Applicability of the P19CL6 cells as a model of cardiomyocytes – a transcriptome analysis

Iraj Khodadadi¹, 2, Nick J. Plant², Vassilis Mersinias³, Alfred E. Thumser²*

¹Department of Biochemistry and Nutrition, Hamedan University of Medical Sciences, Hamedan, Iran
²Division of Biochemical Sciences, University of Surrey, Guildford, United Kingdom
³B.S.R.C. “Alexander Fleming”, Varkiza, Greece; a.thumser@surrey.ac.uk

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ABSTRACT
The P19CL6 cell-line, a clone of the P19 mouse embryonal carcinoma cell-line, has been extensively used as a model for cardiomyocytes as these cells can be differentiated into a cardiomyocyte phenotype upon incubation with dimethyl sulfoxide. Uniquely, these cells can be observed to “beat” when monitored by microscopy. We started investigating the response of P19CL6 cells to fatty acids, but highly variable results lead us to investigate the phenotype of the P19CL6 cells in more depth. In this study we demonstrated that the P19CL6 cells are responsive to adrenaline, but lose the “beating” phenotype after 16 passages in culture. Analysis of specific mRNA transcripts indicated that the P19CL6 cells express both cardiac- and skeletal muscle-specific genes, while global analysis of microarray data showed clear differences between the P19CL6 cells and cardiac tissue of embryonic or adult origin. In conclusion, our observations suggest caution in the use of the P19CL6 cells as a model of cardiomyocytes unless rigorous validation for the intended analysis has been undertaken.

Keywords: Gene expression; Cardiomyocyte; P19CL6 Cell-line

1. INTRODUCTION
There is a requirement for the development of realistic cell culture models both for basic research and the development of novel therapeutic agents. However, for several tissues, including heart, no individual cell line has been successfully validated for these purposes; such a failure is usually the result of the loss of one or more specific phenotypic features associated with the target tissue in the cell line. One approach to mitigate these issues has been the utilisation of chemically-stimulated differentiation of stem cells, with the hope that these cell lines will have a more realistic phenotype than cell lines derived from fully differentiated tissue. Pluripotent embryonic carcinoma cells have been reported as successful in vitro models of cardiac differentiation; for example, the P19 mouse embryonal carcinoma cell-line has been reported to differentiate into an embryonic cardiac-muscle phenotype in vitro [1] upon the addition of dimethyl sulfoxide (DMSO) [2-5]. Differentiated P19 cells have been reported to retain the ability to spontaneously contract and shown to express transcripts in a temporal manner during culture, suggestive of a cardiac-muscle phenotype [5-7], and as such these cells have therefore been extensively used to study cardiac cell physiology [1,2,5,6,8], although with the caveat that these cells are embryonic instead. However, in addition to these cardiac-muscle-specific properties, P19 cells also display pluripotent properties and can be differentiated into cells displaying either a skeletal muscle or neural phenotype [1,3-5]. There has thus been some concern about the homogeneity of DMSO-differentiated P19 cultures, with a heterogeneous cell population following differentiation significantly reducing the utility of these cells as a cardiac-muscle-specific model: There has thus been much interest in identifying subclones of P19 cells that more robustly differentiate into cardiomyocytes. The P19CL6 cell-line, a sub-clone of P19 embryonal cells, has been reported to efficiently differentiate into beating cardiomyocytes upon exposure to DMSO under adherent culture conditions [9] and has been widely used as an in vitro model of cardiovascular cells [1,2,10-16].

It is clear that the P19CL6 cell line has potential as a model system for the study of cardiomyocyte development and differentiation, and indeed they are currently used as such. However, full characterisation and validation is required before they can be used for this purpose with full confidence. A review of the literature, focusing on P19CL6 and P19 cell culture conditions, shows that two separate methods are commonly used, namely adherent and non-adherent culture conditions [1,5,9,10,17].
In addition, vitamins and hormones such as adrenaline have been shown to act as potent inducers of P19 cell differentiation into cardiomyocytes in addition to the aforementioned DMSO [1, 5, 7, 9]. In this study we have characterized the P19CL6 cells in more detail under different culture conditions, focusing in particular on utilising microarray methodologies to compare the P19CL6 transcriptome against native cardiac-muscle and skeletal-muscle transcriptomes. These investigations represent the first robust examination of both P19CL6 transcriptome and cardiac phenotype, and demonstrate that the P19CL6 cell-line displays only a limited cardiomyocyte phenotype that is dependent on passage conditions. As such we would advise caution in the use of this cell line as a ‘complete’ in vitro model of cardiac-muscle cells.

