Assessment of the Tumorigenic Potential of Spontaneously Immortalized and hTERT-Immortalized Cultured Dental Pulp Stem Cells

RYAN WILSON, a NORA URRACA, b CEZARY SKOBOWIAT, c KEVIN A. HOPE, b,d LETICIA MIRAVALLE, e REED CHAMBERLIN, e MARTIN DONALDSON, a TIFFANY N. SEAGROVES, f LAWRENCE T. REITER b,f

ABSTRACT

Dental pulp stem cells (DPSCs) provide an exciting new avenue to study neurogenetic disorders. DPSCs are neural crest-derived cells with the ability to differentiate into numerous tissues including neurons. The therapeutic potential of stem cell-derived lines exposed to culturing ex vivo before reintroduction into patients could be limited if the cultured cells acquired tumorigenic potential. We tested whether DPSCs that spontaneously immortalized in culture acquired features of transformed cells. We analyzed immortalized DPSCs for anchorage-independent growth, genomic instability, and ability to differentiate into neurons. Finally, we tested both spontaneously immortalized and human telomerase reverse transcriptase (hTERT)-immortalized DPSC lines for the ability to form tumors in immunocompromised animals. Although we observed increased colony-forming potential in soft agar for the spontaneously immortalized and hTERT-immortalized DPSC lines relative to low-passage DPSC, no tumors were detected from any of the DPSC lines tested. We noticed some genomic instability in hTERT-immortalized DPSCs but not in the spontaneously immortalized lines tested. We determined that immortalized DPSC lines generated in our laboratory, whether spontaneously or induced, have not acquired the potential to form tumors in mice. These data suggest cultured DPSC lines that can be differentiated into neurons may be safe for future in vivo therapy for neurobiological diseases. Stem Cells Translational Medicine 2015;4:905–912

SIGNIFICANCE

This study demonstrated that immortalized dental pulp stem cells (DPSCs) do not form tumors in animals and that immortalized DPSCs can be differentiated into neurons in culture. These results lend support to the use of primary and immortalized DPSCs for future therapeutic approaches to treatment of neurobiological diseases.

INTRODUCTION

Relative to harvesting of other stem cell types, such as hematopoietic stem cells from the bone marrow, or the generation of induced pluripotent stem cells (iPSCs) from more differentiated cell types like skin, dental pulp stem cells (DPSCs) are relatively easy to obtain from subjects and can be differentiated at high frequency into neurons with functional potential per published protocols [1–3]. Because DPSCs may be harvested from either shed teeth or extracted teeth, and adolescents shed all 20 primary teeth between the ages of approximately 6–12 years, DPSCs are an exciting resource that hold immense potential for the study and treatment of several diseases including the neurogenetic disorders that are the focus of research in our laboratory. Understanding of the behavior of DPSCs both in vitro and in vivo, including their ability to reliably differentiate into multiple neuronal lineages, is paramount to their use in the study of disease states [4]. Ongoing studies in our laboratory have validated the previously published parameters for the harvest and growth of DPSCs, which were developed by other laboratories, and showed that DPSCs have the ability to differentiate into functional neurons [5–7]. In addition, we have observed that DPSCs typically can grow up to 13 passages before complete senescence [8]. Still, questions remain about the use of DPSCs to treat neurogenetic syndromes.

DPSCs possess the ability to be differentiated into a variety of cell types, including odontoblasts, osteoblasts, chondrocytes, and adipocytes [5], making their utility in regenerative medicine a real possibility. Although an obvious use for DPSCs is in regenerative dentistry, there
is also great promise for therapies directed at the repair of damaged or malfunctioning neuronal tissues [3, 5, 9–11]. DPSCs injected into the rat brain have been shown to migrate to the site of neuronal lesions and to respond to the local microenvironment by differentiating into both neurons and glia [12]. These experiments suggested that DPSCs would be therapeutic in cases of traumatic brain injury or spinal cord injuries, but we also envision that DPSCs could potentially be useful in genetic disorders that result in intellectual disability related to deficiencies in neuronal signaling or synapse stability or formation, including psychiatric disorders or autism, which is often considered a synaptic disorder [13].

