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TECHNICAL NOTE

The effect of cell passage number on osteogenic and adipogenic characteristics of D1 cells

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Abstract Cell line passage number is an important consideration when designing an experiment. At higher passages, it is generally understood that cell health begins to decline and, when this occurs, the result can be variable data. However, there are no specific guidelines regarding optimal passage range, and this information is dependent on cell type. To explore these variabilities, low passage D1 cells were thawed (passage 3) and passaged serially until a much higher number (passage 34). Samples were taken every five passages and analyzed for alkaline phosphatase and triglyceride; also, the gene expression of both adipogenic and osteogenic markers was tested. The results indicate that the growth rate of these cells did slow down after passage 30. However, expression of the osteogenic characteristics seemed to cycle, with the highest levels seen at passage 4 and 24. The adipocyte expression levels remained the same throughout the study.

Keywords Adipogenic · D1 cells · Osteogenic · Passage number · Subculture

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Introduction

The passage number of cells is an important factor in all cell culture laboratories. One of the first techniques learned is how to passage, or subculture, cells. Every time a population of cells is split, that is an indication that the cells are aging. Aging may have different meanings, depending on what cell line is being used. Typically, passage is a crucial descriptor for primary cells. These cells have a finite lifespan and it is expected at a certain point the cells will change. Passage number still influences cell lines, but this influence is thought to be less critical than that on primary cells and therefore less defined. Generally it is understood that, even though cell lines are immortal, cell health will decrease as passage number increases (Clynes 1998). At a certain point, many researchers will discard higher passage cells in order to start over with lower passage cells to ensure consistent results. However, even with this understanding, actual studies outlining when to stop using a certain cell line are few and far between (Hughes et al. 2007). The effect of

passage number on primary cells has been documented, but not much has been determined regarding these continuous cell lines (Prasad Chennazhy and Krishnan 2005).

The D1 cell line is commonly used in many bioengineering studies, both with and without differentiation inducers. Depending on the culture conditions, these cells can exhibit osteogenic, chonodrogenic, and adipogenic properties (Diduch et al. 1993; Devine et al. 2002). In our own studies, this cell line is mainly used to investigate questions regarding osteogenesis and adipogenesis (Yang et al. 2007; Maxson and Burg 2008). In every experiment we conduct, consideration is given to the passage number of cells and, after passage 30, the cells are no longer used. This number was not derived from any testing process, but rather a laboratory "urban legend"; that is, no specific scientific rationale underlies this limit.

To clearly define a passage range for D1 cells in our work, early passage D1 cells (passage 3) were continuously subcultured and analyzed for different activity levels. The growth rate was monitored to determine if the generation time would change. Also, different osteogenic and adipogenic levels were monitored to evaluate potential fluctuations in the baseline characteristics, without differentiation inducers. Changes in these levels could indicate possible changes in the differentiation potential at different passages.

Materials and methods

Cell culture

Multipotent murine bone marrow stromal cells, D1 cells, were purchased from ATCC (Manassas, VA, USA) and grown in a standard T75 cell culture flask (Corning, Corning, NY, USA). These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Atlanta Biologicals, Atlanta, GA, USA) supplemented with 10 % fetal bovine serum (Corning, Manassas, VA, USA), 1 % antibiotic/antimycotic (Gibco, Grand Island, NY, USA) and 0.1 % fungizone (Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C and 5 % CO_2 and grown until ~80–90 % confluent.

For new passages, between 1–2 × 10^6 cells were seeded into T75 flasks and grown to 80–90 % confluence. The cells were removed by adding Trypsin–EDTA (Corning, Manassas, VA, USA) and the cell number was counted using a Millipore Sceptor Sensor (Millipore, Billerica, MA, USA).

At passages 4, 9, 14, 19, 24, 29, and 34, in addition to a T75 flask, a six-well plate was seeded by adding 100,000 cells per well. Once the cells reached confluence, all wells were rinsed with phosphate buffered saline (PBS; Sigma, St. Louis, MO, USA).

Cell lysis

A volume of 750 µl of PBS was added to three of the wells from each plate, and then the cells were removed using a cell scraper. The mixture was transferred into a sterile 2.0 ml tube, sonicated for 15 s, and frozen at −80 °C. Once all samples were collected, the lysate was thawn and used for alkaline phosphatase, triglyceride, and PicoGreen assays. The cells in the remaining three wells were used for ribonucleic acid (RNA) extraction.