2. MATERIALS AND METHODS

2.1. Materials

Cell culture media and reagents were obtained from Invitrogen Corporation (Paisley, U.K.) and Sigma-Aldrich Company Ltd. (Poole, U.K.). Materials and kits for RNA extraction, cDNA synthesis and RT-PCR were supplied by Promega Corporation (Southampton, U.K.), Amersham Biosciences (Chalfont St. Giles, U.K.) and Qiagen Ltd. (Crawley, U.K.). Corning Life Sciences (Schiphol-Rijk, Netherlands) supplied the ProntoPlus microarray kit. Ambion Ltd. (Huntingdon, U.K.) supplied mouse heart and embryonic total-RNA, whereas mouse skeletal muscle and embryonic heart total-RNA were purchased from Panomics Inc. (Redwood City, U.S.A.) and Zyagen (San Diego, U.S.A.), respectively.

2.2. Cell culture

P19CL6 cells were purchased at passage 9 from Riken Cell Bank (Ibaraki, Japan) in growing flask and cultured in medium containing α-MEM (minimal essential media) supplemented with 10% FBS (foetal bovine serum) and 1% penicillin-streptomycin (10,000 Units/ml and 10 mg/ml, respectively) [9]. To differentiate P19CL6 cells into cardiomyocytes, cells were plated in 6-well culture plates (10 cm²) at a density of 2×10⁴ cells/cm² in standard medium containing 1% DMSO [9]. Cells were cultured for 15 days at 37°C and 5% CO₂ with medium refreshed every second day. For culturing P19CL6 cells under non-adherent conditions, cells were stimulated to form aggregates by incubation in bacterial petri dishes (1×10⁶ cells/dish; 78 cm²), containing a thin layer of 0.5% agar, for 4 days with standard media containing 1% DMSO, before transfer to regular cell culture flasks for the remainder on the incubation period [5]. Cell aggregates were collected by centrifugation and replated into culture flasks for 15 days at 37°C and 5% CO₂ in the presence of 1% DMSO. The H9C2 (2-1) cell-line, a murine cell-line that expresses a skeletal muscle phenotype, was obtained from the European Collection of Cell Cultures (ECACC; Salisbury, U.K.) and cultured under the same adherent conditions as the P19CL6 cells.

2.3. Determination of mRNA Levels by Reverse Transcripase Polymerase Chain Reaction (RT-PCR) and cDNA Microarrays

Total RNA was isolated from cells with Trizol reagent, as per manufacturer’s instructions (Invitrogen Corporation, Paisley, U.K.). Whole heart and skeletal muscle tissues (upper leg muscle) were dissected from 10 week old male CD1 wild-type mice (+/+) homogenised in Trizol reagent using an Ultra-Turrax T8 homogeniser, and total RNA isolated as per manufacturer’s instructions. Gene-specific forward and reverse primers used in the one-step RT-PCR or nested PCR reactions are shown in Table 1. In samples that did not show detectable cDNA levels after an initial RT-PCR amplification the PCR products were reamplified by nested PCR [18]. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide, and the identity of PCR products verified by sequencing.

Microarray experiments were designed as dual-hybridisation optimal interwoven loops (http://exgen.ma.umist.ac.uk) [19-21]. cDNA was synthesised from purified total-RNA and labelled by a direct labelling method in the presence of Oligo dT, nucleotide mixture, Cy3-/Cy5- dCTP dyes, and SuperScript-II Reverse Transcriptase, based on Human Genome Mapping Project protocols (http://www.hgmp.mrc.ac.uk/). Purified Cy3- and Cy5- labelled cDNA samples (40 pmol each) were mixed in pairs (total volume 40 µl), according to the experimental plan, and hybridised to microarrays by incubation in hybridization chambers for 20 hours at 50°C, before post-hybridization washing and drying. Microarray images were acquired using an Affymetrix 428 scanner (Affymetrix, Santa Clara, USA) and analysed with BlueFuse (BlueGnome Ltd., Cambridge, U.K.), including quantification of pixel intensities of the spots and excluding background intensity and artefact areas on the arrays. The data was filtered by eliminating low quality array spots, as determined by BlueFuse’s spot uniformity and circularity measurements (spot uniformity and circularity >0.5 in at least 50% of the arrays), and normalised by intensity-dependent per spot and per chip (LOWESS) normalisation with GeneSpring-7 data analysis software (Agilent Technologies UK Ltd, Stockport, UK).

