Technical Report

Rapid Selection of Differentially Expressed Genes in TNF α -activated Endothelial Cells

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Abstract

Background: RNA differential display (DD) RT-PCR is a useful method to identify and clone differentially expressed genes. However, the rate of false positives and redundancy associated with this PCR-based method as well as laborious downstream screening steps constitute major limitations. Here we present DD RT-PCR and reverse northern (RN) protocols allowing rapid and accurate identification of genes upregulated in porcine endothelial cells (EC) in response to TNF α .

Materials and Methods: The housekeeping gene β -actin was used to investigate mispriming and to set up optimal conditions for DD-RT-PCR and RN. In this study DD was performed to compare resting and TNF α -activated ECs.

Selection of DD-fragments was performed following 30-cycles of PCR using serial dilutions of template cDNA and regulation of 6 out of 17 candidate genes were first confirmed by semi-quantitative RN.

Results: Using this protocol, 5 out of 6 DD-fragments were further confirmed to be upregulated by Northern blot, and 3 novel porcine cDNAs were cloned including the pro-apoptotic member of the Bcl-2 family, Noxa.

Conclusion: In this study we demonstrate that the combination of DD-RT-PCR and RN, which efficiently reduces the number of false positive candidates derived from mispriming at the screening step, allows a rapid identification of differentially expressed genes.

Introduction

RNA differential display (DD) RT-PCR is a useful tool to identify differentially expressed genes in various biological systems (1–3). This technique has many advantages including the small amounts of RNA samples required, its rapid and easy technical procedure, the possible multiple comparison between samples in one study, the sequence dependent amplification, and its low financial cost, compared to other techniques. Comparison of electronic subtraction, subtractive hybridization and DD in the same cell type has concluded that DD is a straightforward method (4), whereas the fact that no common

positive transcript was found between these three methods remains to be investigated. It was also emphasized that redundancy was not mRNA abundance-dependent for DD-RT-PCR due to the mechanism of primer sequence-dependent amplification in this technique.

Discordant xenotransplantation results in hyperacute rejection and acute vascular rejection (AVR), due to preformed anti-donor antibodies and proinflammatory cytokines. Activated monocytes and macrophages secrete a broad panel of cytokines, including tumor necrosis factor- α (TNF α), which affects the functions of endothelial cells (EC). TNF- α is known to upregulate the expression levels of a variety of genes in ECs to include E-selectin, vascular cell adhesion molecule-1, intracellular adhesion molecule-1 (5–7). As a result of these phenotypic and functional changes, EC activation plays a crucial role in the initiation of AVR in the pig to primate organ xenotransplantation models. Several studies have focused on the activation of porcine

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aortic endothelial cells (PAEC) stimulated by human TNF- α (8). We have studied vascular EC activation in organ transplantation, including the expression of cytokines and adhesion molecules and the effects of immunosuppressive drugs on their expression at the mRNA level (9,10). This directed us to study EC activation on a molecular basis and to employ DD-RT-PCR to identify porcine genes which are differentially expressed upon TNF α -mediated activation as performed in human ECs (11).

A high frequency of false positive, for which differential expression cannot be verified by Northern blot or other methods, is usually associated with DD-RT-PCR and remains problematic. The majority of «false positives» results from the re-amplification of co-migrated surrounding PCR products and several methods have been developed to exclude these contaminants (12). However, co-migration of PCR products alone cannot explain this phenomenon. Frost, et al. have demonstrated that two types of mispriming events can frequently occur during the PCR step; mispriming of the primer in the poly(A) tail anchored region and priming at an internal site on the mRNA rather than the poly(A) tail (13). Mispriming during the reverse transcription (RT) reactions leads to repetitive oversampling in subsequent polymerase chain reactions (PCR), in addition to mRNA abundance-dependent amplification. Consequently a variety of bands amplified from a single mRNA may be visible on gels, leading to DD-fragments which do not reside at the 3'-end of the mRNA. To rule out these possibilities, we present a DD-RT-PCR protocol associated with several criteria for the selection of DD-fragments, aimed to reduce the number of candidate genes selected. Subsequently, reverse northern (RN) was employed as a first screening step to rapidly exclude «false positive» candidates, with an evaluation allowing selection of cDNAs derived from the 3'-end of mRNAs. Finally, gene up-regulation was confirmed by conventional northern blot. As a result, three novel porcine cDNA clones, corresponding to TNF α -responsive genes, were isolated from a cDNA library. One of the genes found upregulated in TNF α -activated ECs was the pro-apoptotic member of the Bcl-2 family, Noxa. In conclusion, this study demonstrated that a combined screening approach including RNA DD and comprehensive RN is a suitable methodology to detect and rapidly clone differentially expressed genes.

