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ABSTRACT:

The study of neurogenetic disorders such as Angelman, Rett and Fragile X syndromes as well as autism spectrum disorders has been challenging because of limited testing models. So far, these studies rely on the analysis of gene/protein expression in non-neuronal biospecimens like lymphoblast and fibroblast cell lines. Here we propose to use dental pulps from extracted primary teeth as a source of dental pulp stem cells (DPSCs) for the study of a variety of neurogenetic syndromes. Currently there are no established protocols to successfully obtain DPSC lines from individuals with neurogenetic syndromes. The aim of this study is to examine the effects of storage temperature, time of processing, immortalization, and cellular senescence of DPSCs to determine an optimal protocol for DPSC culture and maintenance to provide a long term and potentially renewable research resource. To accomplish these aims 23 extracted primary teeth were collected from neurotypical subjects from the UTHSC College of Dentistry clinics. The teeth were stored at 4°C and 72°C and processed at 24 and 72 hours and then processed to obtain DPSCs. The DPSCs were cultured and maintained to examine how long they remain viable and how the storage conditions affected culture success. DPSCs that were kept under different storage conditions were immortalized before passaging and after passaging to observe if there is an optimal time for immortalization and whether or not storage conditions affect success. A senescence assay was conducted on 4 samples to determine the longevity of the DPSCs. A culture success rate of 62.5% was obtained with similar failure rates among the different storage temperatures and processing times. All seven attempts at immortalizing samples at passage 2 from groups 1, 2, and 3 were successful. All six attempts at immortalization before passaging were not successful for all groups. Four DPSC lines that underwent senescence testing at each passage all showed similar rates of increased cellular senescence as the number of passages increased. The average senescence rate peaked at 93.6% at passage 13. Two DPSC lines had a significant reduction of their senescence rate after passage 13 possibly indicating neoplastic changes. Our results reveal that storage at 4°C and processing DPSCs within 24 hours may yield a slightly higher rate of growth. DPSCs did not survive the immortalization process before passaging for any group but were successfully immortalized for all groups after going through one passage. Senescence testing revealed that DPSCs approach 100% senescence after about 13 passages but there may be a chance for them to undergo neoplastic transformation, which causes a dramatic lowering in the senescence rate.
INTRODUCTION:

One of the greatest challenges to the study of neurogenetic syndromes that affect the brain is our inability to access live neurons from individuals with neurogenetic disorders. Understanding the molecular and physiological changes in neurons of the brains of individuals with disorders from mental retardation to autism is hampered by a lack of access to live neurons that accurately represent the conditions in the brain. One recent approach to the problem has been the collection of skin biopsies from individuals with neurogenetic disorders to create fibroblast cell lines which are then induced into pluripotency, becoming induced pluripotent stem cells (iPSCs), and finally differentiated down neuronal lineages in culture (1). However, there are problems with this approach: 1) fibroblasts must be obtained though a fairly invasive skin biopsy which leaves a scar and causes undue pain and distress in developmentally disabled or autistic children; 2) induction of fibroblasts into stem cells and then neuronal lineages is a laborious task that may not maintain epigenetic marks on the DNA that are essential to proper gene regulation in the native neuronal tissue; and 3) viral vectors that are used to induce iPSCs are immunogenic making downstream therapeutic uses for these neurons improbable. It is still not clear if the reprogramming process affects epigenetic marks on the DNA, interferes with proper chromatin conformation or results in neuronal lineages that only superficially represent neurons in the brain.

One solution to this problem is to obtain biospecimens of multipotent neuronal precursor cells, i.e. cells already destined to become neurons and therefore do not need reprogramming. Mammalian dental pulp is a neural crest-derived tissue and has been shown to contain a potent population of stem cells with neurogenic potential in vitro and in vivo (2,3). Since normally exfoliated or extracted primary teeth are a good source for these cells, it is possible to collect discarded teeth from individuals with neurogenetic syndromes in order to generate representative neural precursor and differentiated neuronal cell lines. The process of generating neurons from tooth pulp neural precursors is now well tested, but it has never been used before as a way to study neurogenetic disease and as such there are no protocols for them (2-5). Here we propose that DPSCs collected from exfoliated or extracted teeth will provide a new avenue for the study of neurons in the disease state representing the epigenetic, molecular, and physiological properties of neurogenetic syndromes involving allele specific expression and methylation patterns in neurons.