To convert DPSCs into neurons, DPSCs must be cultured ex vivo after isolation from human subjects and exposed to defined growth factors and supplements for conversion into cells with neuronal physiology. This process takes approximately 4 weeks. Because DPSCs must be cultured before any therapeutic reintroduction into the sites of damage or injury in humans, it is critical to understand whether cell culture conditions induce detrimental changes in DPSCs. One of the biggest concerns is that cells in prolonged culture would acquire spontaneous mutations that may lead to uncontrolled growth or, worse, to aggressive tumors when injected into animals. The spontaneous immortalization of normal cells in culture is a rare but well-established process that can lead to permanent cell lines for study in the laboratory [14]. Several pathways are implicated in regulating spontaneous immortalization and senescence, including loss of tumor suppressors, such as p53, which is necessary but not sufficient to induce immortalization [15]. A more recent technical approach used in research laboratories to efficiently generate immortalized cell lines that are difficult to maintain in culture is to stably express human telomerase reverse transcriptase (hTERT) in these cells; this approach has been used for a variety of cell types, including DPSCs [16].

It is generally accepted for cultured cells that immortalization is a prerequisite of transformation by oncogenes (reviewed in [17]). To rule out the possibility that either spontaneously immortalized DPSC lines or hTERT–immortalized DPSCs could cause tumors in animals and, potentially, in human subjects, we performed both in vitro and in vivo studies to determine whether the immortalized DPSC lines were potentially tumorigenic. These experiments suggested that DPSCs would be therapeutic in neuronal lesions and to respond to the local microenvironment by differentiating into both neurons and glia [12].

Cytogenetic Analysis of DPSC Lines

Genomic stability studies were performed in collaboration with Genetics Associates Inc. (Nashville, TN, http://geneticsassociates.com). DPSCs were grown in T25 flasks until 80%–90% confluent. Flasks were then filled with culture media and shipped to Genetics Associates Inc. for karyotyping. The DPSC cycle were arrested using 20 µl of a 10-µg/ml Colcemid solution (Gibco; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) in 5 ml of culture media. Subsequently, metaphase cells were analyzed using standard Giemsa staining (G-banding). In most cases, 20 metaphase cells were analyzed by G-banding. For cell lines harvested at very early passages (TP–023 and TP–092), metaphase spreads were difficult to obtain. Consequently, a minimum of 10 cells were analyzed. Cell lines TP–023(I) P32, TP–023(SI) P20, and TP–092 P3 showed abnormal karyotypes. These cell lines were also analyzed using high-resolution array comparative genomic hybridization to better characterize the origin of the abnormality and the precise chromosomal break point [20].

Immunohistochemistry of Cultured Cells

DPSCs were grown and differentiated using previously published protocols [3] in four-well poly-d-lysine-coated chamber slides (Lab-Tek; Thermo Fisher Scientific). Cells were then fixed in 4% paraformaldehyde for 20 minutes at room temperature. The neuronal tubulin marker TUJ1 was used to visualize both DPSCs and differentiated neurons. Staining was performed as directed by the manufacturer for anti-human TUJ1 (Millipore, Billerica, MA, http://www.emdmillipore.com) at 1:500 and secondary anti-chicken 488 (Molecular Probes; Thermo Fisher Scientific) at 1:1,000 dilution in phosphate-buffered saline with Tween 20. Slides were mounted using Vectashield HardSet mounting media plus DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, https://www.vectorlabs.com), and images were captured on a Leica DM6000 upright fluorescence microscope (Leica, Wetzlar, Germany, http://www.leica.com) at ×40 magnification.

In Vitro Soft Agar Assay

A mixture of 1.6% agarose (Invitrogen; Thermo Fisher Scientific) and 2 × Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Manassas, VA, http://www.cellgro.com) was prepared and used to coat each well of a 12-well plate cell culture-treated dish (750 µl). Agarose-coated plates were then placed into the refrigerator for approximately 20 minutes to allow the agarose to fully solidify. Next, single cells (1,000–1,500–, and 2,000-cell density) prepared from each DPSC cell line (TP–023(SI), TP–023(I), TP–053(SI), and TP–092) were resuspended in a volume of 250 µl in DMEM and mixed with 250 µl of sterile water and 500 µl of 1.6% agarose. This mixture was then evenly pipetted onto the DMEM/agarose bases.
The plates were again placed in the refrigerator for 20 minutes to allow the agarose to completely solidify. The plates were immediately placed in the incubator at 37°C (5% CO2) for 3–4 weeks, followed by staining colonies with 500 μl of MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] per well. The plates were then returned to the incubator for 24 hours before enumerating colonies containing more than 8 cells per colony under a light microscope at ×10 magnification. The five technical replicates per cell line were averaged, and significant differences in mean colony counts were compared by analysis of variance.

