Type of Serum as a Cell Culture Supplement Influences Regulation of MicroRNA Expression in Breast MDA-MB-231 Cancer Cells

Nihad Al-Othman [1], Hana Hammad [1], Mamoun Ahram [2]

Abstract

Background: When performing cell culture, fetal bovine serum (FBS) is widely used to supplement in culture media. However, FBS may also contain compounds such as steroid hormones that can mask the effect of exogenous hormones under investigation. Processing of FBS with charcoal is a routine practice to remove these molecules; some of which may regulate the expression of microRNAs (miRNAs).

Aim: To analyze the effect of the charcoal stripping of FBS when investigating regulation of miRNA expression by the steroid hormone, 5α-dihydrotestosterone (DHT) in breast cancer cells.

Methods: Breast MDA-MB-231 cancer cells were cultured in media supplemented with either charcoal-stripped FBS (CS-FBS) or standard FBS. Cells were treated with 100 nM DHT for three days. Cell morphology was assessed. In addition, the expression of miRNAs was investigated using PCR arrays.

Results: Alterations in cell morphology are serum-type independent. The basal expression of 13 miRNAs was slightly altered in cells cultured in CS-FBS versus standard FBS. Treatment of cells with DHT alters the expression of only four miRNA when grown in media supplemented with standard FBS. On the other hand, DHT alters the expression of 35 miRNAs when cells were cultured in media supplemented with CS-FBS.

Conclusion: The type of serum influences miRNA expression in cultured cells and unmasks differences in miRNA expression induced by DHT.

Keywords: Charcoal-stripped fetal bovine serum, FBS, PCR array, 5α-dihydrotestosterone, Androgen receptor, Microrna, Breast cancer.

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Introduction

MicroRNAs (miRNAs) belong to a large family of small regulatory RNA molecules through facilitating post-transcriptional gene silencing [(1)]. These short molecules that do not code for proteins form single-stranded, 19–24 nucleotide-long RNA molecules [(1)]. They reduce protein synthesis via the formation of RNA-RNA duplex with complementary mRNA molecules either halting translation or facilitating mRNA degradation[(1)]. An increasing number of studies have revealed an

1. **Department of Biological Sciences, School of Science, Jordan.**
2. **Department of Physiology and Biochemistry, School of Medicine, The University of Jordan, Amman, Jordan.**

**** Correspondence should be addressed to:***
Mamoun Ahram
Department of Physiology and Biochemistry, School of Science, Jordan.
E-mail: m.ahram@ju.edu.jo

effective role of certain miRNAs as either oncogenes or tumor suppressors with a specific role regulating cell invasion and metastasis [2,3]. The effective role of miRNAs as oncogenes arises from studies that revealed their induction of tumor progression[4]. In contrast, miRNA can act as tumor suppressor genes by, for example, down-regulating the expression of oncogenes such as Ras and c-Myc[5,6].

Serum is an essential requirement in cell culture experiments. It contains various molecules that affect cell growth such as steroid hormones, growth factors, and cytokines. To minimize the effect of endogenous steroid hormones on cell behavior, charcoal stripping is frequently performed to reduce the concentration of certain molecules such as steroid hormones, thyroid hormones, peptide hormones, and lipids[7-9]. Yet, the behavior of cells cultured in media containing charcoal-stripped fetal bovine serum (CS-FBS) may differ compared to those grown in media supplemented with standard FBS.

In this report, we aimed to investigate the effect of charcoal stripping of serum used in cell culture as a prerequisite to unravel the role of the steroid hormone, 5α-dihydrotestosterone (DHT), in regulating the expression of miRNA molecules. For this purpose, the breast MDA-MB-231 cancer cells were utilized.

Materials and methods

Cell line

MDA-MB-231 cells were kindly provided by Prof. Said Ismail (School of Medicine, The University of Jordan). The cells were maintained at 37°C, 5% CO_2, and a relative humidity of 95% in Dulbecco's Modification of Eagle's Medium (DMEM)-high glucose without L-glutamine. Media were supplemented with 10% FBS (PAA Laboratories GmbH., Austria) or 10% CS-FBS (PAA Laboratories GmbH.), 1% of L-glutamine, and 1% Penicillin/Streptomycin solution (Euro Clone S.P.A., Lonza, Belgium).

Drug preparation and treatment conditions

DHT was obtained from Tokyo Chemical Industry (Japan) and prepared as a 1 mM stock solution in dimethyl sulfoxide (Sigma, UK). The final concentration of DHT in media was 100 nM. Cells were cultured in media supplemented with 10% CS-FBS for three days prior to any treatment. All preparations of RNA, miRNA, cDNA, and expression level analyses were performed using kits from Qiagen (Germany) according to manufacturer's guidelines.