After Lowess normalisation and filtering of microarray data linear modelling (LLAMA; Live Linear Analysis of MicroArray) was performed (http://exgen.ma.umist.ac.uk) to convert data from the loop experiment into a linear model and generate differential gene expression estimates.

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In a series of separate cultures of P19CL6 cells it was noted that the cultures did not consistently display a “beating” phenotype, a characteristic that has previously been used as evidence of the cardiomyocyte properties of this P19 sub-clone [9].

### 3. RESULTS

A characteristic of the P19CL6 cells is that they display beating in localised nodes following differentiation in the presence of DMSO [9]. The cardio-stimulatory chemical adrenaline elicited a dose-dependent linear increase in the observed pulse rate with statistically significant increases observed between 0-20 μM adrenaline (Figure 1a). However, observation of P19CL6 cells over time in culture (passages 12-22) demonstrated that beating was consistently only observed between passages 12-16, and quantification of this beating showed a significant inverse correlation of the pulse-rate with passage number (Figure 1b). Microscopic analysis of DMSO-differentiated P19CL6 cells demonstrated mono-nuclear cells with no evidence of cell fusion; these characteristics were distinct from the morphological characteristics of skeletal muscle cells, as typified by the H9C2 (2-1) murine cell-line (Figure 2), and added further weight to the assertion that differentiated P19CL6 cells exhibit a cardiac-specific muscle phenotype [5,10,34]. The microscopic analysis and response to adrenaline provided an indication of a cardiac-type phenotype, although the loss of beating with passage number suggested that the phenotype may not be robust with regards to culture duration.
3.2. The P19CL6 Cell-Line Expresses both Cardiac- and Skeletal Muscle-Specific Transcripts

To further examine the reported cardiac properties of the P19CL6 cell-line, mRNA transcript levels of α-MHC, β-MHC, MyoD and myogenin were determined by RT-PCR followed by nested PCR. The expression of these gene products are characteristic of cardiac- (α-MHC, β-MHC) and skeletal muscle (MyoD, myogenin) tissues [35-38]. Figure 3a shows that P19CL6 cells expressed significant levels of α-MHC, β-MHC, MyoD and myogenin transcripts, inconsistent with a cardiac only phenotype: To examine if the unexpected expression of skeletal muscle markers in P19CL6 cells was caused by the culture conditions we also examined P19CL6 under non-adherent culturing conditions; under these conditions we once again observed both cardiac- and skeletal muscle-specific markers (Figure 3b), suggestive of a mixed cardiac/skeletal-muscle transcriptome in P19CL6 cells regardless of culture conditions. The identity of the α-MHC, β-MHC, MyoD and myogenin transcripts was confirmed by sequencing of the nested PCR products (data not shown). The marker transcript specificity was confirmed using both the H9C2 (2-1) cell-line, which only expressed MyoD and myogenin transcripts and mouse cardiac tissue, which only expressed the aforementioned transcript markers of cardiac phenotype, α-MHC and β-MHC (Figure 3c). These data were thus consistent with P19CL6 cells exhibiting a mixed cardiac/skeletal muscle phenotype, and therefore a global transcriptome analysis was undertaken to examine this hypothesis, comparing P19CL6 cells with the H9C2 (2-1) cell-line and mouse cardiac and skeletal muscle tissue.

3.3. Characterisation of the P19CL6 Cell-Line by Microarray Analysis

Transcriptomes from three independent P19CL6 cultures were compared by microarray analysis to the transcript-
tomes of mouse cardiac and skeletal muscle tissue, H9C2 (2-1) cells, and a reference sample, which was a mixture of cDNA from all the samples in equal proportions. An interwoven loop design and data reduction by linear modelling [21-23] was utilized to compare all samples to the reference sample (Figure 4). The transcriptomes of all samples were initially analysed by PCA and showed a low correlation between the P19CL6 cells and other samples (Table 2, Figure 5). Only the embryonic heart sample did not differ significantly from the P19CL6 cells (P>0.05), although even this comparison showed a very low correlation of 0.007 (Table 2). Subsequent PLS analysis, excluding the H9C2 (2-1) samples in order to simplify interpretation, showed that principal component 1 (PC1) accounted for 52% of the total variance and clearly separated the P19CL6 sample from the heart and muscle tissue (Figure 5). The loading factors for PC1 showing a variable importance (VIP) of >1.0 represent those transcripts driving the separation of P19CL6 cells from the other samples; these were put into a biological context by ascertaining Gene Ontology (GO) identifiers that were significantly over-represented in the identified transcript level changes, using the DAVID bioinformatics suite [24]. Such over-representation is often indicative of a significant biological effect in the pathway(s) associated with the GO identifiers. Several annotation clusters with an enrichment score of greater than 1.0, i.e. showing significant enrichment, were identified (502 separate genes) and the five main Biological Processes that were identified are shown in Table 3. A more detailed analysis of the genes identified within the “Regulation of cellular processes” cluster showed that 57% (total number 146 separate gene products) of the identified mRNA levels were up-regulated in P19CL6 cells compared to embryonic heart tissue, whereas the remainder were down-regulated (data not shown). The latter tissue was the primary focus for comparison as P19-derived cardiomyocytes are embryonic in nature and have previously been used as a model system for the embryonic heart [1,6,8]. However, in the original P19CL6 paper this cell-line was proposed to be a good model system for adult heart [9].