Alkaline phosphatase

The cell lysate samples and all reagents were warmed to room temperature. Each sample was combined with a 5 mM phosphate substrate solution and incubated at room temperature for 1 h. The reaction was stopped using 3 M sodium hydroxide. Absorbance was read at 405 nm using an MRX Micoplate Reader (Dynex Technologies, Denkendorf, Germany) and the results were compared to a standard curve with known amounts of the phosphate substrate to determine the enzyme activity. This activity was normalized using the amount of double stranded deoxyribonucleic acid (DNA), determined with the PicoGreen assay. Results were reported as mM alkaline phosphatase (ALP)/µg of DNA.

Triglyceride

The same lysate samples that were used for the alkaline phosphatase assay were used to test for triglycerides. Each sample was combined with 1 % Triton X-100 (Sigma) at a 1:2 ratio and maintained for 15 min. The samples were subsequently combined with Infinity triglyceride reagent (Sigma, St. Louis, MO, USA), also at a 1:2 ratio. The samples were incubated at room temperature for 15 min and absorbance was read at 490 nm using a MRX Microplate Reader (Dynex Technologies, Denkendorf, Germany). The results were compared to a

standard curve that was generated with known amounts of a glycerol standard (Sigma, St. Louis, MO, USA) to calculate triglyceride amount. The amount of triglyceride was normalized by the amount of DNA, which was determined with the PicoGreen assay. Results were reported as mM of triglyceride/μg of DNA.

PicoGreen

Each cell lysis sample used in the alkaline phosphatase and triglyceride assays was also tested using the PicoGreen reagent (Invitrogen). Each sample was combined with PicoGreen reagent and allowed to incubate at room temperature. Fluorescence was then detected at 485 nm excitation and 528 nm emission. Results were compared to a standard curve generated with known amounts of DNA and were reported as μg of DNA. These results were used to normalize the alkaline phosphatase and triglyceride values.

RNA extraction

RNA from cells in the remaining three wells of the samples in the 6-well plate was extracted using Trizol (Ambion, Carlsbad, CA, USA). After the PBS rinse step, 1 ml of Trizol was added to each well. The wells were scraped and the extracts triturated to lyse the cells. Each extract was then transferred to a 1.5 ml tube, and the RNA was extracted according to the manufacturer's instructions.

Real-time PCR

RNA was reverse-transcribed to complementary DNA (cDNA) using the Ambion Retroscript kit two-step protocol. The quantity and quality of the RNA was determined using the Nanodrop 2000. The cDNA was analyzed using the QuantiTech SYBR Green reverse transcriptase polymerase chain reaction (RT-PCR) Kit (Qiagen, Valencia, CA, USA) on the Rotor-Gene light cycler. The markers analyzed were alkaline phosphatase (ALP), runt-related transcription factor 2 (RunX2), osteocalcin (OC), and adipocyte protein 2 (AP2). The primer sequences can be seen in Table 1. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene and the passage 4 cells as the control cells (Livak and Schmittgen 2001).

Table 1 Primers (Integrated DNA Technologies, Coralville, IA, USA) used for real-time PCR

Gene	Sequence
GAPDH	F: 5′-GAACGGATTTGGCCGTATTG-3′
	R: 5′-CGTTGAATTTGCCGTGAGTG-3′
ALP	F: 5′-GTAACGGGCCTGGCTACAAG-3′
	R: 5′-AAAGACCGCCACGTCTTCTC-3′
RUNX2	F: 5′-ACTGGCGGTGCAACAAGAC-3′
	R: 5′-ATAACAGCGGAGGCATTTCG-3′
Osteocalcin (OC)	F: 5′-AGACCGCCTACAAACGCATC-3′
	R: 5′-GATCAAGTCCCGGAGAGCAG-3′
AP2	F: 5′-ATGTGTGATGCCTTTGTGGGA-3′
	R: 5′-TGCCCTTTCATAAACTCTTGT-3′

Statistical analysis

Data were analyzed using Excel 2010 (Microsoft, Redmond, WA, USA) and JMP software (JMP, Cary, NC, USA). The types of statistical tests performed included (1) linear and segmented regression to test for a significant relationship of doubling and passage, and (2) Analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference Test to test for differences. Statistical significance was defined by a hypothesis test producing a p value <0.05.