Total RNA extraction from MDA-MB-231 cell line

In order to detect the expression of AR mRNA, total RNA was extracted from MDA-MB-231 cells using RNeasy Mini kit. Complementary DNA (cDNA) was synthesized from total RNA samples using miScript II RT kit.

Nested PCR for detection of AR

Nested PCR was performed in two rounds as previously described[10,11]. Genomic DNA extracted from a blood sample using DNeasy Blood & Tissue kit was used as a positive control. The detection of amplified AR DNA was analyzed by agarose gel electrophoresis.

Cell morphology

MDA-MB-231 cells were cultured in six-well plates and were treated with 100 nM DHT for three days. Changes in cell morphology were monitored and photographed using Leica inverted microscope (Microsystems, USA).

PCR array

MiRNA was extracted from MDA-MB-231 type using the miRNeasy mini kit using the HiSpec and HiFlex buffers for miRNA and total RNA samples, respectively. Then, cDNA was synthesized from

miRNA samples using the miScript II RT kit. The miScript miRNA human breast cancer PCR array (MIHS-109Z) was used to assess miRNA expression. The array is composed of a panel of 84 cancer-related miRNAs and six housekeeping genes (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6B). Real-time amplification reactions were prepared using miScript SYBR Green PCR kit and the amplification was performed by ABI 7500 Real-Time PCR System (Applied Biosystems, ThermoFisher, USA). Relative quantification for the miRNAs was performed after normalization of the controls based on the $\Delta\Delta C_t$ method using the RT^{2}Profiler PCR Array Data Analysis web portal was provided at Qiagen's web site.

Results

Expression of AR in MDA-MB-231 cells

It was important to ensure that MDA-MB-231 cells expressed AR. Following reverse transcription of mRNA into cDNA, PCR was conducted. A faint band was observed indicating that cells expressed minute amounts of AR (Fig. 1).To confirm the expression of AR and amplify the small amount of amplified DNA, nested PCR was performed using internal primers of AR cDNA. An amplicon was apparent in both samples confirming the expression of AR in the cells.

Effect of DHT and FBS type on cell morphology

To illustrate the effect of DHT treatment and FBS type on MDA-MB-231 cell morphology, cells were grown for 3 days in media supplemented with CS-FBS or standard FBS in the presence or absence of 100 nM DHT. Untreated cells had a spindle-like or fibroblast-like shape. There was no difference between cells cultured in media supplemented with standard FBS or CS-FBS (Figure 2A and B, respectively). Moreover, treatment of cells with 100 nM DHT resulted in a slight decrease in cell size and an increase in cell width in both culture conditions, although they preserved their spindle-like and fibroblast-like shape (Figure 2C and D, respectively).

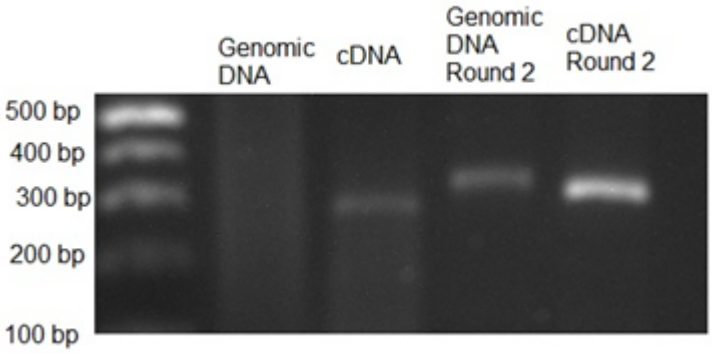

Figure 1. Expression of AR in MDA-MB-231 cells by nested PCR. Lane one represents the DNA ladder (left). Lanes two and four represent the PCR control (blood sample). Lanes three and five represent the samples from MDA-MB-231 cells

