
CEBPA	GCTACGATCGGCAGGTCATGACGCCTGCGCAGCGGGTGGACACTGAGCCGCGACTGGACACGCTGCGCGA
COL1A1	AGAAGCTCAGGTACCATGACCGAGACGTGTGGAACCCGTGCCCTGGCCAGAATCTGTGTCTGCGACAACGGAA
DBI	GCGTAGTAGAACTTAGACAACCAGCAGATGATGAGATGCTGTTCATCTACAGCCACTACAAACAAGCGACA
DGAT2	CGTGTCATACGCGCTCTACTCACTGGCTGGCGTTGACATGGACACGCCAAGAAAGGTGGCCGGAGGCTAT
GAPDH	GCACAGGGTGGTGGACCTCATGGTCCACATGGCCTCCAAGGAGTAAGAG
GTF2H3	GTTACGTAGGCAGAAAAGAACTGAAAAATGTCCCTCATGATAGTAAAAATTTTTTCTCCATCACTGTAGAGCGTGGA

(Table 3) contd….

Gene	Sequence
NUBP1	CTCGGCTGTCTCAGTGTTCTTGGAGACAACTTGGGGGTGATGTCGGTGGGTTTCTTGCTCAGCAGATACCTGAA
PPARG	CGTGATAGAGTACAGCGACCGTGCGATATTTATAGCTGTCATTATTCTCAGTGGAGACCGCCAA
PPP2CB	TCAATATTGAGAGAACACGAGAGCCGACAATTACCCAAGTATATGGCTTTTATGACGAATGTACTGCGCATAA
SCD	CACCGATCTGTGATGTGCTTCATCCTGCCACGATCGTGCCCTGGTATTGCTGGGGTGAAG-CTTTTCCCAAAGCCTGTTCGTCGCCAA
SPARC	CTTTGACTTCCTGCCACTTCTTTGCCACCAAGTGCACACTGGAGGGCACCAAGAAGGGCCACAaGC
SPP1	GCTTCTGGCCGATACTATTTCCTCGGAGGAACGGACGACTTCAACAAGAGACCCTGCCAAGCAAATTT

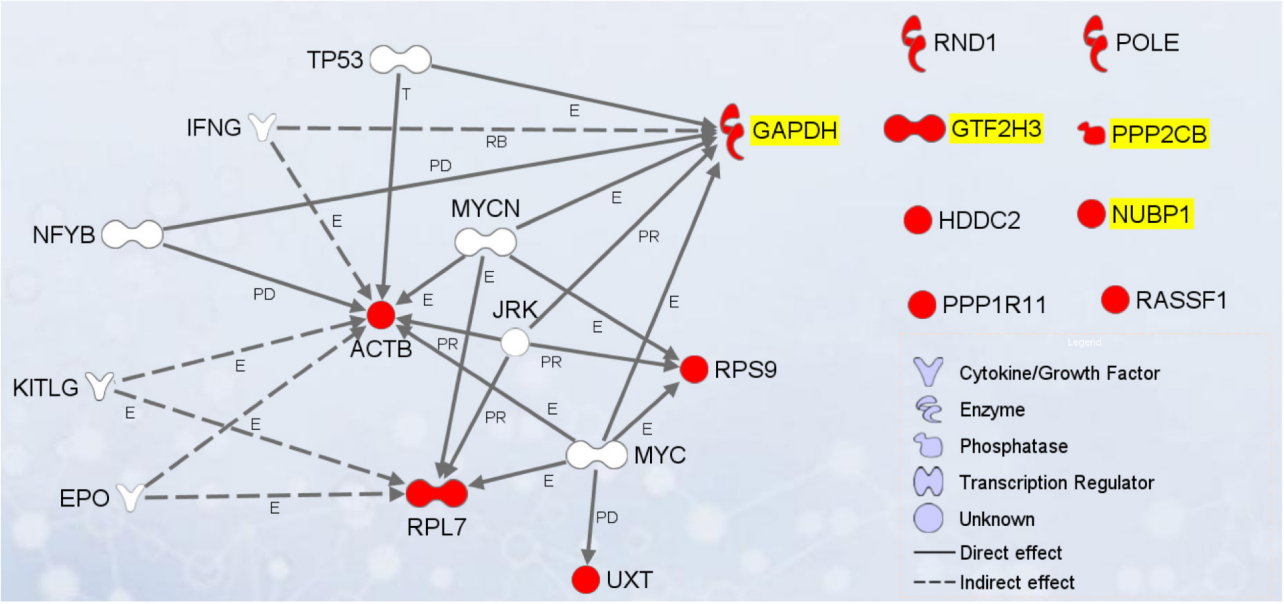

Fig. (S1). Co-regulation among selected internal control genes (ICG). The networks among genes considered all the possible relationships with a direct or indirect effect on transcripts, as denoted by the letter on the edges. For the purpose only effect on expression (E), protein-RNA binding (PR), protein-DNA binding (PD), and effect on transcription (T) were selected. In red are denoted the ICG and highlighted in yellow are denoted the 4 ICG selected for further analysis. Shapes of the nodes are described in the legend. Ingenuity Pathway Analysis® (IPA) uncovered co-regulation (common up-stream regulators) among several ICG, such as *ACTB*, *GAPDH*, *RPL7*, *RPS9*, and *UXT*. The other ICG did not have a known co-regulation.

(Fig. S2) contd….

