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Robust reprogramming of Ataxia-Telangiectasia patient and carrier erythroid cells to induced pluripotent stem cells

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ABSTRACT

Biallelic mutations in ATM result in the neurodegenerative syndrome Ataxia-Telangiectasia, while ATM haploinsufficiency increases the risk of cancer and other diseases. Previous studies revealed low reprogramming efficiency from A-T and carrier fibroblasts, a barrier to iPS cell-based modeling and regeneration. Here, we tested the feasibility of employing circulating erythroid cells, a compartment no or minimally affected in A-T, for the generation of A-T and carrier iPS cells. Our results indicate that episomal expression of Yamanaka factors plus BCL-xL in erythroid cells results in highly efficient iPS cell production in feeder-free, xeno-free conditions. Moreover, A-T iPS cells generated with this protocol maintain long-term replicative potential, stable karyotypes, re-elongated telomeres and capability to differentiate along the neural lineage *in vitro* and to form teratomas *in vivo*. Finally, we find that haploinsufficiency for ATM does not limit reprogramming from human erythroid cells or *in vivo* teratoma formation in the mouse.

1. Introduction

Ataxia-Telangiectasia (A-T; OMIM208900) is an autosomal recessive syndrome caused by compound heterozygous null mutations in the locus encoding the ATM kinase at chromosome 11q22 (Savitsky et al., 1995) ("classical" A-T). Most A-T patients suffer from severe cerebellar degeneration due to Purkinje cell death, B and T cell immunodeficiency and increased cancer predisposition (Boder and Sedgwick, 1958; Lavin, 2008; McKinnon, 2012; Shiloh, 2003). Moreover, haploinsufficiency for ATM, estimated to occur in 1.5-2% of the population worldwide (Swift et al., 1986), increases the risk of cancer and heart disease and decreases lifespan (Su and Swift, 2000; Swift and Chase, 1983). However, how alterations in ATM copy number result in such pleiotropic phenotypes remains incompletely understood.

Nucleoplasmic ATM is activated in response to DNA double-strand breaks (DSB) to orchestrate the cellular DNA Damage Response (DDR) and promote cell cycle checkpoint activation and DSB repair (Paull, 2015; Shiloh and Ziv, 2013). In ATM-deficient developing lymphocytes, broken DNA ends at "programmed" DSBs remain unrepaired or are repaired aberrantly, leading to T and B cell immunodeficiency and oncogenic translocations, respectively (Callen et al., 2007; Franco et al., 2006). In contrast, the contribution of defective DSB repair to the neurodegenerative phenotypes observed in A-T is less well understood. In this context, the finding that ATM may localize primarily to the cytoplasm in post-mitotic neurons (Barlow et al., 2000; Oka and Takashima, 1998), together with its emerging cytoplasmic functions in the regulation of oxidative stress and metabolism (Ambrose and Gatti, 2013; Paull, 2015; Valentin-Vega et al., 2012; Zhang et al., 2015), may suggest a mechanism independent of DSB repair (Carlessi et al., 2013). However, $Atm^{-/-}$ mice (Lavin, 2013) and $ATM^{-/-}$ pigs (Beraldi et al., 2015) do not manifest ataxia or significant cerebellar pathology, underscoring the need for novel human cell-based approaches to model neurodegeneration.

Induced pluripotent stem (iPS) cells derived from human somatic cells by expression of defined transcription factors represent a powerful novel system for disease modeling (Park et al., 2008; Takahashi et al., 2007). Moreover, the recent development of highly efficient genome editing tools has greatly facilitated the use of corrected iPS cell-derived products for autologous tissue replacement (Mali and Cheng, 2012). Most relevant to A-T, preclinical studies have started to define sets of transcription factors that promote differentiation of mouse ES cells (Muguruma et al., 2010; Tao et al., 2010) and human ES and iPS cells (Muguruma et al., 2015; Wang et al., 2015) to Purkinje neurons.

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Furthermore, these cells have some engraftment capability (Muguruma et al., 2010; Wang et al., 2015), suggesting that A-T iPS cells could similarly represent a source of neuronal cells for disease modeling and ultimately for regenerative therapy.

Previous work has shown that A-T iPS cells generated by expression of Yamanaka factors in A-T fibroblasts (Fukawatase et al., 2014; Lee et al., 2013; Nayler et al., 2012) or T cells (Lin et al., 2015) are viable. However, all fibroblast-based protocols employed integrating viral vectors and feeder layers (Fukawatase et al., 2014; Lee et al., 2013; Nayler et al., 2012). Moreover, consistent with impaired reprogramming in fibroblasts deficient for other DSB repair factors (Gonzalez et al., 2013; Tilgner et al., 2013), the efficiency of reprogramming from A-T fibroblasts was decreased about 100-fold relative to control fibroblasts from healthy individuals (Fukawatase et al., 2014; Lee et al., 2013; Nayler et al., 2012). Furthermore, the efficiency of reprogramming from A-T carrier fibroblasts was also markedly decreased in one study (Nayler et al., 2012), suggesting a gene dose effect. Patient-derived circulating T lymphocytes were recently shown to represent an alternative to fibroblasts (Lin et al., 2015). However, the fact that they often harbor clonal pre-leukemic rearrangements involving antigen receptor loci (Taylor et al., 1996) complicates their use for disease modeling and therapy. In this context, reprogramming of nonlymphoid mononuclear cells (Chou et al., 2015; Dowey et al., 2012; Hu et al., 2011) could provide a robust yet safe approach for A-T patients and carriers. More specifically, the erythroid compartment is no or minimally affected in A-T (Boder and Sedgwick, 1958).