Materials and Methods

Cell Culture and RNA Preparation

PAECs were isolated from porcine aortas using collagenase A (1 unit/ml, Boehringer Mannheim, Germany), as previously described (9), and cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco BRL), 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml

streptomycin (Gibco BRL) in gelatin-coated tissue culture plates. PAECs were incubated with recombinant human TNF α (rTNF- α was kindly provided by Dr. Müller Neumann, Ludwigshafen, Germany) for 12 h at 100 units/ml or with culture medium alone as control. Total RNA was isolated by TRIzol (Gibco BRL). Three micrograms of total RNA were treated with DNase I (Boehringer, Mannheim) and precipitated with 2-isopropanol following phenol-chloroform extraction. The quality of RNA preparations was assessed by both OD260 measurement and gel quantification to adjust the volume employed in the following procedures.

RT-PCR for β -actin

RT reactions were performed using 2 μ g RNA from unstimulated PAEC primed with 12.5 μ g/ml oligo (dT)25–30 in a buffer containing 625 μ M dNTP, 10 mM DTT, 40 units RNase inhibitor (LifeScience, Gaithersburg, MD), and 200 units Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco BRL) in a final volume of 20 μ l. PCR was performed on 1 μ l of the final RT reaction mixture using the upper primer (5'-ATGTTTGAGACCTTCAACACGCC-3', nucleotides 1–23 in Genbank accession no. U07786), and the lower primer (5'-GCAGCTCGTAGCTCTTC-TCCAG-3' nucleotides 361–340) in a buffer containing 20 μ M dNTP, 1.5 mM MgCl₂, 0.5 U Taq polymerase (LifeScience), and 0.1 μ l [³²P]dATP in a final volume of 10 μ l. The PCR conditions were as follows: 94°C $\times$ 30 sec, 65°C $\times$ 30 sec, 72°C $\times$ 30 sec for 20, 30 or 40 cycles flanked by a 94°C $\times$ 5 min denaturation and 72°C $\times$ 5 min extension steps using a GeneAmp PCR system 9700 (Perkin Elmer). This reaction was carried out in triplicate with serial ten-fold dilutions of cDNA template. Following the addition of 6 μ l of loading dye, 5 μ l of PCR products were separated on a standard 6% polyacrylamide sequencing gel (7.5 M urea, PAGE) at 60 V for 3 to 4 hrs. The gel was subsequently dried onto Whatman paper and analyzed by autoradiography after exposure to photographic X-ray film.

RNA Differential Display RT-PCR Protocol

RNAimage kits were purchased from GenHunter Corporation (Nashville, TN). RT reactions were performed using 0.2 μ g DNase-treated RNA primed with 0.2 μ M one-based-anchored H-T11V oligonucleotides (V represents A, C, or G), respectively (14), in the same buffer as described above. PCR was performed on 1 μ l of the final RT reaction mixture with 0.2 μ M H-T11V and an arbitrary 13-mer (H-API-H-AP6) in the same buffer as above under the following conditions: 94°C $\times$ 30 sec, 42°C $\times$ 2 min, 72°C $\times$ 30 sec for 30 cycles flanked by a 94°C $\times$ 5 min denaturation and a 72°C $\times$ 5 min extension steps. This reaction was also carried out in triplicate in serial ten-fold dilutions of cDNA template. Different approximate concentrations of DNA templates were derived from 100 ng, 10 ng and 1 ng of

DNase-treated total RNA. H-AP arbitrary primers were as follows:

H-AP1 (5'-AAGCTTGATTGCC-3'),
H-AP2 (5'-AAGCTTCGACTGT-3'),
H-AP3 (5'-AAGCTTTGGTCAG-3'),
H-AP4 (5'-AAGCTTCTCAACG-3'),
H-AP5 (5'-AAGCTTAGTAGGC-3'),
H-AP6 (5'-AAGCTTGCACCAT-3').

