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ANIMAL HUSBANDRY

Single Cell Proliferation to Produce Monoclonal Population of Transfected Caprine Fibroblast Cell

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ABSTRACT

Transfected cell lines are widely used in biological research. Cell heterogeneity within a population can be vital to its unusual function and fate. Mixed mutants may not be as informative regarding which cell responds to our treatment. Cell to cell differences in RNA transcripts and protein expression further complicate the condition. To understand the real affect on the treated cells, researchers want to have single cell monoclonal population to provide correct information for getting truly transfected cell population. With this fact, we focus on the single cell isolation from transfected cells and its proliferation, which include methods of single cell pickup, analysis of media supplements on growth parameters and optimizing the best condition for single cell proliferation. Dilution method and Single cell pickup with mouth pipette method were compared for their efficacy. Rotational media supplement was found better than the regular medium used for cell growth. To explore the best suitable conditions for cell proliferation and survival, the cell viability percentage, total cell count, cell proliferation rate and population doubling time were calculated at different time interval in different media supplement groups.

Highlights

- Single cell pickup is crucial for RNA interference studies to get monoclonal population.
- Variable media supplementations perform better than the routine media.

Keywords: Single cell proliferation, Clonal cell population, Dilution method, Mouth pipette cell pickup, Rotational media

Monoclonal cell population generated by Single-cell has become important to study the correct effect of the treatment. Over the last few years, RNA interference (RNAi) has become a widely used and promising RNA-based technique that permits the knockdown and hence functional analysis of individual genes in vertebrate cells (Agrawal *et al.* 2003). After transfecting the cell population with RNAi molecules like siRNA or shRNA to create the stable cell lines, it is important to identify the cells getting these RNAi molecules. As it is now well recognized that the level of gene expression varies widely at the single-cell level in transfected cells (Jain *et al.* 2015). Several researchers studied different mathematical models for the quantitative analysis of the dynamics and stability of cellular status at the genome, transcriptome, proteome, and/or metabolome levels. This type of single-cell study showed that the mRNA and protein expression levels show extreme fluctuation in not only undifferentiated but also all kinds of cells (Hitoshi *et al.* 2010).

Vector based delivery system support the identification of transfected cells as they contain selection marker, Most of selectable marker's are often drug resistance genes such as *hpt* (Hygromycin phosphotransferase), *neo* (Neomycin phosphotransferase), *pac* (Puromycin

N-acetyltransferase) etc. After selection with these markers further evaluation of each cell are necessary to assess its efficiency. For that Single cell should be collected and proliferate to generate the monoclonal population. The single cell can be isolated either by dilution method, using low melting agarose or single cell pickup by micromanipulator (Nishizawa *et al.* 2012).

In order to ensure the exponential growth of single cell in culture medium, the media conditions were optimized by supplementation for the better cell multiplication. Dulbecco's Modified Eagle Medium (*DMEM*) is a widely used basal medium for fibroblast cells and well recognized for porcine (Steward *et al.* 2008) and caprine (Jain *et al.* 2010) fibroblast cells. Seven media supplements were tried as described by Jain *et al.* (2015) for single cell proliferation. Serum is very important factor of the media which provide all necessary elements for the cell growth. It also helps in better cell surface attachment. Insulin has been found to increase the plating efficiency of several cell types (Hamilton and Ham 1977). Insulin promotes the uptake of glucose and amino acids (Kelley *et al.* 1978). Insulin-like Growth Factor-II (IGF-II) is a factor displaying insulin-like activities and induced cellular division in chick fibroblasts (Jr.,Pierson and Temin 1972) and stimulates glucose uptake (Sinha *et al.* 1990). Fibroblast Growth Factor-Basic (bFGF) is a potent mitogenic agent for a wide variety of mesoderm-derived cells. Glucose is included in most media as a source of energy. It is metabolized principally by glycolysis to form pyruvate, which may be converted to lactate or acetoacetate and may enter the citric acid cycle and is oxidized to form CO_2 and water (Freshney 2000). Medium that has been used for the growth of other cells acquires metabolites, growth factors, and matrix products from these cells. This conditioned medium can improve the plating efficiency of some cells if it is diluted into the regular growth medium (Freshney 2000).