Here, we show that A-T and carrier circulating erythroid cells can be reprogrammed to iPS cells in xeno-free, feeder-free conditions with high efficiency. Moreover, this protocol results in A-T iPS cells with intact replicative potential, stable karyotypes, re-elongated telomeres and capability to differentiate along the neural lineage.

2. Materials and Methods

2.1. Patients

A 12 year-old male diagnosed with A-T and followed at the A-T Clinic at Johns Hopkins Hospital and his parents were consented and enrolled in this study, following protocols approved by the Johns Hopkins Institutional Review Board (IRB#CR00007000). The proband was known to be compound heterozygous for frameshift mutations in exon 23 (3369delA, codon 1123) and exon 26 (3754delTATinsCA in codon 1252) of *ATM*, both resulting in a null allele. Peripheral blood was drawn by venipuncture and subjected to reprogramming, as described below. The iPS cell lines generated from this family were designated as SF-001 (mother), SF-002 (father) and SF-003 (patient). The BC1 iPS cell line was previously generated in the laboratory of L.C. from bone marrow (BM) $CD34^+$ cells of an adult healthy donor (Chou et al., 2011).

2.2. Generation of iPS cells from peripheral blood erythroid cells

Reprogramming was done in the laboratory of L.C. as previously described (Chou et al., 2015; Dowey et al., 2012). In brief, mononuclear cells (MNCs) were isolated from PB (15 to 30 mL per donor) using a Ficoll gradient and cultured for one week in serum-free media containing Stem Cell Factor (SCF; 255-SC, 50 ng/mL, R&D Systems, Minneapolis MN), interleukin (IL)-3 (10 ng/mL, product number 200-03, PreproTech, Rocky Hill NJ) and erythropoietin (2 U/mL; National Drug Code (NDC) 59,676-303-01), to promote erythroblast expansion, as we have previously described (Chou et al., 2015). Cells were then transfected with three episomal plasmids encoding Yamanaka factors (OCT4, SOX2, KLF4 and c-MYC) and BCL-xL and cultured for two additional weeks in reprogramming medium. At day 14 after reprogramming, cells were stained with an antibody to TRA-1-60 and the total number of positive and negative colonies was counted. To enrich cultures for iPS cells, TRA1-60^+ cells were sorted and pooled for expansion.

2.3. Cell culture

Human iPS cells were cultured in Essential 8 Medium (Invitrogen A1517001) in 6-well plates coated with vitronectin (VTN-N; Invitrogen A14700). For passage, cells were disaggregated using StemPro Accutase Cell Dissociation Reagent (Thermo Fisher Scientific, #A11105). A ROCK inhibitor Y-27,632 dihydrochloride (Santa Cruz Biotechnology; sc-281,642 A) was added during passage at a concentration of 10 μM.

2.4. In vivo iPS cell immunostaining

iPS cells were incubated with antibodies to pluripotency markers OCT4, SOX2, SSEA4 and TRA-1-60 using the Pluripotent Stem Cells 4-marker Immunocytochemistry Kit (Life Technologies, A24881), following manufacturer's instructions. Images were taken using an EVOS FL Auto Cell Imaging System.

2.5. Teratoma assay

iPS cells (5-10 million) were mixed 1:1 with Matrigel (Cornig Matrigel hESC-qualified Matrix, #354,277) to a total volume of 200μL and injected into the flanks of NOD scid gamma (NGS) mice subcutaneously. After 2-34 weeks, teratomas were harvested, fixed in formalin and embedded in parafin at the Johns Hopkins Histopathology Core Facility. Five hematoxilyn/eosin (H/E)-stained sections were analyzed for lineage.

2.6. Analysis of mutation by sequencing

For confirmation of ATM mutations, we amplified genomic DNA from iPS cell lines using the following primers: for exon 23, ATM-23F: 5′- TTTGTTCTGGAATATGCTTTGG-3′ and ATM-23R: 5′-TGGTGAAGTAATTTATGGGATATT CA-3′; for exon 26, ATM-26F: 5′- CTT TAATGCTGATGGTATTAAAACAG-3′ and ATM-26-R: 5′-GCCATACCTGTTTTCCCAAT-3′. For mutational analysis of the p53 locus in line SF-001, we amplified genomic DNA at exons 5-8 of p53 using previously described primers (Rechsteiner et al., 2013): ex5-F: 5′-CACTTGTGCCCTGACTTTCA-3′; ex5-R: 5′-AACCAGCCCTGTCGTCTCT-3′; ex6-F: 5′-CAGGCCTCTGATTCCTCACT-3′; ex6-R: 5′-CTTAACCCCTCCTCCCAGAG-3′; ex7-F; 5′-CCACAGGTCTCCCCAAGG-3′; ex7-R: 5′-CAGCAGGCCAGTGTGCAG-3′; ex8-F: 5′-GCCTCTTGCTTCTCTTTTCC-3′; ex8-R: 5′-TAACTGCACCCTTGGTCTCC-3′. For both, PCR products were purified, cloned into TOPO-TA and sequenced.

2.7. Plasmid integration analyses

To exclude plasmid integration, genomic DNA was amplified with primers specific to the common plasmid backbone: EBNA-Fw1: 5′-ACGATGCTTTCCAAACCACC-3′ and EBNA-Rev1: 5′-CATCATCATCCGGGTCTCCA-3′. As a control, we amplified the same DNA with primers to 18S: 18S-F: 5′-GCGAGTACTCAACACCAACATCG-3′ and 18S-R: 5′-TCAAGTCTCCCCAGCCTTGC-3′.

2.8. Short Tandem Repeat (STR) Profiling

To confirm line identity, Short Tandem Repeat (STR) profiling was done for all lines at early passage at the Johns Hopkins Genetic Resources Core Facility, using GenePrint10 (Promega).