Samples from patients with neurogenetic diseases are often hard to obtain and must be collected from around the nation. Currently there are no standard protocols for long-term storage and transportation of extracted teeth. Undoubtedly this ultimately has an effect on how viable the pulp is for DPSC creation. To investigate this issue, teeth were stored and processed at various time intervals. The resultant cultured DPSCs were observed to see how each cell line performs in viability. Immortalization of DPSCs may allow DPSCs to be a viable and sustained study model for use in future research. In this study, DPSCs that have been grown under various conditions are immortalized with the hTERT retrovirus soon after processing and after passaging to assess how storage conditions, processing time, and the time of immortalization affect the success of the immortalization process, determine at which time point we obtain the highest yield of transformed
DPSCs, and how they are affected by senescence. Over time, senescence will render the DPSCs unusable and the sample will be lost forever. Senescence testing was conducted as DPSCs undergo expansion and passaging to examine the longevity of these cells. These parameters will be critical for establishing protocols to successfully obtain DPSC lines from any individual with any neurogenetic syndrome.

METHODS:

Qualifying teeth were obtained through the Department of Pediatric Dentistry at the University of Tennessee Health Science Center. The UTHSC Institutional Review Board approved this study (protocol #10-00878-XP) and informed consent was obtained from the parent or legal guardian of all participants. All subjects were typically developing children with no suspicion of undiagnosed neurogenetic disorders. The teeth included in the study were non-caries, had no previous restorations, and had no reports of prior trauma. All teeth were extracted on the day of tooth collection.

Determining the effects of storage conditions and processing time of DPSC lines.

A total of 23 healthy extracted teeth from neurotypical subjects were processed to grow DPSCs. Seven teeth were extracted, placed in the transportation media at 4°C and processed in the lab after 24 hours. Nine teeth were extracted, placed in the transportation media at 4°C and processed in the lab after 72 hours. Seven teeth were extracted, placed in the transportation media at room temperature (RT) and processed in the lab after 72 hours (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Teeth</th>
<th>Store</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extracted</td>
<td>4°C</td>
<td>&lt; 24 hours</td>
</tr>
<tr>
<td>2</td>
<td>Extracted</td>
<td>4°C</td>
<td>&gt; 72 hours</td>
</tr>
<tr>
<td>3</td>
<td>Extracted</td>
<td>RT</td>
<td>&gt; 72 hours</td>
</tr>
</tbody>
</table>

Table 1. Storage temperature and processing time for each group of extracted teeth.

Transportation and Cell Culture.

The teeth were placed in transportation media immediately following extraction. The teeth were stored at 4°C or RT and were processed in 24 or 72 hours depending on their group. The pulp was obtained from the tooth and minced with a scalpel. The pulp was digested in a solution of 3 mg/ml Collagenase type I and 4 mg/ml Dispase II for one hour at 37°C. The DPSCs were then cultured in DMEM and observed to see which transportation conditions yielded the best results. Cells were only considered viable DPSCs if they grew to confluence after 4 weeks in culture. Cell viability and morphology was checked through a light microscope.

Each group had different subgroups. Three teeth in each group were processed as usual. When the DPSCs became confluent, they were split into three groups. One group was used to expand the cells, another group was used for immortalization and the last group was frozen. For subsequent passages we had two groups, one to expand the cells
and another frozen for storage. The remaining teeth in each group underwent immortalization within one week.

**Transduction of DPSCs with hTERT.**

Immortalization was attempted on seven different samples of DPSCs starting from passage 2. Six different samples were immortalized one week after processing and incubation. DPSCs were plated in a new poly-D-lysine coated 3.8cm² 12-well plate and allowed to attach overnight in regular media. The next day, the DPSCs were infected with pBABE-hTERT-Puro retrovirus. The cells were incubated with the virus overnight. The next day, the media containing the virus was removed and replaced with growth media for a 72-90 hour period. After this period, the infected DPSCs were selected by adding 1 µg/ml puromycin for 7-10 days. The DPSCs that survived this process were immortalized DPSCs and were ready for further experimentation such as differentiation into neurons.