In Vivo Tumor Studies
All animal experiments were conducted in compliance with UTHSC institutional animal care and use committee approval. Male Nod-Scid-γ (NSG, stock no. 005557; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) at 6–8 weeks of age were injected subcutaneously in the right flank with DPSCs harvested from T-75 flasks after culture under standard conditions (37°C and 5% CO2). Cells were detached with HyClone HyQtase (Fisher Scientific, Pittsburgh, PA, https://www.fishersci.com) and resuspended at a density of 1.0 × 106 cells per 100 μl in Dulbecco’s PBS and kept on ice until injection into host mice. Just prior to injection, mice were anesthetized using isoflurane gas. Cells of the same genotype (n = 5 hosts per DPSC line or RT MEF) were injected into each flank per host, except for one mouse inadvertently injected with RT MEF cells in the right flank and the hTERT-immortalized DPSC cell line in the left flank. The same observer palpated the sites of injection twice weekly to monitor tumor growth. When RT MEF tumors reached 1.0 cm2 (assay positive control), mice were euthanized using isoflurane gas. Cells of the same genotype from exfoliated primary teeth from neurotypical individuals have been shown to be spontaneously immortalized. It should be noted that all cells exhibited contact inhibition at confluence.

Two of nine primary DPSC lines (TP-023 and TP-053) spontaneously immortalized during cellular senescence studies and continued to divide past P30, well beyond the passage at which the other lines had stopped growing (P13). To compare transformation potential of these spontaneously immortalized cells, we used a low-passage primary DPSC line from an idic(15) subject (TP-092 P3) and a known tumorigenic mouse cell line (RT MEF) [19] as well as an hTERT-immortalized version of TP-023 cells (TP-023[I]). The hTERT-immortalized lines we made in the laboratory are still able to differentiate into neurons just as efficiently as the nonimmortalized DPSCs. These neurons are positive for the neural β-tubulin marker TUJ1 and show morphological properties of neurons in culture (Fig. 1).

Because the spontaneously immortalized and hTERT-immortalized DPSCs may have acquired gross chromosomal changes in culture due to genomic instability, we performed karyotype analysis on the lines used in this study. Giemsa staining (G-banding) of metaphase chromosomal spreads from the two spontaneously immortal lines, TP-023(SI) and TP-053(SI), showed a normal 46,XY male chromosomal complement with no indication of chromosomal instability, as did the early passage TP-023 line (Fig. 2). The hTERT-immortalized line TP-023(I), which was derived from an early passage of TP-023, showed some underlying instability. A derivative chromosome 9 resulted from an unbalanced translocation between chromosomes 1q and 9q with concurrent duplication of chromosomal material from 1q42.3 to 1q-telomere [46,XY,der(9)t(1;9)(q42.3;q34.3)]. The hTERT-immortalized line TP-023(I) cell line but not the parent TP-023 or the spontaneously immortalized TP-023(SI) cell line. The low-passage control line TP-092 was from a known isodicentric chromosome 15q subject and showed an abnormal but stable isodicentric chromosome 15 female karyotype [47,XX,+idic(15)(q13.3)]. These results (Fig. 2) indicate that although spontaneously immortalized DPSCs do not appear to show genomic instability, the process of immortalization using hTERT may result in some chromosomal changes that are visible by G-banding.

Soft Agar Colony-Formation Assay
One of the most common assays to score for characteristics typical of transformed cells is the ability of normally adherent cells to grow from single cells into colonies when embedded in soft agar. This assay scores for the ability to grow in an anchorage-independent manner [21]; however, growth in soft agar assays is not sufficient to define a cell line as tumorigenic. This can be determined only by the injection of suspected tumorigenic cell lines into animals and observation of subsequent tumor formation [22].

Because hTERT-immortalized cells have been shown to have increased soft agar colony-forming unit potential relative to parental lines [23–25], the TP-023(I) cell line was used as the assay internal control. Although RT MEFs formed large colonies within 10 days of plating [19], DPSCs were derived from a different cell lineage, so we rationalized that they served only as an internal assay technical control. hTERT-immortalized DPSCs reliably grow in an attachment-independent manner in our laboratory, so they were used as a positive control because of their cellular origin similar to the experimental lines. A very low-passage (P3) primary DPSC line, TP-092, was used as the anticipated negative control. Representative photos of soft agar assays plated at 2,000 cells per well are shown for each genotype in Figure 3. There were no significant differences in colony formation among cell lines when plated at a density of 1,000 or 1,500 cells (Table 1); however, at a density of 2,000 cells per
well, both the TP-053(SI) and the TP-023(I) (hTERT-immortalized) cells formed significantly more colonies compared with the low-passage TP-092 cells (Table 1). These results indicate that either an hTERT-immortalized or a spontaneously immortalized DPSC line derived from the same subject can form colonies in soft agar, although the frequency of colony formation is enhanced approximately 68-fold in response to stable expression of hTERT (0.05% TP-023 parental vs. 3.4% TP-023[I]).