All above facts make the single cell pickup crucial. Therefore in this study we compare two different methods of single cell pickup i.e. Dilution method and Single cell pickup by mouth pipette method. Furthermore the rotational media supplementations were analyzed for better cell proliferation to get monoclonal population in minimum time.

METHODOLOGY

Single cell pickup

The suspected positive clones were selectively overgrown. For this purpose two different methods had been adapted: (a) *Dilution method* - The cells were trypsinized to produce a single-cell suspension. When the cells rounded up and started to detach, the monolayer in 5 mL medium containing serum was dispersed. The cells were counted and diluted in the cell suspension (1×10^5 cells/mL), and finally 10 cells/mL suspension was prepared. A 96 well plate were seeded with diluted cell suspension by adding 100 µl medium per well. The 2 fold serial dilutions were made to get single cell in each well. The plate was incubated in humid CO_2 incubator at 37°C. Plate was observed every 3rd day and media was changed as per necessity. (b) *Single cell pick-up by holding pipette* - The targeted cell monolayer was trypsinized and cell suspension was prepared in 1 mL medium containing serum. A drop of cell suspension was prepared on a petri plate and a single cell was picked by the holding pipette by creating negative pressure using micro-manipulator. The individual cell was then dispensed in a well of 96 well plate containing 100 µl media. Finally these single cells were incubated in CO_2 incubator till colony formation.

Cell proliferation and viability assay

The conditions were optimized for cell growth and multiplication efficiency. For this purpose seven different medium suppliments as described by Jain *et al.* (2015) were tested to study the various quality parameters of caprine fibroblast. These were tested for single cell proliferation assay with one cell per well seeded in 96 well plate. After 24 hrs of cell plating the normal media were replaced with different medium supplement groups. Media were replaced after every 3rd day. The readings were taken on the 11th day of cell seeding for cell viability, proliferation rate and population doubling time.

Cell viability were calculated by staining of cells with 0.4% trypan blue, the cells with blue stain were counted as dead and viability percentage was calculated using hemocytometer *Cell Viability* = (Viable cell count/ Total Cell count) × 100. Cell proliferation rate were calculated by *Proliferation rate (r)* = $3.32 \times \{(\log N_2 - \log N_1)/ t_2 - t_1\}$, where r = cell

Table 1: Compositions of media during Rotational media supplementation assay at different time interval

		Media Type				
Time Interval	**Cumulative Time**	**Control-I**	**Control-II**	**Variable-I**	**Variable-II**	**Variable-III**
0	0	10% FBS	10% FBS	10% FBS	10% FBS	10% FBS
24	24	10% FBS	15% FBS	20% FBS + 10mM Glu	20% FBS + 10mM Glu	20% FBS + 10mM Glu
48	72	10% FBS	15% FBS	15% FBS	15% FBS + FGF	10% FBS + IGF-II
72	144	10% FBS	15% FBS	20% FBS	20% FBS	20% FBS
72	216	10% FBS	15% FBS	15% FBS	15% FBS	15% FBS
48	264	Cell Counting				

proliferation rate, $\log N_2$ = total cell count at time of harvesting, $\log N_1$ = number of cells seeded initially, t_2-t_1 = time interval between seeding and harvesting of cells in days. The population doubling time was calculated by *Population doubling time (hr) = 24/r.*

Rotational media supplement assay

On the basis of our previous work (Jain *et al.* 2015), in this study we have tried an innovative combination of media supplements to provide the optimum growth conditions to the cells. The best media supplement groups observed was combined in a rotational manner to generate an effective and optimal cell growth conditions. The media supplement group which perform best upto a particular stage of cell proliferation during single cell pick-up assay in transfected cell were used during that particular time interval, so that maximum cells will multiply and produce clonal population (Table 1). 10% FBS and 15% FBS supplemented media was taken as control group. The three different variable media groups (namely Variable-I, II and III) were used to test the efficiency of these combinations and to evaluate the optimal conditions (Table 1). After 11th day cells were harvested and evaluated for growth parameters.

