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TET2 haploinsufficiency alters reprogramming into induced pluripotent stem cells

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ABSTRACT

The discovery of the Ten-Eleven Translocation (TET) protein family was initiated by the identification of the MLL partner TET1, and of mutations in the *TET2* gene in hematological malignancies including myeloproliferative neoplasms (MPN). TET1, 2 and 3 proteins hydroxylate 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC) and further oxidize 5-hmC into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Previous studies highlight the involvement of TET proteins in somatic cells reprogramming into induced pluripotent stem cells (iPSC), particularly Tet1 and 2 in mouse and TET1 in human. Here, we asked whether endogenous TET2 knockdown also displays this function. Using different shRNA against *TET2*, we provide evidence that TET2 strongly decreases the reprogramming of human hematopoietic progenitor cells into iPSC. Importantly, using 2 MPN patients, we observed that *TET2* mutations affecting catalytic domain allowed iPSC generation. Instead, using another *TET2* and *TET3*-mutated patient, we could only reprogram IPSC with *TET3* mutation alone, suggesting that the type of *TET2* mutation and/or the cooperation with *TET3* mutations may alter the reprogramming activity. Altogether, this work highlights the importance of endogenous TET in the reprogramming process of human hematopoietic progenitors.

1. Introduction

The family of TET proteins (TET1, 2 and 3) was first discovered with TET1 as an MLL fusion partner in the translocation t(10;11) (q22;q23) in leukemia (Lorsbach et al., 2003; Ono et al., 2002). Later, *TET2* mutations were identified in both myeloid and lymphoid hematological malignancies. Particularly, they were identified with a high frequency in chronic myelomonocytic leukemia (CMML) (>50%) and also in classical myeloproliferative neoplasms (MPN) (~15%) including essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) (Abdel-Wahab et al., 2009; Delhommeau et al., 2009; Langemeijer et al., 2009; Quivoron et al., 2011). Classical MPN are clonal hematological malignancies that are due to the acquisition of a genetic abnormality at the level of hematopoietic stem cells. It results in overproduction of red blood cells in PV, platelets in ET and a deregulation of megakaryocytic and granulocytic lineages in PMF. These diseases are due to mutations in 3 genes including *JAK2* (*JAK2*V617F), calreticulin (*CALR*) and the thrombopoietin receptor (*MPL*) leading to an increase in the JAK2/STAT signaling pathway (Vainchenker and Kralovics, 2017).

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All three TET proteins display a 2-oxoglutarate/Fe(II)-dependent oxygenase catalytic activity at their C-terminus, converting 5-methylcytosine (5-mC) into 5-hydroxymethylcytosine (5-hmC), and subsequently into 5-formylcytosine and 5-carboxylcytosine (Ito et al., 2010; Ito et al., 2011; Tahiliani et al., 2009). Studies have shown that TETs can activate or repress gene expression either through the generation of 5-hmC or independently (Pastor et al., 2013; Wu and Zhang, 2014; Williams et al., 2011). Active gene expression regulation takes place through the generation of 5-hmC which in turn leads to DNA demethylation, including of regulatory elements such as the pluripotency gene promoters in embryonic stem (ES) cells (Williams et al., 2011; Wu et al., 2011). The 5-hmC can also enhance gene expression by promoting the interaction with specific factors and/or by preventing the interaction between the 5-mC and certain proteins such as DNA methyltransferase (DNMT1), the methyl-binding proteins (MBD1/2) and methyl CpG binding protein 2 (MeCP2) and/or transcription factors (Pastor et al., 2013; Wu and Zhang, 2014; Lafaye et al., 2014; Spruijt et al., 2013). In contrast, the TET proteins also inhibit gene expression independently of 5-hmC generation *via* the regulation of the Sin3A repressor and or the recruitment of the transcriptional repressor polycomb repressive complex 2 (PRC2) (Williams et al., 2011; Wu et al., 2011). Alternatively, the TET-mediated recruitment of β-D-N-acetylglucosamine (O-GlcNAc) transferase (OGT) to chromatin leads to the activation of gene expression (Chen et al., 2013; Deplus et al., 2013; Vella et al., 2013).