PCR using only one of two primers, H-T11V or H-AP, was performed in parallel as a control to exclude PCR fragments flanked by the H-T11V or H-AP primer only. After the same procedure of separation on PAGE and gel drying as described above, putative differentially expressed DD-fragments were selected according to the following parameters: PCR product between 250–500 bp in length amplified in at least two lanes of the triplicate reactions. Fragments of cDNA were then cut out of the gel and eluted as described previously (15). Reamplification of DD-fragments was performed under the same conditions as above except that 5 μ l of the template was used from 10 μ l of the eluted solution and that [32 P]dATP was not present. The PCR products were cloned into pCR2.1 vector (Invitrogen, Groningen, The Netherlands) by TA cloning according to manufacturer's instructions.

Reverse Northern Blotting Analysis

Fifty microliters of PCR products amplified using M13 forward and reverse primers of pCR2.1 (the same condition as above), which contained inserted DD-fragments, were separated on a 1% agarose gel, followed by transfer in duplicate onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech Inc., les Ullys, France). Radiolabeled cDNA probes were generated by RT reactions for each cell batch using 5 μ g of total RNA primed with 2.5 μ M H-T11V oligonucleotides in a buffer containing 500 μ M of dATP, dTTT and dGTP, 10 mM DTT, 5 μ l [32 P]-dCTP (3000 Ci/mmol), 40 units of RNase inhibitor (Rnase Out, LifeScience), and 200 units of M-MLV reverse transcriptase in a final volume of 20 μ l. Radiolabeled RNA/DNA hybrids were hydrolyzed by 3 μ l of 3 N NaOH for 30 min at 65°C, neutralized by adding 10 μ l of 1 M Tris-HCl, pH 7.4 and 3 μ l of 2 N HCl, and extracted with phenol-chloroform as described previously (15). Unincorporated [32 P]dCTP was removed using ProbeQuantTM G-50 Micro Columns membranes (Amersham Pharmacia Biotech Inc.). Hybridization, washing and film exposure were performed as described (15).

3'-RACE of Porcine β -actin cDNA

To evaluate the quality and quantity of radiolabeled cDNA probes, two PCR products of β -actin cDNA were applied as controls onto the same agarose gel as that used for RN, which were derived from the

3'-terminal region of cDNA and the middle coding region of porcine β -actin. The former was produced by 3'-rapid amplification of cDNA ends (3'-RACE) using an upper primer (5'-ggatCCTTCCAGCAGATGT-3') whose sequence resides in the 3'-terminal coding region of the porcine β -actin cDNA (nucleotides 686–700 from Genbank accession no. U07786), in combination with H-T11C, H-T11A and H-T11G primers, respectively. The latter was the PCR product amplified using the upper primer (5'-ATGTTTGA-GACCTTCAACAC-3', nucleotides 1–20) and the lower primer (5'-CACGTCGCACTTCATGATCGA-3', nucleotides 495–475) from the same sequence.

Sequencing of DD-fragments and Bioinformatic

Sequencing was performed using the Applied Biosystems 373A DNA sequencer with the M13 forward and reverse primers according to the manufacturer's protocol. Random EST sequences were analyzed using BLASTN for searching the Genbank nucleotide database (<http://www.ncbi.nlm.nih.gov/blast/>), and BLASTX for searching non redundant protein database (16,17). Detailed sequence analysis including protein alignments and hydrophobicity, and phylogenetic evaluation were performed using DNASTAR (Madison WI).

Northern Blotting Analysis

Ten micrograms of total RNA were separated on a 6% denaturing agarose gel, transferred onto a Hybond N⁺ membrane, and subjected to hybridization using [32 P]-labeled cDNA fragments isolated from DD-RT-PCR as probes. The protocols for UV-crosslinking, prehybridization, hybridization and washing were as described (15).

cDNA Cloning from cDNA Library

A cDNA library from activated porcine ECs (1×10^6 plaques, kindly provided by Dr. Rainer de Martin, University of Vienna, Austria) was screened with DD-fragments as probes. Duplicate filters (Hybond N⁺) were hybridized under the same conditions as for RN and exposed to Kodak XAR autoradiography film at –80°C overnight. Double positive phage clones were plaque amplified, subjected to several screening procedures and converted into plasmid by *in vivo* excision, followed by sequencing by the Applied Biosystems 373A DNA sequencer as above. For confirmation, cDNA clones were also sequenced commercially (MWG Biotech AG, Germany).