**Determining the longevity of non-immortalized DPSC lines.**

Senescence testing was performed on 4 DPSC lines from passages 2-15. Each DPSC line was washed twice with 0.5 ml of 1X PBS per well. 400 µl of 1X fixation buffer was added to each DPSC line and incubated for 7 minutes at room temperature. The staining mixture was prepared just prior to use. A total of 10 ml's of the staining solution consisted of 1 ml staining solution (10X), 125 µl of reagent B, 125 µl of reagent C, 0.25 ml of X-gal solution, and 8.5ml of Ultrapure water. The cells were rinsed three times with 0.5 ml of 1X PBS. 400 µl of staining mixture was added to each sample. The samples were incubated at 37°C without CO2 until the cells were stained blue. This took approximately 18 hours. The plates were sealed with parafilm to prevent drying. The cells were rinsed twice with 1 ml of 1X PBS then counterstained with Eosin for 8 minutes. The cells were rinsed in distilled water. The cells were mounted with vectashield and stored in the dark overnight. This procedure resulted in the application of a blue stain to Beta-galactosidase, the marker for cellular senescence. The senescent cells were then counted under a light microscope, twice and independently to ensure accuracy of the results. Preliminary senescence testing was also conducted on an immortalized cell line to evaluate the immortalized DPSCs.

**RESULTS:**

Teeth were collected from 7 female and 5 male subjects. The subjects’ ages ranged from 6-13 years old with an average age of 9. Seven out of twelve subjects had mild medical conditions such as asthma or eczema and took associated medications. No observable difference was found between DPSCs from subjects with no medical conditions compared to those with mild medical conditions. We attempted to culture DPSCs from 23 extracted teeth. Two samples from group 1 became contaminated. One sample from group 2 became contaminated and two samples did not grow. In group 3, two samples did not grow and one sample became contaminated. This gave us a total of 8 samples out of 23 that did not succeed in being cultured, which led to a 62.5% success rate in culturing DPSCs. Out of the 8 samples that failed, 50% were due to contamination and 50% were due to no growth overall. Group 1 did not have any failures due to lack of
growth of the cells, while group 2 and 3 had a mixture of failures. Only one sample failed from a subject that had active caries while many samples from subjects with active caries were successful. Nine out of 12 samples from subjects with good oral hygiene succeeded while 6 out of 11 samples from subjects with fair oral hygiene succeeded. All remaining samples are growing and two samples have reached passage 15 and are still growing (Figure 1).

All seven attempts at immortalizing samples at passage 2 from groups 1, 2, and 3 were successful. All six attempts at immortalization before passaging were not successful for all groups (Table 2).

<table>
<thead>
<tr>
<th>Extracted Sample Contamination Rate</th>
<th>17.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracted Sample Growth Failure Rate</td>
<td>17.4%</td>
</tr>
<tr>
<td>Culture Success Rate</td>
<td>65.2%</td>
</tr>
<tr>
<td>Immortalization Rate After Passaging</td>
<td>100%</td>
</tr>
<tr>
<td>Immediate Immortalization Rate</td>
<td>0%</td>
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</table>

Table 2. Success and Failure Rates.

Four samples underwent senescence testing. Senescence testing indicated that as time progresses, cellular senescence increases until about passage 13 where senescence reached a peak (Figure 2). The mean senescence rate for the four samples at passage 2 was 22.8%. At passage 13, it was 93.6%. The senescence rate of two samples significantly lowered on passage 15 and growth of cells in those samples suddenly increased in speed and number.
Figure 2. Senescence Assay by Beta-Galactosidase Staining: Four samples of extracted teeth underwent senescence testing from passage 2-15. As time went on senescence increased and reached a peak at passage 13 with an average of 93.6%. Two samples had undergone a significant reduction in senescence rate after passage 13.

DISCUSSION:

The general objective of this project is to optimize a DPSC study model that can be used for future research of neurogenetic disorders. We chose to evaluate DPSCs from primary teeth because studies have shown that they have the potential for long-term cultivation, research, and possible tissue banking in the future (6). Currently neurogenetic studies rely on the analysis non-neuronal biospecimens like lymphoblast and fibroblast cell lines. This process requires induction of fibroblasts back to stem cells and then to a neuronal lineage. This is labor intensive, adds to the cost, and requires modification of the fibroblasts that may alter gene expression. The viral vectors that are used limit the use for transplantation of these cells. The skin biopsy procedure is also traumatizing for children, especially those with intellectual disabilities. Numerous studies have shown that DPSCs can be differentiated into several tissue types, including neurons (2,4,5). We are now at the early stages of this field with regards to neural differentiation and therefore it is critical to understand the basic properties of DPSCs. Successful immortalization of these cells will mean that we have a sustainable source of pluripotent cells that we can differentiate into various lineages for study or therapeutic applications. Our broad long-term goal is to create an ex vivo study model that can provide neurons which accurately represent neurons in the brains of individuals with neurogenetic disorders.