High levels of 5-hmC are present in embryonic stem cells (ES) associated with the expression of both Tet1 and 2 in mouse and mostly TET1 in human cells (Ito et al., 2010; Langlois et al., 2014). TET proteins have been shown to play a role during the development, probably with redundant functions. Indeed, *tet1* or *tet2* knockout (KO) mice and a fraction of *tet1/tet2* double KO mice can survive while triple *tet1/2/3* KO ES could not support embryonic development (Quivoron et al., 2011; Dawlaty et al., 2014; Dawlaty et al., 2013; Dawlaty et al., 2011).

TET proteins, and especially their catalytic activity, have been implicated in reprogramming of mouse and human somatic cells into induced pluripotent stem cells (iPSC) using knockdown or KO (Costa et al., 2013; Doege et al., 2012; Hu et al., 2014; Wang et al., 2013). Triple *tet*-deficient mouse embryonic fibroblasts (MEF) could not be reprogrammed into iPSC due to a block in the mesenchymal-to-epithelial transition step (Hu et al., 2014). Reprogramming of MEF was also completely abrogated after *Tet2* knockdown as a result of *Nanog* deregulation (Doege et al., 2012). Conditional *tet2* KO in mouse B cells also evidenced its role in the C/EBPα-enhanced reprogramming into iPSC (Di Stefano et al., 2014; Sardina et al., 2018). Embryonic germ cells-mediated reprogramming of human B cells was decreased upon *tet2* knockdown through *Oct4* downregulation (Piccolo et al., 2013). Moreover, Nanog was found to interact with both Tet1 and Tet2 to enhance iPSC reprogramming of MEF through regulation of *Oct4* and *Esrrb* loci (Costa et al., 2013). In addition, Tet1 was demonstrated to enhance Oct4 expression and could even replace Oct4 during reprogramming of MEF (Gao et al., 2013). In human, *TET1* knockdown strongly decreases the reprogramming efficiency of fibroblasts into iPSC through a 5-hmC-dependent mechanism (Wang et al., 2013).

Given the role of TET proteins in pluripotency and TET2 in human normal and malignant hematopoietic cells, here we asked whether it could be involved in reprogramming human hematopoietic progenitor cells into iPSC, using both a knockdown strategy of *TET2* and *TET2*-mutated patients with MPN.

2. Experimental procedures

2.1. Patients, cell purification and progenitor cultures

Peripheral blood samples were collected from MPN patients. Written informed consent was obtained from patients in accordance with the Declaration of Helsinki and the study was approved by Comité de Protection des Personnes (CPP) Ile-de France IV- Institutional review board (agreement from US Department of Health and Human Services (n°IRB 00,003,835- Protocol 2015/59-NICB) and Commission Nationale de l'Informatique et des Libertés (CNIL) (authorization #915,663), in France and by the ethics committee of National Cancer Institute (CEP/CONEP N° 62/08), in Brazil. Mononuclear cells and granulocytes were separated over a Ficoll density gradient. Granulocytes were obtained after lysing the red cells and $CD34^+$ cells were purified by a double-positive magnetic cell sorting system (AutoMACS; Miltenyi Biotec) from mononuclear cells. To establish hematopoietic progenitor cell architecture, $CD34^+CD38^+$ cells were further sorted using anti-CD34-FITC and anti-CD38-PE (Becton Dickinson, BD) on a BD Influx cell sorter (supplemental Fig. S1). They were cloned at 1 cell/well and cultured in serum-free medium (Iscove Modified Dulbecco Medium with penicillin/streptomycin/glutamine, alpha-thioglycerol, Bovine Serum Albumin (BSA), a mixture of sonicated lipids, insulin-transferrin in presence of a cocktail of human recombinant cytokines containing EPO (1 U/mL) (Amgen Thousand Oaks, CA), TPO (20 ng/mL) (Kirin, Japan), SCF (25 ng/mL) (Biovitrum AB, Sweden), IL-3 (10 ng/mL), FLT3-L (10 ng/mL), G-CSF (20 ng/mL) and IL-6 (100 U/mL) (MiltenyiBiotec). Fourteen days later, individual colonies were plucked and lysed with proteinase K and 0.2% Tween 20 (Sigma) at 65° for 60 min and 95 °C for 15 min. To identify somatic mutations, DNA from progenitor-derived colonies were genotyped for *JAK2*V617F by Taqman allelic discrimination assay and for *TET2* mutations by Sanger sequencing using primers described in Supplemental Table S1.