Results

Differential Display of mRNA

Primarily, we evaluate whether PCR conditions, including number of amplification cycles and template concentration, could influence the detection of a highly expressed housekeeping gene, β -actin. [32 P] dATP-labeled RT-PCR of porcine β -actin was performed with a specific primer set in a high stringency condition

Fig. 1. RT-PCR analysis of porcine β -actin using specific primers. Two micrograms total RNA from PAEC were used for reverse transcription. PCR was performed for 20, 30 or 40 cycles. The cDNA templates were diluted 0-fold (lane 1), 10-fold (lane 2), and 100-fold (lane 3) in each cycled PCR. The expected size of the PCR product is shown by an arrow. Marker sizes are indicated on the left.

(65°C as annealing temperature) as compared with DD-PCR protocol (42°C as annealing temperature) and electrophoresed on a 6% acrylamide sequencing gel (Fig. 1). We observed that many, lower sized, PCR products were produced in addition to the amplicon of the expected size. Presence of the contaminating PCR products was dependent on the number of PCR cycles and the concentration of cDNA template. Only one PCR product of the expected size was obtained by a 20-cycles PCR using a hundred-fold diluted cDNA template (Fig. 1, lane 3). We considered that one important factor for the exclusion of «false positive» would depend on how to rule out the PCR products amplified from the abundantly expressed genes.

Display of mRNA from resting porcine ECs was compared with mRNA from ECs treated with TNF α for 12 h. Six 5' arbitrary 13-mer primers (H-AP-1 to H-AP-6) in combination with three 3' one-base-anchored primers (H-T11C, H-T11G and H-T11A) were tested. We showed above that excessive number of amplification cycles could increase appearance of contaminating PCR products which could affect the amplification of other DD-Fragments. Therefore, 30 cycles of PCR was performed instead of 40. Moreover, each reaction was carried out in triplicate in serial ten-fold diluted concentrations of cDNA

templates (derived from 100 ng, 10 ng and 1 ng of total RNA) in order to evaluate the reproducibility of differential display. This protocol produced 50 to 150 visible bands on the gel in all primer combinations according to the template concentration (Fig. 2A), whereas difference in the number of cDNA fragments obtained were found to exist between the 18 primer sets used. The signal-to-noise ratio was reduced according to the template dilution. The lower template concentration allowed a better separation and selection of DD-fragments from the surrounding PCR products. In general, a roughly similar pattern of bands was observed (Fig. 2A, lane 1–3). Therefore, we choose to select cDNA fragments which appear at least in two of triplicate assays. Moreover, cDNA fragments longer than 250 bp in length were selected to allow their use as probes for the following procedures. With this procedure, seventeen candidates corresponding to mRNAs upregulated in ECs in response to TNF- α were selected. Representative cDNA fragments selected are shown in Fig. 2B. Candidates 10, 13, and 17 were not amplified in lane 1 corresponding to undiluted cDNA template, PCR condition consistent with the original protocol described by Liang, et al. (18).

Rapid Screening by Reverse Northern

Expression of the seventeen selected DD-fragments in resting *versus* TNF-activated ECs was further analysed by Poirier, et al. have confirmed reproducible differential expression pattern by spotting DD-fragments at serial concentrations ranging from 6 μ g to 60 ng onto membranes followed by hybridization with radiolabeled cDNA probes derived from amplified RNA (19). Alternatively, we evaluate the optimal probe design and labeling, which was produced by a [33 P]dCTP labeled RT reaction of non-amplified RNA, and its ability to hybridize to cDNA fragments from DD-RT-PCR. Firstly, we investigated the ability of this radiolabeled cDNA probe to detect the PCR products of porcine β -actin, which were used as a control in the RN screening step. Two PCR products of porcine β -actin were produced; a middle fragment (M) of the open reading frame (ORF) and a 3'-end fragment of the mRNA that was obtained by 3'-RACE with a specific upper primer and H-T11C, H-T11A, and H-T11G lower primers in three different reactions (3'C, 3'A and 3'G, respectively). The nucleotide sequence of 3'G was identical to that of 3'C, but 21 nucleotides shorter (Fig. 3C). These results indicated that β -actin mRNA seems to use several different polyadenylation sites or that mispriming of the H-T11V primers in the poly(A) tail anchored region occurred during the RT reaction. The hybridization pattern of M and 3'C fragments with radiolabeled cDNA probe is shown in Figure 3B. The optimal concentration of total RNA template for the RT reaction was determined by evaluation of radioactive intensity hybridized to M and 3'C fragments which were slot-blotted onto the membrane. Aliquots of radiolabeled cDNA probes derived from