**In Vivo Tumor-Formation Studies**

Although escape from cellular senescence and the ability to grow in soft agar are phenotypes of transformed cells, it is the ability of cells to form tumors in animals that defines a cell line as tumorigenic. To determine whether either spontaneously or hTERT-immortalized DPSC lines could form tumors in mice, immunocompromised mice were injected with cell suspensions and observed for tumor growth over a 6-week period. As expected, all mice injected with RT MEF cells rapidly developed tumors greater than 1.0 cm² within the first 14 days (n = 5 mice with 9 tumors) [19]; however, none of the mice injected with spontaneously immortalized cells (TP-023[I] and TP-053[I]), hTERT-immortalized cells (TP-023[I]), or the low-passage cell line (TP-092) grew tumors by 6 weeks after injection when all non-tumor-bearing animals were euthanized for necropsy (n = 5 mice per genotype) (Table 2). Analysis of H&E-stained sections derived from RT MEF tumors or flank skin of non-tumor-bearing mice revealed that the RT MEF cells formed poorly differentiated fibrosarcomas, whereas the skin preparations from mice injected with all DPSC cell lines showed no signs of tumor growth (Fig. 4).

**DISCUSSION**

The potential treatment of human neurological diseases using stem cells or stem cell-derived progeny is opening an exciting new field of regenerative medicine. Several options are currently available as potential sources for therapeutic stem cells. Induced pluripotent stem cells are generated from terminally differentiated cells, such as fibroblasts, that are then infected with viral vectors containing conversion factors Oct-3/4, Sox-2, Klf4, and c-Myc to generate cells with pluripotent potential, albeit at a low frequency, that can be differentiated into a variety of cell types [26]. Peripheral blood hematopoietic stem cells (HSCs) have also been explored as a source of cells for reprogramming into somatic stem cells because they have the ability to differentiate into mesenchymal stem cells, cardiomyocytes, and hepatocytes [27]. Nonetheless, challenges are associated with both sources of stem cells. For iPSCs, viral integration has been associated with mutations, induction of tumor formation, and interference with differentiation [28–32]. The potential oncogenic and genomic instability observed for cultured iPSCs also limits their use in therapeutics [33]. In contrast, DPSCs harvested from the primary or permanent dentition are not only multipotent but often can be obtained more easily in the clinic than the use of skin punch biopsies to collect fibroblasts. When DPSCs are cultured in adipogenic, chondrogenic, neurogenic,
osteogenic, or myogenic media, DPSCs exhibit the ability to differentiate and express markers indicative of the differentiated cells. They are also able to differentiate into chondrocytes, osteocytes, neurons, and muscle cells that exhibit the morphology and functions of mature cells [5, 34].

DPSCs have potential applications in several areas of regenerative medicine and therapeutics. The most obvious direct application is for regenerative dentistry. When porcine DPSCs, for example, are implanted in the presence of a BMP2-treated scaffold, they have the ability to regenerate pulpal tissue and to form functional odontoblasts that produce reparative dentin [35]. In addition, DPSCs transplanted in the presence of granulocyte colony-stimulating factor into pulpectomized dog teeth were shown to regenerate pulpal tissue that completely filled the root canal and produced dentin and associated neural and vascular tissues [36]. DPSCs also exhibit the ability to differentiate into a variety of other cell lineages. With appropriate supplements and growth factors, cultured DPSCs have even been shown to form clusters of pancreatic β islet cells. When these cells were then introduced into diabetic mice, the mice became normoglycemic within 2 weeks; that condition was maintained for 2 months, and body weight and urine glucose levels returned to normal levels [37].