Results

Cell doubling rates

The doubling rate of the cells stayed fairly constant through the passage study, until passage 30 when the rate increased. A linear regression was performed to assess the relationship between passage number and doubling time. It was determined that the doubling time was related to passage number and increased significantly as passage number increased. A segmented regression was also performed and showed, while there was an increase in doubling time from passages 4–25, the doubling time increased more dramatically starting at passage 26 (Fig. 1; Table 2).

Alkaline phosphatase

The amount of alkaline phosphatase was higher in the earliest passage (passage 4) compared to the later

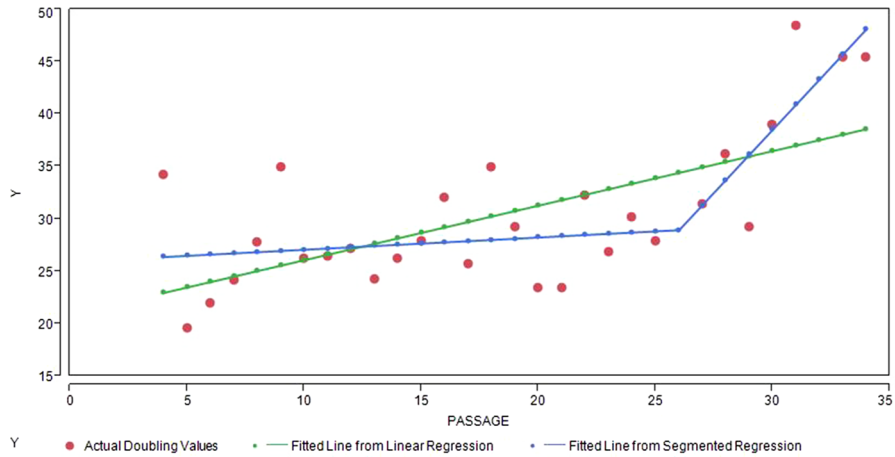

Fig. 1 Doubling time by passage number. Both a linear regression and a segmented model were performed. Both indicated that doubling time increase with passage number

passages. The ALP activity declined until passage 24, and then increased slightly before decreasing again. Data stemming from this assay were analyzed using the one-way ANOVA in JMP. When the means were compared for each passage, ANOVA suggested significant differences among the responses. Specific comparisons were performed for each passage number using Fisher's Protected Least Significant Difference test. Results of this test indicated that only passage 4 was significantly different from other passages (Fig. 2).

Triglycerides

Data from this assay was analyzed using the one-way ANOVA in JMP. However, in this assay, the means were not determined to be significantly different (Fig. 3).

RT-PCR

Data from the $2^{-\Delta\Delta Ct}$ method calculations were analyzed using the one-way ANOVA in JMP. The means of the adipogenic gene (ap2) were not significantly different with passage; however, there was significant difference in the means of the osteogenic genes (ALP, RunX2, and OC) from passage to passage. Specific comparisons were then made using Fisher's Least Significant Difference test. Expression of ALP and OC genes at passage 4 was shown to be significantly different from the expression at other passages. Passage 9, 14, 19, 24, 29, and 34 were not shown to be significantly different.

When considering the RunX2 gene, multiple groups of passages could be identified. Passages 4 and 24 were shown to be statistically different from the other passages, but not from each other. The same was true for passages 4 and 19. The remainder of the passages (passages 9, 14, 29, and 34) as well as passage 19 could also be grouped together as being statistically similar, and statistically different from passages 4 and 24 (Figs. 4, 5, 6, 7).

Discussion

One of the first cell culture techniques taught is cell passaging. Passaging is important for many reasons, the most obvious being to create a critical number of cells. The immortal cell line is vital to obtain enough cells to perform experiments and studies and to provide consistency. Cells are passaged to maintain cell health; once cells are overgrown, their activity can change, thus potentially affecting experimental outcomes. Cells are passaged for storage; large batches of cells can be frozen and stored for future use (Freshney 2005). However, with all this good come the bad. The more cells are passaged, the more they can change (Clynes 1998).