4. DISCUSSION

The P19CL6 cell-line, a derivative of P19 embryonal carcinoma cells, is widely used as an in vitro model of cardiovascular cells [1,2,10,16], and has been shown to differentiate into a beating phenotype that is reminiscent of cardiomyocytes upon exposure to DMSO [9]. Data presented here confirm that differentiated P19CL6 cells do exhibit some markers of a cardiac phenotype in the P19CL6 cells: a beating phenotype that is positively responsive to adrenaline; expression of transcript markers of cardiac phenotype (α-MHC, β-MHC); microscopic analysis showing mono-nuclear cells with no evidence of cell fusion [1,5,8,25]. However, closer examination of these features raises some concerns, and is suggestive of an unstable, mixed, cardiac/skeletal muscle phenotype. We demonstrated that the pulse-rate for beating was negatively correlated with the passage number of the cells, with no beating observed after passage 16, suggesting

**Figure 5.** Analysis of transcriptomic expression data (microarrays) by PLS analysis. Gene expression data from microarray analysis was reduced by linear regression, using the LLAMA algorithm [19-21]. The data was further analysed by PCA and subsequently PLS to demonstrate discrimination between the samples (R²X for principal components 1 and 2 is 0.52 and 0.33, respectively) and generate a list of variable importance that could be used in the DAVID analysis.
Table 1. Sequences of oligonucleotide primers used for PCR.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequences</th>
<th>%GC</th>
<th>Tm (°C)</th>
<th>Application</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-F</td>
<td>ATT CAA CGGCAC AGT CAA GGG AGC AGT ATT GGGGTT AGG AAC AC</td>
<td>52</td>
<td>59.8</td>
<td>RT-PCR</td>
<td>556 bp</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>AGC TTG TCA TCA AGC GGA AGC AGC TGA AGC TGG TGC AG</td>
<td>55</td>
<td>59.4</td>
<td>nested PCR</td>
<td>275 bp</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>GAC TCA ACT CAC GAC AAA TCC AGT CTT CGT</td>
<td>52</td>
<td>59.8</td>
<td>nested PCR</td>
<td>413 bp</td>
</tr>
<tr>
<td>α-MHC-R</td>
<td>ATG GAG CAG ACC ATC AAG GAC TGG TGT ATT</td>
<td>52</td>
<td>59.8</td>
<td>RT-PCR</td>
<td>491 bp</td>
</tr>
<tr>
<td>α-MHC-R</td>
<td>GGC CAC AGC GAG AAG AGT GAG CGG CAC ATC</td>
<td>55</td>
<td>59.4</td>
<td>nested PCR</td>
<td>302 bp</td>
</tr>
<tr>
<td>β-MHC-F</td>
<td>AGT GGA GAT CTT ACC GCC CCA CCA CCA CCA GAC</td>
<td>50</td>
<td>60.3</td>
<td>RT-PCR</td>
<td>587 bp</td>
</tr>
<tr>
<td>β-MHC-R</td>
<td>GAG ACT TGG TCT GAG GGC ATT GAG TGG ACA TTC</td>
<td>52</td>
<td>59.8</td>
<td>nested PCR</td>
<td>412 bp</td>
</tr>
<tr>
<td>β-MHC-R</td>
<td>CTT TCT TGG TCT TGC C CTC TAC CCA AGG TGG AGA TCC TGG TGT CAT TGT</td>
<td>55</td>
<td>59.4</td>
<td>nested PCR</td>
<td>392 bp</td>
</tr>
<tr>
<td>MyoD-F</td>
<td>AGC TTG ATG AGA CAT CCC CCT ACG ATG GAC GTA AGG GAG ACC AGG AGC</td>
<td>52</td>
<td>59.8</td>
<td>RT-PCR</td>
<td>608 bp</td>
</tr>
<tr>
<td>MyoD-R</td>
<td>AGC CCC ACT TCT ATG TGC</td>
<td>55</td>
<td>59.4</td>
<td>nested PCR</td>
<td>337 bp</td>
</tr>
<tr>
<td>Myo-G-F</td>
<td>GCG CAG GAT CTC CAC TTT AG</td>
<td>55</td>
<td>59.4</td>
<td>nested PCR</td>
<td>337 bp</td>
</tr>
</tbody>
</table>

F and R indicate forward and reverse primers, respectively. Tm: annealing temperature (°C); bp: base pair.

