Exequibility of differential gene expression analysis by DDRT-PCR in murine bone marrow cells

Exequibilidade do DDRT-PCR para análise da expressão gênica diferencial em células de medula óssea murina

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ABSTRACT:

Model of study: Experimental study. Introduction: Recently, stem cell research has generated great interest due to its applicability in regenerative medicine. Bone marrow is considered the most important source of adult stem cells and the establishment of new methods towards gene expression analysis regarding stem cells has become necessary. Thus Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) may be an accessible tool to investigate small differences in the gene expression of different stem cells in distinct situations.

Aim: In the present study, we investigated the exequibility of DDRT-PCR to identify differences in global gene expression of mice bone marrow cells under two conditions.

Methods: First, bone marrow cells were isolated fresh and a part was cultivated during one week without medium replacement. Afterwards, both bone marrow cells (fresh and cultivated) were submitted to gene expression analyses by DDRT-PCR.

Results: Initially, it was possible to observe in one week-cultured bone marrow cells, changes in morphology (oval cells to fibroblastic-like cells) and protein profile, which was seen through differences in band distribution in SDS-Page gels. Finally through gene expression analysis, we detected three bands (1300, 1000 and 225 bp) exclusively expressed in the fresh bone marrow group and two bands (400 and 300 bp) expressed specifically in the cultivated bone marrow cell group.

Conclusions: In summary, the DDRT-PCR method was proved efficient towards the identification of small differences in gene expression of bone marrow cells in two defined conditions. Thus, we expect that DDRT-PCR can be fast and efficiently designed to analyze differential gene expression in several stem cell types under distinct conditions.

Key-words: DDRT-PCR. Gene Expression. Bone Marrow Cells.

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Introduction

In the recent past, stem cell research has received great interest in medicine principally due to the fact that stem cells possess the ability to differentiate into several cells and participate in the regenerative process of damaged tissues.[1]

Bone marrow is considered the major source of adult stem cells and the establishment of new methods in basic research involving these cells has become extremely necessary. The application of molecular techniques towards the study of gene expression in models of cell culture and co-culture, presents itself as a promising tool for the analysis of potential gene pathways related with cell proliferation and differentiation. The Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR), originally developed by Liang & Pardee2, presents itself as a useful tool for the detection and understanding of gene expression.[2]

This procedure allows the identification of differentially expressed genes by comparative analysis between two populations of RNA transcripts in different tissues or cell types or, in different situations that received individual environmental influences. Due to these aspects, DDRT-PCR can also be considered useful as an efficient method for cloning and construction of cDNA libraries.[3]

Since its development, variations of the DDRT-PCR method have been observed in many different biological systems, such as to identify new markers important for neuronal development in mammals and investigate gene expression in lung tissues, especially those expressed during organ development and pathophysiological processes, including injury and repair, tumorigenesis and response to particular treatment.[3,4] This technique also played an important role in the search for genes involved in regulating embryonic development in rats and in their responses to inflammatory processes.[5,6] In addition, DDRT-PCR also was efficient to identify genes differentially expressed between metastatic tumor and normal prostate cells.[7]

Thus, in the present work, we investigated the exequibility of DDRT-PCR in identifying differences in gene expression of bone marrow cells under different conditions (freshly isolated and one week cultivated bone marrow cells). Through DDRT-PCR, it was possible to verify some small differences in the gene expression profile of fresh and cultivated bone marrow cells. Three bands, identified with 1300, 1000 and 200 bp, were exclusively present in the fresh group and two bands, with 400 and 300 bp, were exclusively present in the cultivated group. All together these results can demonstrate that DDRT-PCR may be an efficient technique , being accessible to identify differences in the gene expression profile for different cells under distinct conditions.

Material and Methods

Animals

Swiss mice with 8 to 10 weeks were kept in polypropylene boxes with controlled lighting (12/12 hours) and temperature (22 °C). The animals in the experimental procedure were given solid diet and water ad libitum. All animal handling protocols were approved by the local Animal Care Committee.

Cell Isolation and Culture

In order to collect and culture bone marrow cells, the methodology described by Minghell and co-authors was used.[8] Male Swiss mice were used to obtain adult bone marrow cells. The femurs and tibias of each animal were extracted to obtain bone marrow cells by flushing.[9] After, the cells were washed by centrifugation at 400g for 10 minutes and cell viability was assessed with a Neubauer chamber. Subsequently the cells were suspended in fresh medium at a final concentration of approximately 13 x 10^6 cells/μL. These were then transferred to culture flasks of 25 cm^2 containing 5.5 mL DMEM medium supplemented with 10% fetal bovine serum (GIBCO-BRL). Cultures were maintained in an incubator with a humid atmosphere with 5% CO_2, at 37ºC for 1 week.