All the cell proliferation experiments were repeated thrice and every time 12 wells per plate in 96 wells plate were treated with same media (N=36). All the generated data were pooled and analyzed. Average and standard deviation were calculated to compare the performance among different media groups.

RESULTS AND DISCUSSION

Interest in single cell molecular analysis has risen dramatically over the last couple of years, chiefly because single cell molecular analysis is the only way to research genetic heterogeneity. During the course of preparing stable transfected cell line, single cell selection is very important step to generate clonal population. This will help in obtaining the true monoclonal population, as the selective overgrowth of cells of the wrong lineage remains a major problem. The aim of stable, long-term transfection is to isolate and propagate individual clones containing transfected DNA that has integrated into the cellular genome. Various single-cell isolation techniques, including dilution, micromanipulation, flow cytometry, microfluidics, and compartmentalization, have been developed to get a single cell for producing clonal population. Various other techniques, such as viable staining, in situ hybridization, and those using auto fluorescence proteins, are frequently combined with these single-cell isolation techniques depending on the purpose of the study (Ishii *et al.* 2010). Different advanced tool like modular Single-Cell Pipette are also available to get single cell (Zhang *et al.* 2016). All the advanced techniques have their limitation in terms of equipments and specific materials. Further they may have off target effects, which may interfere with normal cell proliferation. Hence we had adapted chemical free methods for obtaining single cell.

To explore the better method for selecting single cell and its efficient multiplication, two methods were tried, dilution method and single cell pick-up by mouth pipette. In our experiment, use of dilution method revealed better cell proliferation in wells (82.54%), but it was difficult to get a single cell in each well (32.15%). On the other hand, although by the use of mouth pipette technique resulted in lower number of well showing cell proliferation (46.04%) but assured monoclonal cell population (100%). By these results it is hypothesized that mouth pipette

Table 2: Comparison of Dilution method and single cell pick-up by mouth pipette method

	Dilution Method					Single cell pick-up by mouth pipette		
	Total wells seeded	Wells with Proliferation		Wells with single cells		Total wells seeded	Wells with Proliferation	
		No.	Percent	No.	Percent		No.	Percent
GT	252	208	82.54	81	32.15	252	116	46.04

Table 3: Cell growth parameters in Single cell proliferation assay on 11th day (N=36)

*Media	1	2	3	4	5	6	7
Cell viability percentage	84.26 ± 19.74	98.43 ± 3.14	99.2 ± 1.61	97.18 ± 4.74	97.24 ± 3.02	98.35 ± 2.21	98.95 ± 1.72
Total cell count	528 ± 264	4028 ± 2116	10056 ± 4902	8862 ± 4418	6945 ± 4359	7556 ± 3135	9473 ± 4253
Cell Proliferation Rate [r]	0.81 ± 0.07	1.08 ± 0.07	1.2 ± 0.07	1.18 ± 0.08	1.14 ± 0.09	1.16 ± 0.06	1.19 ± 0.07
Population Doubling Time (hrs)	29.98 ± 2.61	22.46 ± 1.39	20.14 ± 1.03	20.53 ± 1.37	21.24 ± 1.6	20.75 ± 1.06	20.3 ± 1.18

**Media numbers (1 to 7) indicate DMEM media supplemented with:*

1. *10% FBS,*
2. *15% FBS,*
3. *20% FBS,*
4. *10% FBS and 10 mM extra glucose*
5. *10% FBS and 33% Conditioned Medium,*
6. *10% FBS and Fibroblast growth factor*
7. *10% FBS and Insulin like growth factor-II*

technique take more time during single cell transfer, may be the cause of lower cell proliferation (Table 2). Mouth pipette method was adopted for all the further experiments as it provide assured clonal population.