This idea that, over time, subcultures of cells can change is one that is accepted as fact. Cells age, even immortal cell lines, and at a certain point should not be used any more (von Zglinicki et al. 1995). But what is the cut off? Other researchers have found that cell activity levels depend on passage number (Hughes et al. 2007). Many of the previous studies focus on

Table 2 Start and end number of cells, with calculated doubling rates

Passage	Start #	End #	Time (h)	Doubling
4	1,000,000	7,000,000	96	34.20
5	1,000,000	5,500,000	48	19.52
6	1,100,000	5,000,000	48	21.97
7	1,000,000	7,900,000	72	24.15
8	1,000,000	6,020,000	72	27.80
9	1,000,000	6,700,000	96	34.98
10	1,000,000	6,700,000	72	26.24
11	1,000,000	6,590,000	72	26.47
12	1,000,000	6,300,000	72	27.12
13	1,500,000	5,900,000	48	24.29
14	1,000,000	6,700,000	72	26.24
15	1,000,000	6,000,000	72	27.85
16	1,000,000	8,000,000	96	32.00
17	1,000,000	7,000,000	72	25.65
18	1,000,000	6,700,000	96	34.98
19	1,000,000	5,500,000	72	29.28
20	1,000,000	8,400,000	72	23.45
21	1,000,000	8,400,000	72	23.45
22	1,000,000	7,900,000	96	32.19
23	1,000,000	6,400,000	72	26.89
24	1,000,000	9,100,000	96	30.13
25	1,000,000	6,000,000	72	27.85
26	1,000,000	4,600,000	120	Not calculated
27	1,000,000	4,900,000	72	31.40
28	1,000,000	6,300,000	96	36.15
29	1,000,000	5,500,000	72	29.28
30	1,000,000	3,600,000	72	38.96
31	2,000,000	5,600,000	72	48.47
32	2,000,000	3,300,000	72	99.66
33	2,000,000	6,000,000	72	45.43
34	2,000,000	6,000,000	72	45.43

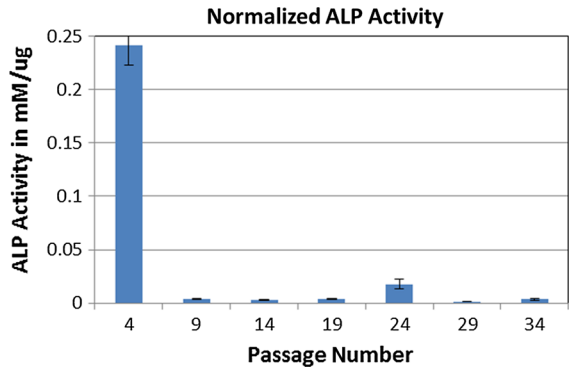

Fig. 2 ALP activity by passage number. The highest ALP activity was seen at passage 4

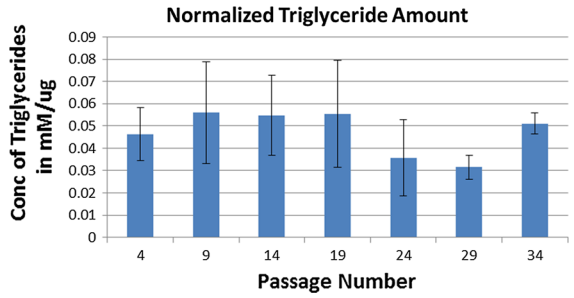

Fig. 3 The concentration of triglycerides by passage number. The amount did not significantly change

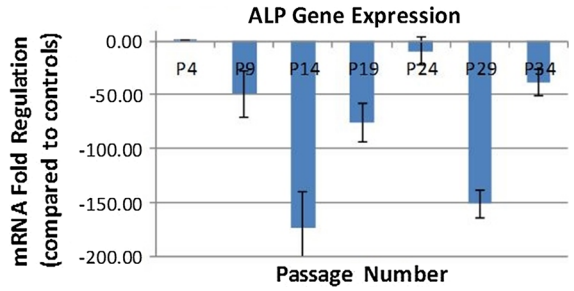

Fig. 4 The relative expression levels of the ALP gene by passage number. The highest expression levels were at passages 4 and 24

primary cells. Because of their finite life span, it is critical to estimate when primary cells might change behavior and function (Prasad Chennazhy and Krishnan 2005; Wall et al. 2007). There have been some continuous cell lines studies; however, many of the studies in the literature focused on a particular cell line. Although only a limited number of cell lines have been studied, some general characteristics have been determined. Cell proliferation decreases at high passages (Peterson et al. 2004); depending on the passage, different factors may have more or less of an impact on high passage cells than on low passage cell counterparts (Chung et al. 1999). It has also been found that cell passage number can affect how easily cells may be virally infected (Jensen and Norrild 2000). In some cases, the studies were able to reinforce the particular laboratory's current guidelines of appropriate passage number of cells (Haghi et al. 2010).