2.2. Constructs and production of viral particles

Short hairpin (sh)RNA strategy to regulate human TET2 expression based on lentivirus was used as previously described (Langlois et al., 2014). Sequences for sh*TET2*, sh*TET2.b* and sh*TET2.c* were (5′GGGTAAGCCAAGAAAGAAA3′), (5′AGAAAGAAATCCAGGTGAA3′) and (5′AAACAAAGAGCAAGAGATT3′), respectively. Lentivirus particles containing pRRLsin-PGK-eGFP-WPRE vector (Genethon, Evry, France) were produced as previously described (Plo et al., 2008). $CD34^+$ progenitors were transduced with lentivirus containing human sh*SCR*, sh*TET2* and sh*TET2.b* or sh*TET2.c* and GFP^+ cells were sorted on a FACSDiva cell sorter (BD) (supplemental Fig. S2).

2.3. ES and iPSC generation

$CD34^+$ cells from healthy donors or MPN patients were purified from peripheral blood mononuclear cells. $CD34^+$ cells from healthy donors were transduced with lentivirus expressing shRNA and sorted on GFP selection marker 3 days later. They were cultured in serum-free medium with cytokines for 5 days before being transduced either with CytoTune-iPS Sendai Reprogramming Kits v1 or v2 (sendai virus (SV), Thermo Fisher Scientific) following the manufacturer´s instruction or with VSV-G pseudotyped retroviruses encoding *Oct4, c-Myc, Klf4* and *Sox2* (Takahashi et al., 2007). Alternatively, $CD34^+$ cells from MPN patients were cultured in serum-free medium with cytokines for 5 days before being transduced with SV. Six days later, cells were seeded on irradiated murine embryonic fibroblasts (MEF) in ES medium (Mali et al., 2008). Colonies with an ES-like morphology were picked from day 20 to day 30 and expanded. The use of hESC (H9) was approved by Agence de la Biomédecine, No. R04-0020 and No. C04-0019. The cultures of pluripotent iPSC and hESC (H9) were performed in Essential 8 medium on matrigel-coated dishes.

2.4. Teratoma assays and embryoid bodies

iPSC cells (1×10^6) were scrapped and resuspended in 140 µL ES medium. Undiluted matrigel (60 µL) was added prior to subcutaneous injection into NOD/SCID/$\gamma c^{-/-}$ (NOG) mice. After 8–12 weeks, tumors were isolated and fixed in formalin (10%). Sections were stained for

germ layers analysis. Embryoid body (EB) formation assay was performed to address spontaneous differentiation *in vitro* into the 3 germ layers.

2.5. Flow cytometry analysis

iPSC colonies were dissociated and the iPSC pluripotency was evaluated using directly conjugated monoclonal antibodies (Human Pluripotent Stem Cell Transcription Factor Analysis Kit, SSEA3-PE, eBioscience, San Diego, CA) and TRA-1–81-APC, (Becton Dickinson, le Pont de Claix, France) (supplemental Fig. S3). Cells were analyzed by flow cytometry using CANTO XI BD Bioscience and analyzed using FlowJo, LLC v8 software.