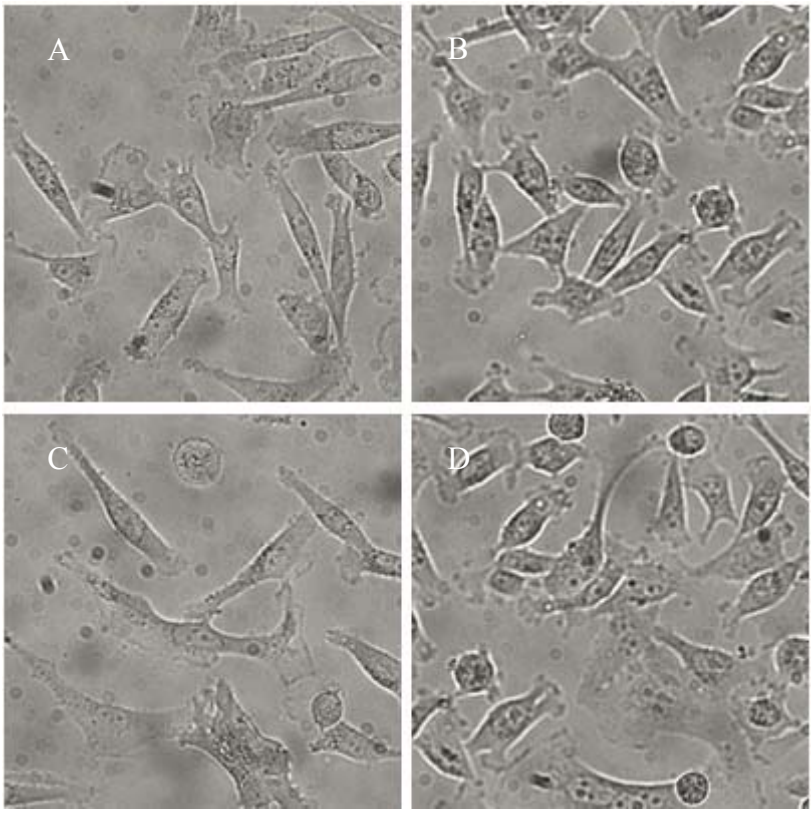

Figure 2.Effect of DHT and FBS type on MDA-MB-231 cell morphology. (A) Untreated cells cultured in media supplemented with 10 % standard FBS. (B) Untreated cells cultured in media supplemented with 10% CS-FBS. (C) Cells cultured in media supplemented with 10% standard FBS and treated with 100 nM DHT. (D) Cells cultured in media supplemented with 10% CS-FBS and treated with 100 nM DHT. Magnification: 40×

Effect of FBS type on miRNA expression

The effect of serum type on the expression levels of miRNA was first investigated. Cells were grown in media supplemented with either FBS or CS-FBS for three days and basal expression of miRNA molecules was assessed using PCR arrays. Of the 84 tested genes, 64 genes had reasonable expression where they were detected at cycles below 30 in both treatments. When comparing the detection level of miRNA in both culture conditions, 13 miRNA molecules had an altered expression (Table 1). Ten miRNA molecules were expressed at lower levels in cells cultured in media supplemented in CS-FBS versus standard FBS, and three genes were up-regulated. It is important to note that these changes in expression were all below 5 folds suggesting modest alteration of expression. It is also intriguing to note the change of expression of members of three families, namely let-7, miR-181, and miR-26. The expression of 5 of the 6 housekeeping genes was not altered and one had a difference of expression of 2.8 folds. However, the latter change would not affect the measurement of differential gene expression since the average change of the expression of all housekeeping genes was considered.

Table 1. Effect of charcoal stripping of serum on basal expression of miRNA in MDA-MB-231 cells (only miRNAs with a change of expression of 2 folds and more and those detected at cycles below 30 in either treatment are shown)

Mature miRNA	Fold-change of expression
let-7a-5p	-4.4
let-7e-5p	-3.9
miR-181b-5p	-3.3
miR-181b-5p	-3.3
let-7f-5p	-3.2
miR-222-3p	-3.2
let-7i-5p	-2.3
miR-100-5p	-2.2
miR-204-5p	-2.1
miR-200c-3p	-2.1
miR-27b-3p	2.0
miR-26b-5p	2.2
miR-26b-5p	2.2
Housekeeping genes	
SNORD61	-1.4
SNORD68	2.8
SNORD72	-1.9
SNORD95	1.3
SNORD96A	-1.1
RNU6-6P	-1.3

Effect of FBS type on DHT regulation of miRNA expression

In order to investigate the effect of DHT on miRNA expression, PCR arrays were utilized. The profiling experiment was first performed by using cell culture media supplemented with 10% standard FBS. Four miRNAs had a difference in regulation by more than two folds. Three were up-regulated: miR-328-3p, let-7f-5p, and miR-27b-3pby 2.6, 2.7, and 2.1 folds, respectively. A fourth miRNA, miR-210-3p, was down-regulated by two folds.

The experiment was repeated using cell culture media supplemented with CS-FBS. Overall, 35 miRNA molecules had a difference in expression in cells treated with DHT versus untreated by more than two folds. One miRNA, namely miR-328-3p, was up-regulated by more than 10 folds, two miRNAs, let-7f-5p and miR-10b-5p, were up-regulated by five to ten folds, and 28 miRNA were up-regulated by more than two and less than five folds. On the other hand, three miRNAs were down-regulated by more than two folds including miR-141-3p, miR-19a-3p, miR-19b-3p, miR-98-5p, and miR-96-5p.

Discussion

The existence of different BC subtypes poses a challenge in their diagnosis and treatment. It is mainly true of the type known as triple negative breast cancer (TNBC) that lacks the diagnostic and therapeutic targets. This necessitates identifying novel targets that may also play regulatory roles in the progression of biological behavior of BC. This was done to explore the expression of different types of miRNAs that probably plays a significant role in TNBC progression. However, it was necessary to optimize for the preferred conditions of cell culture.