Cell culture medium is used to support the growth of cells in-vitro and generally comprised of energy source and compounds which regulate the cell cycle. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose and serum as a source of growth factors, hormones. In addition to nutrients, the medium also helps in maintaining pH and osmolality (Arora 2013). In general, the choice of the medium mostly depends on the type of the cells to be cultured, and the purpose of the culture (growth, differentiation, and production of desired products). The major components of media were naturally derived products such as serum (Yao and Asayama 2017). Fetal bovine serum (FBS) is the most essential supplement in culture media for cellular proliferation, metabolism, and differentiation (Cho *et al.* 2018). Several workers tried different media supplementation to get more viable and functional cells with enhanced cell proliferation rate (Cho *et al.* 2018; Li *et al.* 2017; Singh and Sharma 2011).

With this context the optimum conditions for fibroblast cell growth were standardized for single cell proliferation in this study. All the seven groups of media supplements suggested by Jain *et al.* (2015) were tested on single cell seeded in 96 wells cell culture plate by mouth pipette method. It was observed that cell proliferation was visible after 5-6 days in different densities in different groups. On the 11th day almost 50-60% surface of 96 well was covered by the cell population. At this point the cells were evaluated for different cell proliferation parameters in different groups. In this experiment cell proliferation rate was highest in 20% FBS supplemented media followed by IGF-II, Glucose enriched and FGF containing media (Table 3).

The rotational media supplementation for single cell proliferation was the innovative step of this study. The best media supplement group was used for a particular time interval in a rotational manner to generate an effective and optimal cell growth conditions. On the 11th day, the cells were analyzed and found that all the variable media groups performed better than both the control groups (Table 4). All three variable media groups showed lower population doubling time with respect to both the control groups. Among the variable groups, the Variable-III group was better in single cell proliferation with 23.52 hours population doubling

Table 4: Effect of different media on cell growth parameters at single cell proliferation

	Control-I	Control-II	Variable-I	Variable-II	Variable-III
Viable cell percentage	80.56 ± 17.35	100 ± 0	100 ± 0	97.78 ± .85	90.66 ± 13.65
Total cell count	750 ± 250	1250 ± 662	2334 ± 1942	3917 ± 3503	3834 ± 3876
Cell proliferation rate [r]	0.87 ± 0.05	0.93 ± 0.07	0.99 ± 0.12	1.03 ± 0.19	1.04 ± 0.14
Population doubling time	27.88 ± 1.5	26.09 ± 1.83	24.65 ± 2.95	24.09 ± 4.75	23.52 ± 3.11

time. The total cell count was also 5 fold higher to that of control groups, which is indicative for good cellar health as desired.

The culture conditions and healthy cells are very important for any cell proliferation assay. The healthy cell can produce a good clonal cell population for further investigation and can provide an active stably transfected nucleus for pronuclear transfer to produce SCNT clone. In order to ensure the exponential growth of cells in culture medium, the percentage of live and dead cells were detected by using viability stains (Kaltenbatch *et al.* 1958). Increased vesiculation, irregular shape, and presence of black dots were taken as criteria of poor development of cell line which were discarded for subsequent passages (Freshney 2000). Gupta *et al.* (2003) reported mean population doubling time to be varied from 28.57 to 35.29 hrs in different passages. Likewise Hill *et al.* (2001) reported the mean population doubling time of bovine dermal fibroblast over 120 days of culture to be 30.7 hrs but in our experiment it was only 23.5 hrs with variable III media. All these findings support that if medium conditions were not optimized; exert negative effect on cell culture.

CONCLUSION

The production of stably transfected cells was a crucial step for the gene knockdown studies. The RNAi molecules cannot propagate in eukaryotic cells unless they are integrated in the genome. Integration is a rare phenomemon (1 in 10^4 for mammalian cells), but if there are markers for it, one can select cells with stable transfection. Hence the proper growth of these precise cells is very important to generate clonal population. The present study thus optimized the proliferation conditions of these transfected fibroblast cells. The study concluded that single cell pickup with mouth pipette gives surety of clonal population. The rotational media supports cell multiplication at a faster rate with excellent total cell count. Media supplementation helped to produce monoclonal cell population at a faster rate with optimal growth parameters. Furthermore it was demonstrated that a particular media supplementation performed better at a particular time interval.

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