Our primary interest in DPSCs is related to their ability to form neurons and provide a new source of cells that can be used as tools for the study of neurogenetic syndromes without the need to harvest neurons from patients [1, 2, 38]. It has been shown previously that murine DPSCs cultured in a neural differentiation media exhibited neural phenotypes and expressed L-type voltage-gated Ca2+ channels [39]. Moreover, human DPSCs can differentiate into mature neurons and express functional sodium and potassium voltage-gated ion channels [3]. When injected into a rat model of Parkinson’s disease, DPSC-treated animals showed significant behavioral improvement compared with control cohorts [40]. Perhaps one of the most exciting discoveries from a therapeutic perspective is that, when injected into rats with brain lesions, DPSCs can migrate to the site of injury and differentiate into functionally active neural tissues, effectively repairing the neural tissue in vivo [12].

To date, no one has addressed whether DPSCs exposed to prolonged culture conditions can acquire oncogenic potential when introduced back into animals. In this study, we tested the tumor-forming potential of DPSCs generated from neurotypical pediatric participants and that either spontaneously immortalized in vitro or were immortalized with an hTERT viral vector. We found that low-passage parental DPSCs did not form colonies in soft agar, whereas spontaneously immortalized DPSCs had minimal soft agar colony-forming potential, and that ectopic expression of hTERT increased colony-forming potential by approximately 68-fold, although colonies were below 200 μm in diameter. The immortalization of DPSCs with hTERT also increased genomic instability to some degree because the one line tested by karyotype showed a clonal rearrangement between chromosomes 1 and 9; however, it must be pointed out that neither of the spontaneously immortal lines that were analyzed above passage 20 showed these types of chromosomal events (Fig. 2). We also showed that immortalization does not appear to affect the ability of DPSCs to differentiate into neurons (Fig. 1). Most important, none of the DPSC lines tested formed tumors in immunocompromised mice, indicating that they are not oncogenic. These data provide strong evidence that DPSCs grown in culture, even those lines that spontaneously immortalize, appear to be safe as therapeutic agents in humans, at least in terms of their lack tumor formation potential. Because mice injected with DPSCs did not exhibit tumor growth within 6 weeks, which is a relatively small
fraction of the lifespan of the average mouse, future studies should be performed to assess tumor potential up to 1 year after injection. The RT MEFs reliably grew tumors in all of our experiments; however, these cells grow very rapidly and may not be equivalent to the growth rates of DPSCs in vivo. Consequently, the use of a cell line with a slower but reliable latency of tumor formation in mice may help establish the appropriate parameters for long-term growth study. We are also planning to test whether DPSCs labeled with a fluorescent reporter or long-term retention label would be useful to determine whether DPSCs injected into the brain divide after injection into the mouse, remain dormant, or are capable of differentiation in vivo without forming tumors. These types of long-term studies using human-derived DPSCs implanted into immunocompromised mice will be required to verify the stability and safety of DPSCs in vivo.

Many choices currently exist for obtaining stem cells that may be suitable for therapy, including skin-derived iPSCs, blood derived HSCs, and the tooth-derived DPSCs that we used in these experiments. Because DPSCs are easily accessible, do not appear to have oncogenic potential, and do not show genomic instability associated with other stem cell types when they spontaneously immortalize, we feel that they have great potential both in research and as therapeutics. The experiments described provide evidence of the safety of these cells in an animal model and open possibilities for therapeutic applications of these cells to treat disease and injury in humans.

**CONCLUSION**

In this study, we demonstrated that spontaneously immortalized and hTERT-immortalized DPSCs do not demonstrate tumorigenic potential in animals. We also showed that immortalized DPSCs can be differentiated into neurons in culture. These studies lend support to the use of primary and...
immortalized DPSCs for future in vivo therapy for neurobiological
diseases.

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Figure 4. Histology of RT MEF tumors and flank skin encompassing injection site. Representative hematoxylin and eosin-stained sections of tissues harvested from mice at the flank injection site (A, B, C, E) or from a ras-transformed mouse embryonic fibroblast (RT MEF) tumor (D) are shown: skin TP-023(I), ×10 magnification (A); skin TP-023(SI) (B); skin TP-053(SI) (C); RT MEF tumor, ×20 magnification (D). Sections show proliferation of undifferentiated spindle-shaped cells arranged with herringbone pattern. The tumor involved dermis and subcutis and shows high mitotic activity: skin TP-092, ×10 magnification (E) and ×20 magnification (E’. The RT MEF cells are known to form fibrosarcomas (D). In contrast, no tumor cells or dental pulp stem cells (DPSCs) were noted in either the Epi, Derm, or subcutaneous fat and muscle from mice injected with DPSCs. Abbreviations: Derm, dermis; Epi, epidermis.
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