2.6. Immunofluorescence analysis

iPSC or EBs were fixed with paraformaldehyde (4%), permeabilized with Triton-X (0.3%) and blocked using BSA (3%). Cells were incubated with primary antibodies to confirm the presence of pluripotent markers: SOX2, SSEA-4, TRA-1–81 (Merck Millipore), OCT-4 (Santa Cruz Biotechnologies) and the germ layer markers: AFP (Santa Cruz Biotechnologies), SMA (Sigma-Aldrich) and TuJ1 (Merck Millipore). Cells were then incubated with secondary antibodies such as CF488A Goat anti-rabbit IgG or CF564A Goat anti-mouse IgG (Thermo Fisher Scientific) (Gomez Limia et al., 2018).

2.7. Sequencing and genotyping

*JAK2*V617F genotyping was performed by Taqman allelic discrimination on the ABI Prism GeneAmp 7500 Sequence Detection System (Applied Biosystem, Thermofisher Scientific). *CALR* genotyping was performed by PCR using fluorescent primer, followed by fragment analysis on the ABI3130xL (Applied Biosystems, Thermofisher Scientific) (Klampfl et al., 2013). *TET2* mutations were sequenced using specific primers (Table S1).

2.8. Gene expression analysis

Quantitative real time PCR (qRT-PCR) was carried out using specific primers and the ABI Prism GeneAmp 7500 Sequence Detection System (Applied Biosystem, Thermofisher Scientific). In iPSC, *TET2* and the pluripotent transcription factors *NANOG, SOX2* and *OCT4* were analyzed. After *in vitro* differentiation through EB, the genes of the germ layers markers *FOXA2* (endoderm) and *T* (mesoderm) were quantified. The expression levels were normalized with *PPIA* and results are relative to expression levels in hESC (H9 cell lines) or to the expression in undifferentiated state. The absence of SV transgenes expression was confirmed by RT-PCR using specific primers for *SeV, KOS, KLF4* and *c-Myc* (Table S1).

3. Results and discussion

3.1. Impact of TET2 knockdown during reprogramming of CD34$^+$progenitors into iPSC

In order to understand if human TET2 could impact reprogramming of CD34$^+$progenitors into iPSC, we purified CD34$^+$ progenitors from peripheral blood of healthy donors and transduced them with lentiviral vectors encoding the GFP reporter, and either 3 different shRNA for *TET2* sequence (Langlois et al., 2014; Pronier et al., 2011), or a scramble sequence (SCR) (Fig. 1A). We obtained nearly 80% transduction efficiency. After sorting GFP$^+$ cells, we observed around 40% reduction in *TET2* mRNA in CD34$^+$ progenitors with sh*TET2* compared to *SCR* by qRT-PCR, as previously published (Langlois et al., 2014; Pronier et al., 2011) (Fig. 1B). The two other sh*TET2*, sh*TET2.b* and sh*TET2.c* also elicited a *TET2* knockdown, but at different level in human CD34$^+$ progenitors (Fig. 1B). Sorted or unsorted cells (100,000) were transduced with Sendai viruses containing OKSM (Oct4, Klf4, Sox2, c-Myc) reprogramming factors (experiments 1 and 2 with v1 kit and experiment 3 with v2 kit). After 3 weeks, we were able to generate iPSC colonies in each condition based on the ES-like morphology (Fig. 1C), but with a 75% decrease in reprogramming efficiency for sh*TET2* cells. iPSC reprogramming was also decreased with the two other sh*TET2.b* and sh*TET2.c* (Table 1 and Fig. 1D). GFP$^+$-iPSC (7 for *SCR* and 4 for sh*TET2*) were studied. We confirmed the decrease in *TET2* levels in only 2 clones (shTET2-1 and shTET2-2), while shTET2-3 and shTET2-4 clones expressed similar *TET2* amount compared to SCR clones (Fig. 1E). In parallel, after 10 passages, GFP$^+$-colonies were characterized by qRT-PCR for the expression of endogenous *POU5F1 (OCT4), NANOG* and *SOX2.* As expected, there were comparable levels of the pluripotent transcription factors in ES cells, SCR conditions, as well as in shTET2-3 and shTET2-4 clones, but levels were lower for shTET2-1 and shTET2-2 clones (Fig. 1E). Moreover, these 2 latter clones were not able to self-renew after 14 passages. With sh*TET2.b*, we had a 50% inhibition of *TET2* expression in CD34$^+$progenitors, while after 10 passages, iPSC presented similar *TET2* amount compared to SCR (Fig. 1B and G), suggesting that low *TET2* levels are insufficient to induce iPSC reprogramming. Indeed, with sh*TET2.c* that exhibited more *TET2* expression than with sh*TET2.b* in CD34$^+$progenitors, iPSC could eventually be reprogrammed with such *TET2* level (Fig. 1G). As expected, the pluripotent genes were relatively well expressed excepted for *SOX2* that was decreased for sh*TET2.b* and sh*TET2.c* iPSC compared to SCR iPSC (Fig. 1H).