Protein Evaluation

Total protein of all samples was extracted using Ripa buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Sample protein was quantified using a BSA curve (Figure 2). Approximately 50 μg of each sample was loaded onto a SDS-PAGE gel 12,5 % (30% acrylamide, 1.5 M Tris, pH 8.8, 10% SDS, 10% Ammonium persulfate, 30 μL of TEMED, SIGMA) with sample buffer (0,125 mM Tris-HCl pH 6.8, 4% SDS, 20% Glicerol, 0,02% bromofenol blue). It was utilized two pool of tree samples per experiments for each conditions evaluated (n=2).

mRNA Isolation

Total mRNA was extracted from cell pellet using TRIZOL reagent protocol, according to the manufacture's recommendations (GIBCO-BRL). In order to verify the quantity and the purity and quality of total mRNA, processed samples were then analyzed using a spectrophotometer at wavelengths of 260 nm and 280 nm. mRNA purity was determined by the absorbance ratio (A260/A280 nm) and visualization of bands in 1.5% denaturant agarose gel (Table 1 and Figure 4). mRNA from both cell types was transcribed into cDNA by using the Thermo Script System kit, according to the manufacturer's specifications (GIBCO-BRL). It was utilized two pool of tree samples per experiments for each conditions evaluated (n=2).

DDRT-PCR

Gene expression profile of fresh and cultivated bone marrow cells was performed by DDRT-PCR, using a single random primer with 10 bp (`5`-CCTTGACGCA-`3).[10] cDNA of each cell type was subjected to amplification using the primer described above. Each reaction consisted in the use of μ2 L of cDNA, 70 pmol of primer, 100 μM of each dNTP, 1.5 mM MgCl2, 1X PCR buffer (200mM Tris-HCl pH 8.4, 500 mM KCl) and 2 U Taq DNA polymerase (INVITROGEN). Reactions with bone marrow cells were performed using the following cycle: Cycle 1 (1x) incubated at 95°C for 30 seconds, 30°C for 2 minutes and 72°C for 1 minute. The next step was performed for over 33 cycles under the following conditions: 95°C for 30 seconds, 35°C for 2 minutes and 72°C for 1 minute. The annealing temperature was changed to 60°C and extension time increased for 5 minutes in the final cycle. Later, the samples were stored at -20°C. An amplification without cDNA was performed under the same conditions as a negative control for possible contamination with exogenous DNA. As an endogenous control, amplification with a housekeeping gene (β-actin) was performed for each sample as mentioned above.[11]. It was utilized two pool of tree samples per experiments for each conditions evaluated (n=2).

Electrophoresis in 8% polyacrylamide gel and silver staining

For electrophoresis assays, the protocol described by Dias Neto and collaborators was adopted.[12] Each amplification product was added to sample a buffer (0.125% bromophenol blue, 0.125% xylene-cyanol, and 15% glycerol) and subsequently loaded onto a 8% polyacrylamide gel (acrylamide-bisacrilamide 29:1) with TBE buffer (90 mm Tris-borate, 1 mM EDTA, pH 8.0).[13] Next, the gel was subjected to electrophoresis in a vertical cube at 60V, for 6 h and after, the gel was fixed in a solution of 10% ethanol and 0.5% acetic acid (v/v), stained with 0.2% silver nitrate and washed with deionized water. Finally, in order to visualize the bands, the gel was incubated with a solution containing 0.75 M NaOH and 0.1 M formaldehyde.[13]

Table 1
Measurement of mRNA index.

Samples	260 nm	280 nm	260/280	[RNA] μg/μL
MO_F	0,390	0,233	1,873	1,559
MO_{1w}	0,092	0,076	1,910	0,360

MO_F: Freshly isolated Bone Marrow; MO_{1W}: One week cultured Bone Marrow

Results

Bone marrow cell culture in distinct conditions presented differences in cell morphology

We observed the general morphology of bone marrow cells under different conditions, analyzed fresh or after one week in culture. It was demonstrated that fresh bone marrow cells represented in their majority oval cells while cultivated bone marrow cells showed, at least, two distinct morphologies, representing oval and fibroblastic-like cells (Figure 1).