Fig. 2. Differential display patterns in TNF α -activated ECs. (A) DD patterns for the primer sets including H-T11C, H-T11A and H-T11G, as 3'-primers and H-AP1, H-AP2, and H-AP3 arbitrary 13-mers 5'-primers. The cDNA templates were diluted 0-fold (lane 1), 10-fold (lane 2), and 100-fold (lane 3) for each sample. PCR were performed using only the upper primer (lane U) or the lower primer (lane L), as controls. Marker sizes are indicated on the left. (B) Representatives differentially amplified DD-fragments which were selected for further analysis. DD-fragments were chosen according to the criteria described in the *Materials and Methods* section.

serial dilutions of total RNA were then hybridized as shown on the left panel of Figure 3A, demonstrating that RT with 0.2 μ g RNA template did not allow detection (Fig. 3A). The signal of the 3'C fragment was stronger than that of M using more than 5 μ g RNA template, suggesting that the more RNA template reverse-transcribed, the more predominantly DD-fragments derived from the 3'-end of the mRNA could be detected (Fig. 3B). Therefore, cDNA probes were produced by RT reactions using 5 μ g RNA template and employed for RN screening to confirm the differential expression of the 17 selected DD-fragments.

Seventeen selected DD-fragments were divided into three groups according to the H-T11V primer used during the DD-RT-PCR, separated through three agarose gels, and transferred onto membranes. Control fragments of M and each 3'-end of β -actin mRNA (3'C, 3'A, and 3'G, respectively) were also transferred onto the membranes as controls.

Each membrane was hybridized with the radio-labeled cDNA probe derived from the RT reaction primed with the corresponding H-T11V primer. Expression of six out of seventeen candidates was shown to be regulated after hybridization with radiolabeled probes from 5 μ g reverse-transcribed RNA from EC treated with or without TNF- α for 12 h. Probes derived from 0.5 μ g RNA template in the RT reaction primed with the H-T11C primer did not show differential hybridization (Fig. 4B), which was consistent with the result in hybridization to the M and 3'C fragments of the β -actin control between the upper panel in Figure 4A and Figure 4B.

Comparative Screening by Northern Blot and Sequencing

To confirm RN analysis, we performed Northern blot for the six candidates selected by RN (Fig. 5A). Differential expression was confirmed for five

Fig. 3. Semi-quantitative evaluation of radiolabeled cDNA probes. (A) PCR products of the middle region (M) and the 3'-end region (3'C) of porcine β -actin cDNA were transferred onto the membrane and hybridized with radiolabeled cDNA probes. cDNA probes were reverse transcribed with H-T11C, a one-base anchored oligonucleotide and [33 P]dCTP using serial dilutions of RNA templates. Aliquots of total radiolabeled RT reaction were slot-blotted onto filters and subjected to autoradiography (left-hand panel). (B) Schematic representation of cDNA probing. M fragment positions at 850 bp upstream of the 3'-end of the cDNA. Nucleotide sequence of the 3'-end of porcine β -actin cDNA obtained by 3'-RACE with alignment of the partial human β -actin sequence (Genbank accession no. NM-001101). PCR products (3'G, 3'C and 3'A, respectively) generated with each primer set were aligned. Identical nucleotides to 3'G sequence are indicated by asterisks. These fragments were used as positive controls in RN. The upper and lower primers (H-T11G, H-T11C and H-T11A) for 3'-RACE are indicated by arrows; stop codon by boxes and potential polyadenylation sites are underlined.

candidates with an estimation of their length. No differential expression between resting and TNF α -treated PAECs was observed for candidate 15. Sequencing was performed concurrently with Northern blot. Homology search revealed that candidates 4 and 10 were identical molecules, encoding the