This study clearly shows that the effect of DHT on treated cells is FBS-type dependent. The use of CS-FBS in media unmasks the effect of DHT on the regulation of miRNA expression. Endogenous components of FBS that are not present in CS-FBS clearly have an effect on the basal expression of miRNA in cultured cells. Various alterations in cell signaling and differentiation have previously been reported in cells cultured in CS-FBS in comparison with their counterparts grown in standard serum[12,13]. Furthermore, CS-FBS modifies the cellular bioenergetics of breast cancer cells[14]. Charcoal processing of serum influences the effects of active substances. For example, enzyme activity may be influenced by the presence of enzymatic cofactors[15].

Some miRNAs are associated with androgen expression or the status of cells according to the expression of AR. In one study, androgen activation resulted in up-regulation of miR-125b[16]. In addition, miR-34 has been reported to be absent in androgen-refractory cell lines[17]. There are very few studies on AR-associated miRNAs in breast cancer.

A study was conducted on androgen-inducible miRNAs in AR-positive BC MCF-7 cells and revealed that DHT treatment altered miRNA levels; 13 miRNAs were up-regulated and 28 miRNAs were down-regulated[18].Another study was performed on the global miRNA expression in DHT-treated MDA-MB-453 cells and found that four miRNAs were up-regulated whereas six miRNAs were down-regulated[19]. Our results are consistent with the latter two studies in which DHT alters miRNAs expression levels in BC cell lines. This is the first study to identify androgen-regulated miRNAs in MDA-MB-231 cells.

In conclusion this study offers an insight into the significance of culture conditions on molecular regulation of gene expression, specifically miRNAs. Although it is expensive relative to standard FBS, it is recommended to use CS-FBS in analyzing the molecular effect of DHT, i.e. regulation of miRNA expression. It is also imperative to further analyze androgen-induced alterations in miRNA expression and their biological implications.

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نوع المصل كمكمل لزراعة الخلية يؤثر على تنظيم التعبير عن الحمض الريبي القصير في خلايا الثدي MDA–MB–231 السرطانية

نهاد العثمان[1]، هنا حمّاد[1]، مأمون أهرام[2]

1– قسم علوم الأحياء، كلية العلوم، الجامعة الأردنية، الأردن.

2– قسم الفيزيولوجيا والكيمياء الحيوية، كلية الطب، الجامعة الأردنية، الأردن.

الملخص

الخلفية: عند زراعة الخلايا، يستخدم المصل الجنيني البقري بشكل واسع لتزويد الوسط الخلوي بالمواد الضرورية. فقد يحتوي مركبات مثل الهرمونات الستيرويدية والببتيدية وهرمونات الغدة الدرقية ويقوم بإخفاء تأثير بعض المركبات تحت الدراسة. معالجة المصل الجنيني البقري بالفحم يزيل هذه المركبات والتي يمكن لبعضها أن ينظم تعبير الحمض الريبي القصير (miRNAs).

الهدف: تحليل تأثير نوع المصل الجنيني البقري عند دراسة تنظيم تعبير (miRNAs) عن طريق الهرمون الستيرويدي (DHT) في خلايا الثدي السرطانية.

الطرق: تم زراعة خلايا MDA-MB-231 في وسط استخدم فيه المصل البقري الجنيني المجرد بالفحم أو بالمصل البقري الجنيني العادي. وبعد معاملة الخلايا بـ (100 nM DHT) لمدة ثلاثة أيام، تم تقييم شكل الخلية بالإضافة إلى تغيير تعبير (miRNA) والذي تمت دراسته باستخدام تقنية (PCR arrays).

النتائج: لا يتغير شكل الخلية بتغير نوع المصل الجنيني البقري. تغير التعبير عن 13 (miRNAs) بشكل طفيف في وسط مزود بالمصل البقري الجنيني الذي جرد بالفحم بالمقارنة مع المصل العادي. من ناحية أخرى، غير هرمون (DHT) في تعبير أربعة جزيئات (miRNA) فقط في وسط مزود بالمصل البقري الجنيني العادي، في حين غير في التعبير لـ 35جزيء (miRNAs) عند زراعة الخلايا في وسط مزود بالمصل الجنيني البقري المجرد بالفحم.

الخلاصة: يؤثر نوع المصل على تعبير الـ (miRNAs) في الخلايا المزروعة ويكشف كذلك الاختلافات في تعبير الـ (miRNAs) المحفز بهرمون (DHT).

الكلمات الدالة: المصل الجنيني البقري الجرد بالفحم، المصل الجنيني البقري العادي، خلايا الثدي السرطانية، الحمض الريبي القصير، هرمون.