With these studies in mind, we assessed one of our most heavily used cell lines, D1, to determine passage

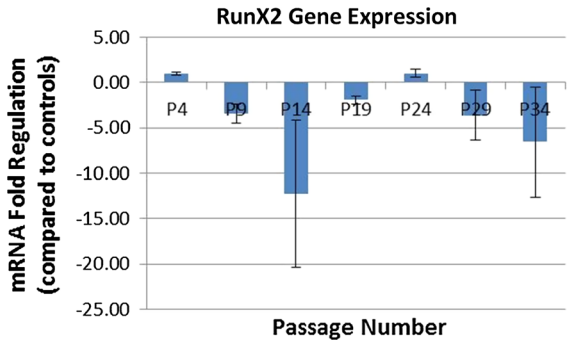

Fig. 5 The relative expression levels of the RunX2 gene by passage number. The highest expression was measured at passages 4 and 24

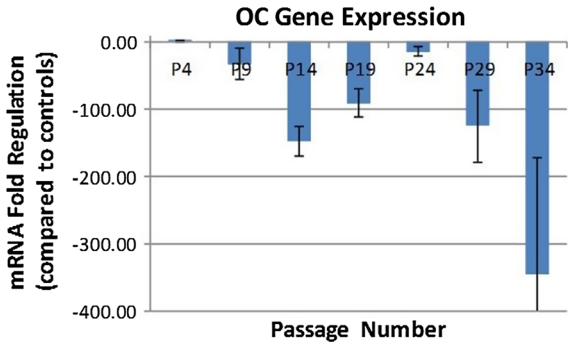

Fig. 6 The relative expression levels of osteocalcin (OC) by passage number. The highest levels were seen at passage 4 and passage 24

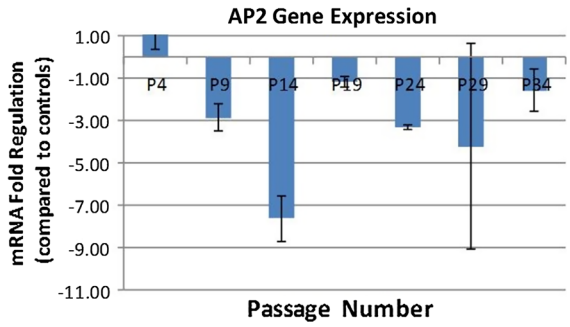

Fig. 7 The relative expression levels of AP2 by passage number. The highest expression levels were seen at passages 4 and 19 but these changes were not significant

limitations. Because we often differentiate these cells to osteocytes or adipocytes, we tested different passages using the same assays with which we test our differentiated cells (Cui et al. 1997; Li et al. 2005). The goal was to use these studies to develop passage specifications for the D1 cells.

As expected, the growth rate of the D1 cells did slow in the later passages. Our pre-existing "cut-off" of passage 30 was proven to be valid for this reason. It was also shown that the adipogenic properties remained consistent through all passage numbers; however, osteogenic characteristics changed significantly, and not by constantly increasing or decreasing, but in a cyclical pattern.

It is interesting to note that the significant variations were mainly seen in the osteogenic characteristics. The levels of ALP and the expression of the osteogenic genes started off high at passage 4, then decreased until passage 24, at which time gene expression cycled up and then down again. D1 cells are unique in that they can be pushed down an osteogenic or adipogenic pathway. It has been documented that some cell lines with the ability to differentiate in these two directions can lose the adipogenic pathway at high passage numbers, while the osteogenic pathway remains intact (Wall et al. 2007). However, other studies have shown that adipogenic potential increases as cells age, which mirrors what is seen in the development of osteoporosis (Kim et al. 2012). If these cells had been allowed to continue past passage 34, perhaps there would have been an increase in the adipocyte characteristics. With the data that were observed, perhaps these D1 cells are able to retain some of their initial characteristics as bone marrow cells and therefore some bone expression levels are more susceptible to changes in the microenvironment.

Based on these results, it is advised that a basic passage study be done on all cell lines to set individual limits, corresponding to the needs of each particular laboratory. From these results, our laboratory guidelines have been amended to reflect D1 cell usage between passage 9 and passage 30.

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