Protein profile of fresh and cultivated bone marrow cells

In order to evaluate if SDS-PAGE gel can be designed to visualize the protein profile of fresh bone marrow cells and one-week cultured cells, we performed a 12,5% acrylamide gel. First, both sample types of bone marrow were submitted to protein quantification using the BSA method (Figure 2 and Table 2). After, the samples were visualized in 12,5% polyacrylamide gel and a distinct distribution was identified on their band profile. Fresh bone marrow cells presented exclusively two bands (one with ≈170 and another with ≈90 kDa) while cultured bone marrow cells did not show any difference in their profile (Figure 3).

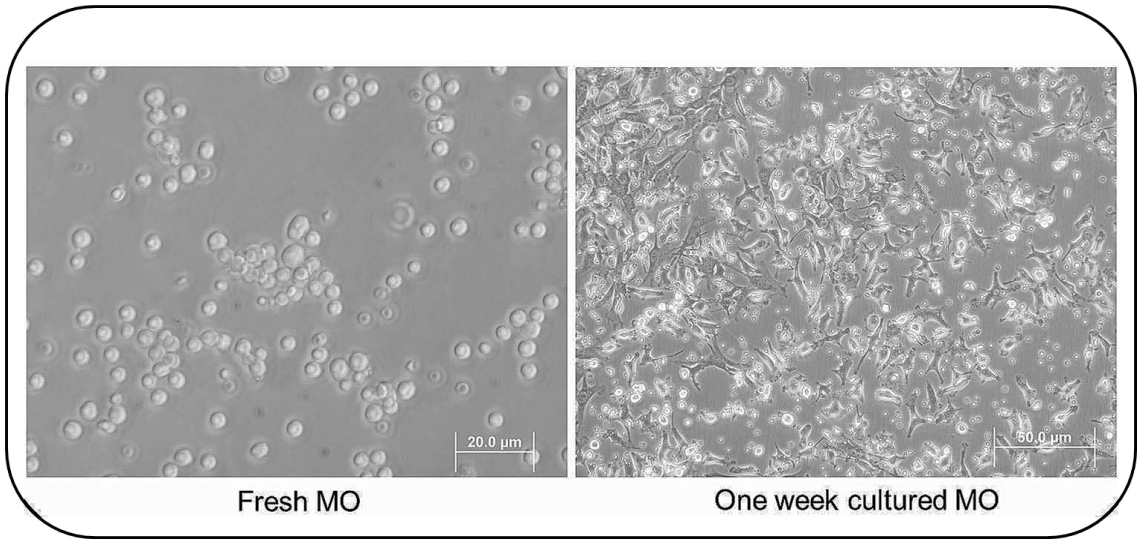

Figure 1. Morphologic aspect of bone marrow cell culture.
Fresh bone marrow cells (Fresh MO) possess an oval or circular shape with rare incidence of fusiform cell forms. After one week in culture, bone marrow cells (One week cultured MO) are more heterogeneous with a higher incidence of fusiform or spindle-shaped cells.

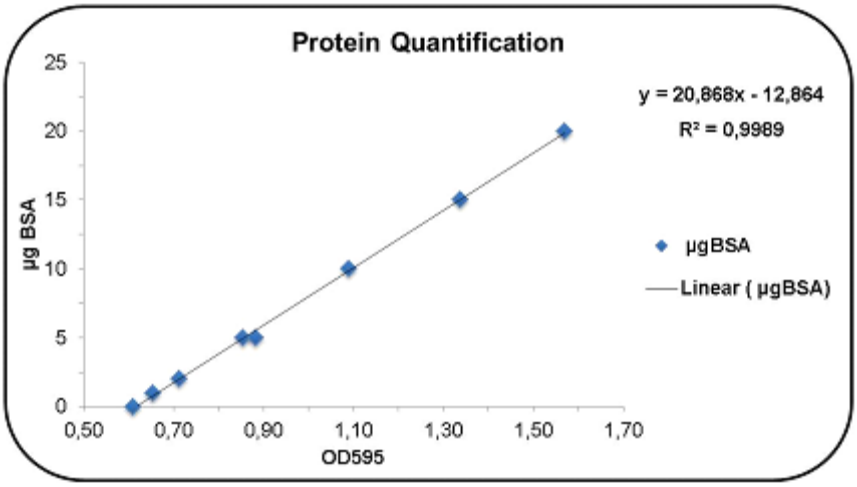

Figure 2. Standard curve of protein quantification.
Using the BSA method, a standard curve was performed to identifify the amount of protein in each sample of mononuclear bone marrow cells. Thus, using the curve, it was possible to find the adequate value to perform protein quantification for bone marrow cell samples.