Table 2. Correlation matrix of mRNA expression levels in P19CL6 and H9C2 (2-1) cells, and skeletal muscle, adult heart and embryonic heart tissue samples, as analyzed by microarray analysis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>H9C2 (2-1)</th>
<th>Skeletal muscle</th>
<th>Adult heart</th>
<th>Embryonic heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19CL6</td>
<td>-0.058*</td>
<td>-0.154**</td>
<td>-0.210**</td>
<td>0.007</td>
</tr>
<tr>
<td>H9C2 (2-1)</td>
<td>--</td>
<td>0.031</td>
<td>0.175**</td>
<td>-0.023</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>--</td>
<td>--</td>
<td>0.299**</td>
<td>-0.024</td>
</tr>
<tr>
<td>Adult heart</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.373**</td>
</tr>
</tbody>
</table>

Microarray data was reduced by linear analysis using LLAMA and the data analyzed by PCA, with the data fitted to 5 components (explaining 100% of the cumulative variance). "P<0.05; "**P<0.001.

In conclusion, we have both undertaken physiological and transcriptome analysis of P19CL6 cells, assessing their suitability as models of cardiomyocytes for in vitro experimentation. Our data suggests that whereas the P19CL6 cells are subject to phenotypic drift with time in culture, and hence may not represent a stable cardiomyocyte phenotype. In addition, while P19CL6 cells expressed the aforementioned transcript markers of cardiac phenotype (α-MHC, β-MHC), they also expressed transcript markers for a skeletal muscle phenotype (MyoD and myogenin), again suggestive of a mixed cardiac/skeletal muscle phenotype. Whereas examination of single markers can give an indication of the potential phenotype for a cell-line they are not necessarily indicative of a fully functioning biological system. For example, liver cell-lines such as HepG2 have been validated for use in drug screening as hosts for both reporter genes and marker transcripts [26,27]; however, in-depth analysis demonstrates that this validation is simplified, with HepG2 cells unable to support some genome-based transcriptional activation and for the transcriptome to be markedly affected by culture conditions [28,29]. Therefore whereas low complexity measurements may be suitable for validation of cell-lines for use in individual assays, approaches such as transcriptome/proteome analysis are more appropriate to fully characterize cell-lines [30]; anchoring this data to known phenotypic markers then allows an accurate assessment of the appropriateness of any cell-line to the in vivo system they are supposed to be modelling [31]. Transcriptome analysis of P19CL6 cells demonstrated significant differences in the transcript profile between these cells and other samples, including importantly both embryonic and adult cardiac cells. Gene Ontology over-representation analysis suggests that these transcripts are linked to biological pathways associated with cellular metabolism, an interesting observation since it is generally accepted that cardiac muscle cells have specific metabolic processes that differentiate embryonic and adult cardiomyocytes, and also skeletal and cardiac muscle cells.

In conclusion, we have both undertaken physiological and transcriptome analysis of P19CL6 cells, assessing their suitability as models of cardiomyocytes for in vitro experimentation. Our data suggests that whereas the
P19CL6 cell-line has some phenotypic similarities to cardiomyocytes (e.g. the ability to pulse) there exist significant differences between these cells and the in vivo situation. Our observations clearly demonstrate that the P19CL6 cell line does not maintain a robust cardiac phenotype as shown by the loss of cell beating with time in culture. In addition, transcriptome analysis clearly shows that even freshly differentiated cells do not exhibit a clear cardiac or muscle transcript profile, further questioning the utility of P19CL6 as a model system for the study of cardiomyocyte physiology.

5. ACKNOWLEDGMENTS

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REFERENCES


ABBREVIATIONS

ANOVA: Analysis of variance;
DMSO: Dimethyl sulphoxide;
RT-PCR: Reverse transcriptase polymerase chain reaction;
PCA: Principle components analysis;
PLS: Partial least squares regression;
LLAMA: Live Linear Analysis of MicroArray.