Fig. 4. Reverse northern blot analysis. (A) Differential hybridization using radiolabeled cDNA probes reverse-transcribed from 5 μ g of RNA template primed with each H-T11V one-base anchored oligo(dT) primer. In each panel, upper; photograph of PCR products on 1% agarose gel, middle or lower; hybridization with radioactive cDNA probe using RNA from PAEC treated without or with TNF α for 12 h. Lanes on the left-hand showed controls performed with porcine β -actin as described in Figure 3. (B) Hybridization using a radiolabeled cDNA probe obtained by retrotranscription of 0.5 μ g of RNA primed with H-T11C. porcine MHC (SLA) class II DQ-alpha chain (Table 1). Candidates 4 and 10 resulted from DD RT-PCR with primer sets H-AP4/H-T11C and H-AP4/H-T11A, respectively. The 3'-terminal eight nucleotides of the

Fig. 5. Northern blot analysis. (A) Each lane contains 10 µg total RNA from PAECs incubated without (-) or with (+) TNF α for 12 h probed with each DD-fragment. β -actin was used as an internal control (upper panel). The approximate length of the transcripts was determined from the position of 28S and 18S ribosomal RNA. The estimated length of the mRNA is indicated on the right. (B) Kinetic analysis for candidate 17 expression in response to TNF α .

H-AP4 primer were identical to its putative binding site (nucleotides 895–902 in Genbank accession no. M29939). This revealed that H-T11C primer anchored at the nucleotides immediately prior to polyadenylation site for candidate 4, but at the nucleotides two

bases prior to the polyadenylation site for candidate 10. This is evidence of the mispriming in the poly(A) tail anchored region (13). Previous studies have demonstrated the role of TNF- α in the regulation of the SLA class II expression on porcine ECs (8,20). Therefore, expression of these two candidates was not further investigated. Two sequences, candidates 3 and 13, showed no sequence homology with sequences present in databases with the sequences of DD-fragments that reside in the 3'-terminal mRNA. Candidate 17 was found to be the porcine homologous of an human G protein coupled receptor ((21), Genbank accession no. AF181862) (Fig. 5B).

Cloning of Three Novel Porcine cDNAs

cDNA fragments, corresponding to candidates 3, 13, and 17, were used to screen a cDNA library prepared from activated porcine ECs. This resulted in the isolation of partial cDNA clones for each candidate. Sequence homology research was then repeated for gene identification. cDNA sizes of the partial clones are indicated in Table 1. The clone for candidate 17 was short and, indeed contained a partial cDNA corresponding to the 3'-end of the putative ORF and 3'-untranslated region (UTR), and again found to be homologous to the human G protein-coupled receptor. Cloning allowed to identify candidate 3 as the porcine homologous of human ATL-derived PMA-responsive peptide (22) and mouse Noxa (23) genes (Table 1). Candidate 13 was found to be a part of porcine RNA helicase ((24), RHIV-1, Genbank accession no. AF181119).

Discussion

RNA DD and random arbitrarily primed (RAP)-PCR (2,3) have been used to isolate a variety of novel

Table 1. DD-fragments screened by DD and RN, and gene identification

Candidates	DD-fragment Size (bp)	Transcript Size (kb)		Sequence Homology	Accession No.
		*	**		
3	437	3.0	2.6	Human ALT-derived PMA responsive (APR) peptide Mouse Noxa TN3 (Porcine Noxa)	D90070 AB041230 AF319660***
4	301	1.0	—	Procine MHC class II SLA-DQ α chain	M29939
10	296	1.0	—	Procine MHC class II SLA-DQ α chain	M29939
13	317	4.0	3.0	Procine RNA helicase (RHIV-1) Procine RNA helicase (partial)	AF181119 AF319661***
17	264	3.5	1.8	Human G protein-coupled receptor (GPCR5B) Procine GPCR5B (partial)	AF202640 AF319662***

*Length of mRNA roughly estimated by Northern blot using DD-fragment as a probe.

**Length of cDNA screened from an activated PAEC cDNA library. Candidates 4 and 10 were not screened because their differential expression was already confirmed.

***Genbank accession number which were newly registered in this study.

genes in a large number of experiments. However, major criticisms include the rate of false positives, redundancy, and time-consuming downstream verification process (25). Wan, et al. reported that DD was not mRNA abundance-dependent due to the mechanism of primer sequence-dependent amplification (4). However, McClelland, et al. have pointed out the probability of mRNA abundance-dependent amplification, suggesting that rare differentially expressed genes will be under-represented among other PCR products on the gel (26). The effect of these parameters on the DD patterns in these methods has been investigated in early studies. The DD pattern was shown to be consistent for serial concentrations of cDNA templates between 7.5 ng and 30 pg in a RAP-PCR system (1,27). On the other hand, the DD pattern with serial dilutions of total RNA before RT was compared between 2 ng and 2 μ g in a RNA-DD protocol (28), and showed that many DD-fragments derived from 2 ng RNA template were not visible in the gel. The different results in the DD pattern affected by the template concentration between these two methods could be explained by the differences in the titration of cDNA or total RNA, that is the titration of the template before or after the RT reaction, and by the conditions of other parameters.