Table 2
Index of protein concentration.

Samples	OD595	[Protein] µg/µL
MOF	0,390	1,559
MO1w	0,092	0,360

MOF: Fresh isolated Bone Marrow; MO1W: One week cultured Bone Marrow

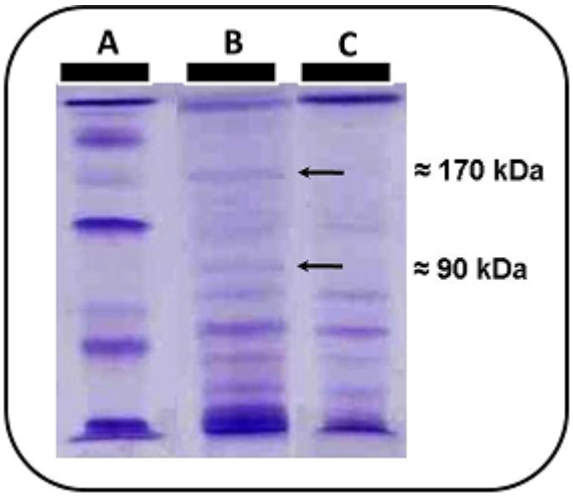

Figure 3. Profile of protein banding using SDS PAGE gel.
(**A**) Standard molecular weight refers to 220 KDa (Myosin), 170 KDa (á-2-Macroglobulin), 116 KDa (â-Galactosidade), 76 KDa (Transferrin) and 53 KDa (Glutamate Dehydrogenase). (**B**) Refers to fresh bone marrow cells and (**C**) indicate bone marrow cells cultured during one week. After the SDS page process, one differential profile was observed among the two samples (**A** and **B**). The fresh bone marrow cells presented two bands (170 and 90 kDa), that were not identified in cultured bone marrow cells.

Differences in gene expression analysis of bone marrow cells can be detected by DDRT-PCR

To investigate if DDRT-PCR can detect differences in the gene expression profile of bone marrow cells, we carried out an experiment with two conditions: freshly isolated cells and cells after one week being cultivated. The purity of both the quantities of RNA in each sample is shown in Table 2 and Figure 4. The RNA extraction process produced ideal values (1.9-2.1 for), indicating low contamination of DNA, proteins, polypeptides and phenol.[13] During electrophoresis in a 1.5% agarose gel, there is a regular quality of samples, and two bands can be observed referring to the 18S and 28S ribosomal RNA subunits (Figure 4). In sequence, by DDRT-PCR, it was possible to identify in a 8% polyacrylamide gel a distinct profile in gene expression for both samples. Fresh bone marrow cells presented three specific bands with approximately 1300, 1000 and 225 bp while cultivated bone marrow cells showed only two bands differentially expressed one with ≈400 bp and another with ≈300 bp (Figure 5).

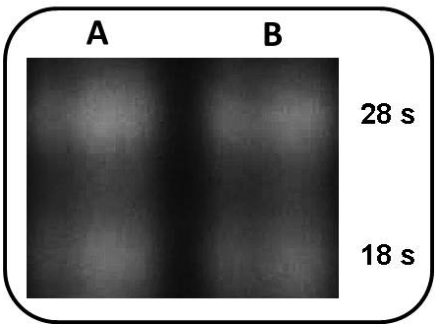

Figure 4. Visualization of subunits of ribosomal RNA in agarose gel.
It is possible to observe the presence of two band referring to the 28s and 18s subunits of ribosomal RNA.

Discussion and Conclusions

The application of molecular techniques to evaluate differences in gene expression is very important in stem cell research. In this sense, recent literature has described several methodologies to perform gene expression analyses. Philips and co-authors, for example, using a similar technique elegantly described several gene products differentially expressed in hematopoietic stem cells and in fetal liver stem cells.[14] Later, Ramalho-Santos and colleagues, using subtractive hybridization and microarray analysis, performed a comparison of the transcription profile of three strains of stem cells (Embryonic, Neural and Hematopoietic).[15] These authors observed the presence of specific genes present in each population of stem cells, and demonstrated a cell-specific gene expression profile.[15] In addition, in another study, through hybridization techniques and advanced bioinformatics, an analysis of the gene expression in three distinct types of hematopoietic stem cells within a homogeneous population was performed.[16]

Alternatively, here we described DDRT-PCR as a quick, easy and efficient method to detect differential gene expression in bone marrow cells. We chose two conditions to analyze gene expression: fresh cells and one week cultivated cells. Initially, changes were observed in cell morphology and in protein profile using SDS-PAGE gel. To demonstrate the applicability of our methods, DDRT-PCR was performed in this same condition. Confirming the findings observed in the SDS-PAGE gel, DDRT-PCR also showed differences among the groups studied (fresh cells and cultivated cells) but more details may be observed.