Many primary teeth collected from patients with neurogenetic disorders are often mailed to our lab from all around the country. The transportation temperature and time before processing can vary greatly between samples. Currently there is no information on whether or not this affects the outcomes of the samples. To investigate this matter, teeth were stored at 4°C and 72°C and processed at 24 and 72 hours. Our results indicated
that there was one less failure in group 1. It is also interesting to note that group 1 only had failures due to contamination and none due to a lack of growth. Group 2 and 3 both had one more failure than group 1 and had both failures due to contamination and lack of growth. This may mean that higher temperatures and longer times before processing can lead to a lower success rate when attempting to culture DPSCs, but a higher number of samples need to be tested to make this a significant result.

Another interesting aspect to note is failures with respect to the subjects’ oral hygiene. A total of 8 samples failed in this study, 3 from subjects with good oral hygiene and 5 from subjects with fair oral hygiene. Good oral hygiene was considered to be present when plaque was not visible and there was no bleeding during a dental prophylaxis. Fair oral hygiene was considered to be present when there was generalized plaque at the gingival margins that did not extend on the axial surfaces of the teeth with or without bleeding during a dental prophylaxis. There were almost twice as many failures from samples with fair oral hygiene verses subjects with good oral hygiene. The mouth has hundreds of species of bacteria, all of which may have the potential to contaminate the media during storage (7, 8). It is also well known that the more poor the oral hygiene, the more pathologic the flora of the mouth becomes (8). Therefore, it is not surprising that a poorer oral hygiene would lead to more failures but further investigation is needed to validate this theory. Another interesting point is that a similar trend was not noted in regards to caries status. This may indicate that periodontal microbes play a larger role in determining DPSC culture success rate than microbes involved with caries.

Studies show that DPSCs can be cultured for several passages without cellular senescence but do not specifically look at their longevity (9,10). For this reason, our study examined cellular senescence in 4 DPSC lines. Senescence testing revealed that two samples reached 100% senescence on passages 13 and 15. The two other lines increased in senescence and approached 95% during passages 13 and 14 but then the senescence rate dropped to approximately 35% for both lines in their next passage. This may be is due to a neoplastic change or simply due to the selection of a healthy and viable DPSC. Further study is needed to determine why the senescence rate of two samples decreased at that time. It is interesting to note that Suchanek reported culturing DPSC lines to the 9th passage with a 90% viability and continued culturing to passage 20, but it appears that the determination of their viability rate was not done by senescence testing, nor was their definition viability made clear (9). We also noted that as the number of passages increased, cell morphology became poorer and the density of cells while culturing decreased, which is also contrary to Suchaneks findings (9).

Immortalization testing with the hTERT retrovirus revealed that DPSCs could be immortalized successfully after passage 2. All seven attempts with different DPSC lines were successful. Our results confirm the ability of DPSCs to immortalize but it remains to be seen whether or not immortalization causes negative cellular and genetic changes or a lowering of their differentiation potential which might prohibit their use for further research (11-13). If this is the case after further analysis, future studies may attempt immortalization using a different method that might be more compatible with DPSCs such as using a different virus (14). If further testing reveals that immortalization does not allow for future differentiation, differentiation of primary DPSCs may be our only option. Attempting to immortalize after 1 week of culturing failed on all six attempts. This may simply be a matter of an increased number of cells providing a higher chance for
immortalization. However, it may also mean that DPSCs need time to attach to the culture plate and stabilize before immortalization can work.

The study of DPSCs is a new and groundbreaking field that will facilitate the study of neurogenetic disorders. We hope to contribute to this research by optimizing a study model for all to use in further research. However, DPSC research is not just limited to genetic disorders. DPSCs are unique in that they are a readily available reserve of postnatal proliferative stem cells. DPSCs have been shown to be very proliferative and able to differentiate into many different lineages (9,10,15-17). This opens many fields of tissue engineering, possible treatment of neural tissue injury, degenerative diseases, and even regenerative dentistry (18-22). DPSCs even have the ability to integrate into host brain tissue in the mammalian brain, migrate to injured areas and express neuronal specific markers as well as voltage dependent sodium and potassium channels (1). Understanding the culture conditions, characteristics, and potential of DPSCs will not only advance the field of neurogenetic disorders, but it will also be beneficial to all fields of health care. By examining the technical boundaries of this renewable resource, we refine testing models for future studies. In the future it may become common practice to retain primary or extracted teeth for the production of personalized stem cell reservoirs for everything from dental implantation to treatments for Alzheimer’s or Parkinson’s disease.

* Note: The authors decided that they would like to update this paper by further studying the effects of immortalization on neural differentiation and epigenetics, and the effects of senescence on DPSCs before submitting for publication.
LITERATURE CITED