Primarily, we used porcine β -actin to test whether specific primers could amplify the PCR product of the expected size in a high stringency condition as compared with PCR protocol for DD as shown in Fig. 1. Considering the fact that abundantly expressed genes will be overamplified even in a 30 cycle-PCR, this would be still not sufficient to exclude false positive PCR products. In another hand, rarely expressed genes would not produce a visible band in a 20 cycles PCR, as experienced in real-time quantitative RT-PCR. Therefore, to improve selection, only PCR products which were reproducibly amplified through serial dilutions of template were selected. As previously reported (26,27), the later criteria (ie., 30 cycle PCR and pattern reproducibility) enable us to considerably decrease the number of candidate genes from 100 to 17 for further investigation. In our study, we selected DD bands of interest from the lanes corresponding to the higher cDNA template dilutions. This selection allowed us to minimize the possibility of contamination with surrounding cDNA fragments during band recovering. Semi-quantitative RN confirmed the upregulation of six out of seventeen candidate genes. According to hybridization signal obtained with the 3'-end fragment of β -actin (3'C, 3'A and 3'G, Fig. 4A), 5 μ g RNA template appeared to be sufficient to produce 3'-end predominant radiolabeled cDNA probes. The fact that only six out of seventeen candidates were differentially expressed suggests that several candidates have been derived from internal sites on the mRNA by mispriming, despite the strict selection criterion applied, and also underline the limit of

detection related to RN. It should be emphasized that DD-fragments do not constitute optimal probes for hybridization analysis, because these cDNA fragments are derived from the 3'-UTR of mRNAs, which are rich in A, T nucleotides and repetitive sequences. Moreover, amplified PCR products might contain misreading nucleotides.

In our study differential expression was confirmed for candidates 10, 13, and 17 by northern blot, whereas DD-fragments were not visible in lane 1 in Fig. 2B. As far as this lane is concerned, it could be said that these DD fragments were false negative, which Welsh, et al. referred to as «under-represented» DD-fragments of rare differentially expressed gene (27). We confirmed differential expression for five out of six candidates by northern blot. Thus, RN screening seems to be efficient in reducing the number of candidates before northern blotting. Two candidates, 4 and 10, were found to be identical and candidate 10 was derived from the misprimed one-base anchored 3'-primer. Thus, it appeared to be impossible to rule out the DD-fragments derived from this type of mispriming through both the DD and RN steps. Although there is a possibility of overlooking the slightly differentially expressed genes by RN, it will also be difficult to detect these genes by northern blot. Confirmation of rare transcripts regulation will require quantitative PCR (29). Only one candidate positive in RN was not confirmed by northern blot. A novel approach was recently reported to select gene corresponding to a specified stimulus. In this study the standard DD-RT-PCR was modified by exchanging the arbitrary upstream primers for sequence-specific primers (30). Such innovative approaches using the DD-RT-PCR are further expected.

The goal of our project was the identification and characterization of novel genes modulated during EC activation. Our data confirmed that in porcine ECs, TNF α induces MHC class II expression (20,8,31). Here, by means of RNA differential display, we identify the pro-apoptotic Noxa protein as a TNF-responsive molecule in ECs. Genes that are directly involved in the initiation of mitochondria-induced apoptosis include the Bcl-2 family members, Noxa, PUMA, and Bax. The Bax protein belongs to the multidomain Bcl-2 family, while Noxa and PUMA are BH3-domain-only proteins (for review see (32)). Noxa encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins; this member contains the BH3 region but not other BH domains. When ectopically expressed, Noxa underwent BH3 motif-dependent localization to mitochondria and interacted with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 (23). Noxa has been mainly reported as a mediator of p53-dependent apoptosis. To our knowledge, this is the first report of a transcriptional upregulation of Noxa in endothelial cells. Furthermore, our findings suggest that Noxa could be involved in the intracellular signaling pathway mediated by TNF α in endothelial cells.

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