The success of this method is related with the choice of primer designated as L5. In this work L5 was defined from previous work from the Jing and Li group,

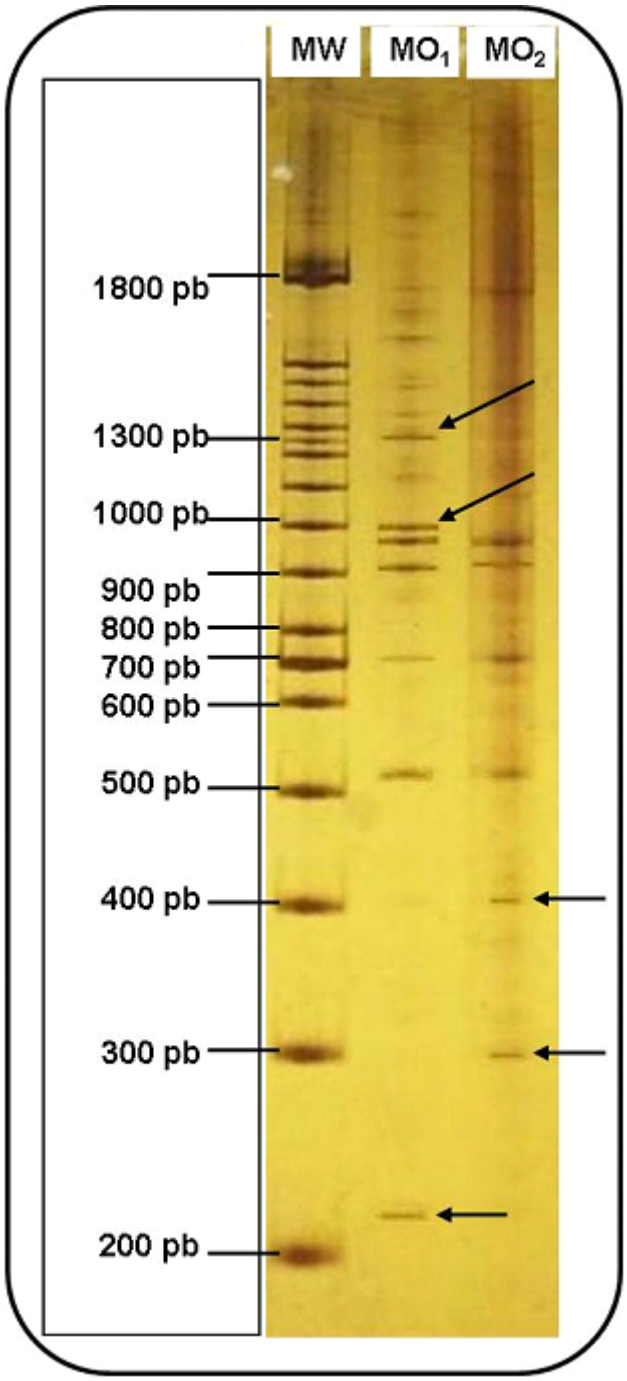

Figure 5. Differential display layout of gene expression analysis of bone marrow cells.

Through the DDRT-PCR method it is possible to observe a distinct profile in gene expression of fresh bone marrow cells (MO1) and bone marrow cells cultured during one week (MO2). In fresh bone marrow cells, there are three fragments (1300, 1000 and 200 bp, respectively) differentially expressed. On the other hand, for cultured bone marrow cells, two exclusive bands (400 and 300 bp) were identified. This result may indicated that during one week in culture, bone marrow cells may shift their gene expression profile due to the proliferation of different cells types, a natural differentiation process or a response to metabolic events such as stress. MW refers to standard molecular weight with 1.800, 1.300, 1.000, 900, 800, 700. 600. 500. 400. 300 and 200 bp.

in which good results were obtained.[17] These authors, using this primer and others, found 299 fragments that determined genetic similarity in three families of *Anguilla*.[17] Promising results were also obtained by Bae and co-authors, using this same primer.[18] In Bae`s work, such result, by the RAPD method, made it possible to identify several random primers including L5, related with the identification of new retro-transposons fragments in *Clonorchis sinensis*.[18] The use of two time points for evaluation, also was established from good results in previous work from Xu and collaborators.[19] These researchers achieved encouraging results using 0h-4h-8h and 12h for the verification of differential gene expression during the regeneration of hepatocytes in rat liver.[19] Following the same methodology, Miles and co-authors also used 1h and 24h to demonstrated great differences in gene expression of rat bone cells before and after an osteogenic stimulus.[20]