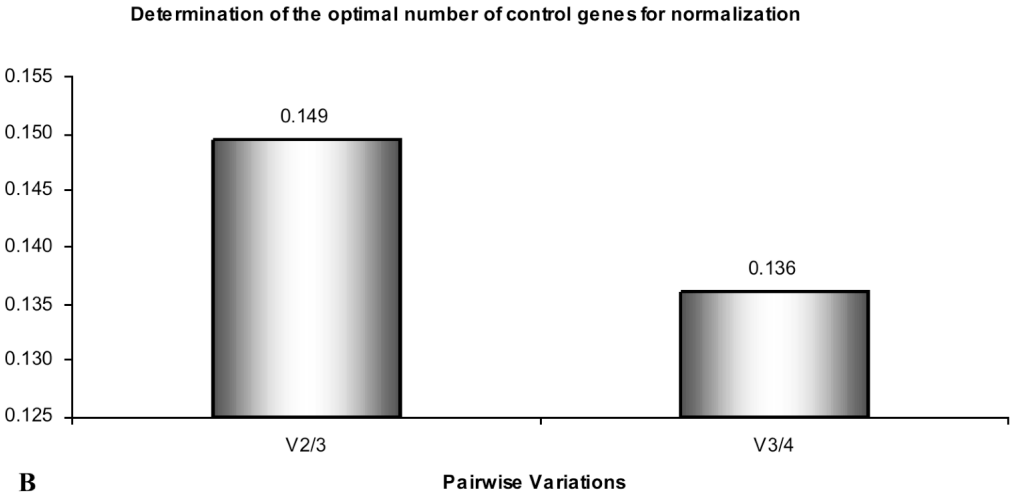

Fig. (S2). A - Average stability of expression ratio values (M = gene-stability measure) of remaining genes tested during pairwise comparison in **osteogenic differentiation**. Values are reported as stepwise exclusion of the least stable control gene. **B** - Determination of the optimal number of control genes for normalization. Y-axis, pairwise variation V (V_n/n+1) between the NF_n (normalization factor) and NF_{n+1}. X-axis, comparison between the use of *n* or *n*+1 genes to calculate the NF, i.e., V2/3 is the comparison between the use of 3 vs 2 ICG to calculate the NF. Figures generated by geNorm.

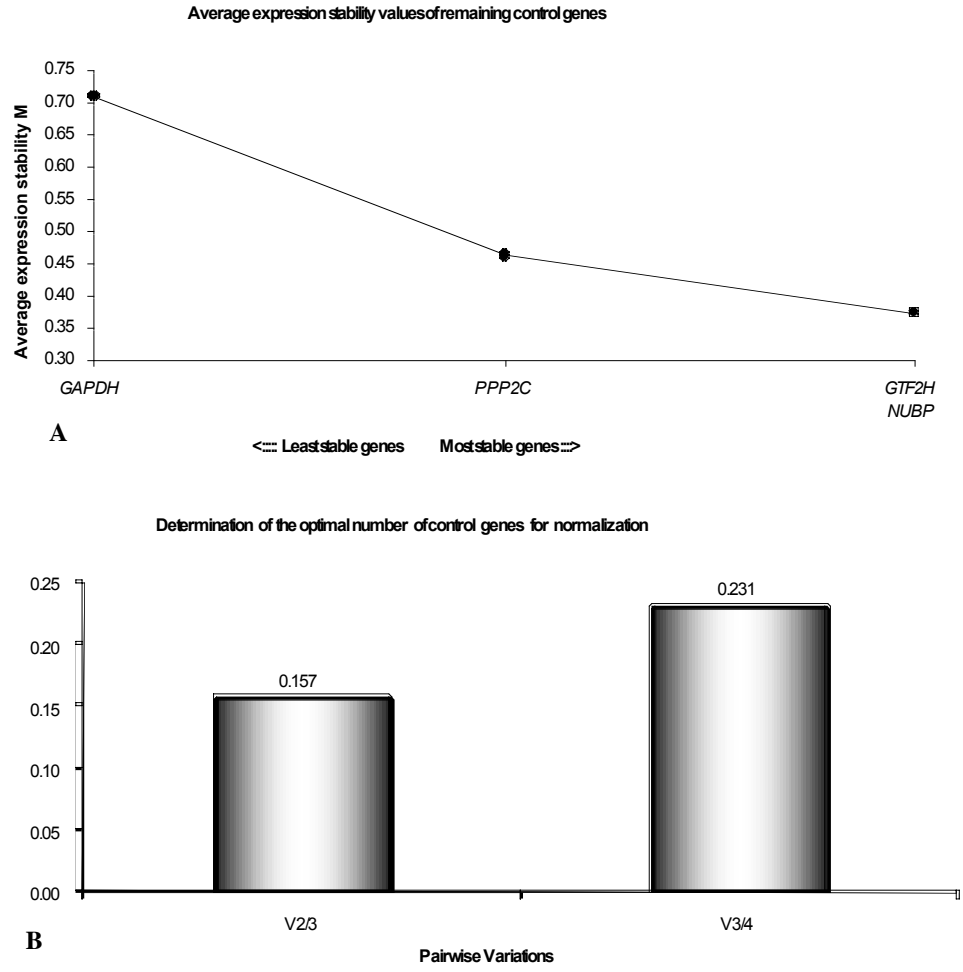

Fig. (S3). A - Average stability of expression ratio values (M = gene-stability measure) of remaining genes tested during pairwise comparison in **adipogenic differentiation**. Values are reported as stepwise exclusion of the least stable control gene. **B** - Determination of the optimal number of control genes for normalization. Y-axis, pairwise variation V (V_n/n+1) between the NF_n (normalization factor) and NF_{n+1}. X-axis, comparison between the use of *n* or *n*+1 genes to calculate the NF, i.e., V2/3 is the comparison between the use of 3 vs 2 ICG to calculate the NF. Figures generated by geNorm.