In the present manuscript, the analysis through DDRT-PCR showed a clear difference in gene expression profiles between the cell types used. Both samples were amplified using the L5 primer and the determination and confirmation of potential polymorphic markers between the cell types in the study were made from the analysis of electrophoretic profiles and band analysis in polyacrylamide gel (Figure 5). The presence of three bands was verified in fresh bone marrow cells (1300, 1000, 225 bp) and two bands were present in the cultivated group (400 and 300 bp).

The bands found in the first group may be related to specific genes restricted to progenitor stem cell pools (hematopoietic, endothelial and mesenchymal cells), besides lymphocytes, monocytes and other myeloid cells present within the bone marrow environment. On the other hand, the two bands observed for the cultivated cells may be related with the process of cellular differentiation in vitro, and enrichment of mesenchymal cells due to culture conditions (DEMEN 10% SBF).[21,22,23] However, other studies suggest that the loss of constitutive expression for some genes by *in vitro* culture methods, which would be normally expressed *in vivo*, may be occurring.[20] In this work, the authors showed in their results that the gene for myeloperoxidase (bactericidal enzyme found primarily in neutrophils and monocytes) is expressed in bone *in vivo*, but its expression was not detected in *in vitro* culture of osteoblasts.[20] In addition, we could not exclude the possibility that these two bands found for cultured cells could also be related to a differential expression of bone marrow cells

in response to the change of microenvironment by activating on an alternative metabolic pathway, principally due to "stress" provided by new conditions such as acidophilic medium, lack of nutrients and imbalance of electrolytes.[24]

Regardless of these questions, DDRT-PCR was reliable, accessible and efficient to detect small differences in gene expression on two populations of bone marrow cells. In conclusion, together these results demonstrated that DDRT-PCR is potentially feasible for application in studies specifically formulated to evaluate large and small differences in the gene expression of distinct cells or the same cells in several conditions. This study represents a work that serves as a basis for the generation of future studies with stem cells in different approaches. We believe that DDRT-PCR is a simple and quick method to evaluate gene expression and can be used for other stem types for differential gene expression analyses in the area of cell-based therapy.

RESUMO

Modelo do estudo: Estudo Experimental. Introdução: Atualmente a pesquisa com células-tronco tem gerado grande interesse devido a sua aplicabilidade no campo na medicina regenerativa. A medula óssea é considerada a maior fonte de células-tronco adultas e o estabelecimento de novos métodos para a análise da expressão gênica torna-se estritamente necessário. Desse modo, o "Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR)", pode ser uma ferramenta acessível para a investigação de pequenas diferenças no nível de expressão gênica em diferentes tipos celulares, sob distintas condições.

Objetivo: Neste presente trabalho nós investigamos a exequibilidade do DDRT-PCR na identificação de diferenças no nível de expressão gênica global em células da medula óssea de camundongos sob duas condições. Métodos: Primeiramente, a medula óssea foi isolada frescamente e uma secunda parte foi cultivada por uma semana sem troca de meio. Posteriormente, as células da medula (fresca e cultivada) foram submetidas a análise da expressão gênica, seguindo a metodologia de DDRT-PCR.

Resultados: Inicialmente, foi possível identificar em células da medula óssea com uma semana de cultivo, pequenas alterações morfológicas (células ovais para fibroblastóides) e no perfil de proteínas, por meio da visualização de bandas em SDS-Page gel. Finalmente, a análise da expressão gênica por DDRT-PCR, mostrou uma expressão diferencial com a presença de três bandas (1300, 1000 and 225 pb) exclusivamente expressas na medula óssea fresca e mais duas bandas (400 and 300 pb) presentes somente nas células de medula cultivadas.

Conclusões: Em suma, a metodologia de DDRT-PCR mostrou-se eficiente para a identificação de pequenas diferenças no nível de expressão gênica em células da medula óssea sob duas definidas condições. Portanto, nós acreditamos que o DDRT-PCR possa ser designado de forma rápida e eficiente para a análise diferencial de expressão gênica em diferentes tipos de células-tronco, sob diferentes condições.

Palavras-chave: DDRT-PCR. Expressão Gênica. Células da Medula Óssea.

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