Altogether these results show that *TET2* knockdown strongly reduces the reprogramming efficacy of human CD34$^+$progenitors into iPSC and if successful, they display a diminished levels of pluripotency master genes. According to the importance of epigenetic changes in the reprogramming (Apostolou and Hochedlinger, 2013), these results are also in line with reduced or abrogated reprogramming activity after *tet2* depletion by shRNA or *tet2* KO in mouse embryonic fibroblasts (MEF) (Doege et al., 2012; Hu et al., 2014). C/EBPα-enhanced reprogramming of mouse B cells into iPSC was mediated by Tet2 overexpression (Di Stefano et al., 2014; Sardina et al., 2018). Moreover, Tet2 overexpression was shown to exert a synergistic effect with Nanog in reprogramming (Costa et al., 2013). Finally, embryonic germ cells-mediated reprogramming of human B cells was decreased upon *tet2* knockdown through OCT4 downregulation (Piccolo et al., 2013).

3.2. Impact of TET2 mutations in the catalytic domain during reprogramming of MPN derived-CD34$^+$progenitors into iPSC

In order to further explore the role of TET2 during reprogramming, we tested if CD34$^+$progenitors obtained from patients with classical MPN exhibiting *TET2* mutations and also *JAK2*V617F or *CALR* mutations were able to generate iPSC (Delhommeau et al., 2009; Vainchenker and Kralovics, 2017). Three patients harboring different *TET2* mutations were reprogrammed using CD34$^+$ progenitor cells (Fig. 2A). First, we successfully reprogrammed one patient (P1), carrying a missense mutation in the catalytic domain. In fact, we had previously described this patient as harboring lower TET2 enzymatic activity and less 5-hmC than TET2 $^{wt/wt}$ MPN patients (Pronier et al., 2011). For patient P1, we reprogrammed not only 23 *JAK2*$^{VF/VF}$ *TET2*$^{wt/MUT}$ clones, but also 3 clones harboring *JAK2*$^{VF/VF}$ *TET2*$^{MUT/MUT}$ that were undetectable in CD34$^+$ progenitors, as determined by their clonal architecture (Table 2, Fig. 2B). One JAK2$^{VF/VF}$/TET2$^{wt/wt}$ iPS clone, 2 JAK2$^{VF/VF}$/TET2$^{wt/MUT}$ iPS clones and 2 JAK2$^{VF/VF}$/TET2$^{MUT/MUT}$ iPS clones were *bona fide* iPSC, as evidenced by the expression of pluripotent markers and transcriptional factors (Fig. 2C/D). The *JAK2*$^{VF/VF}$ *TET2*$^{MUT/MUT}$ (HO) iPS clones were able to give the 3 germ layers. The expression of *FOXA2* (endoderm) and *T* (mesoderm) genes were increased after spontaneous differentiation by EB formation assay compared to undifferentiated state. Endoderm and neuroectoderm were

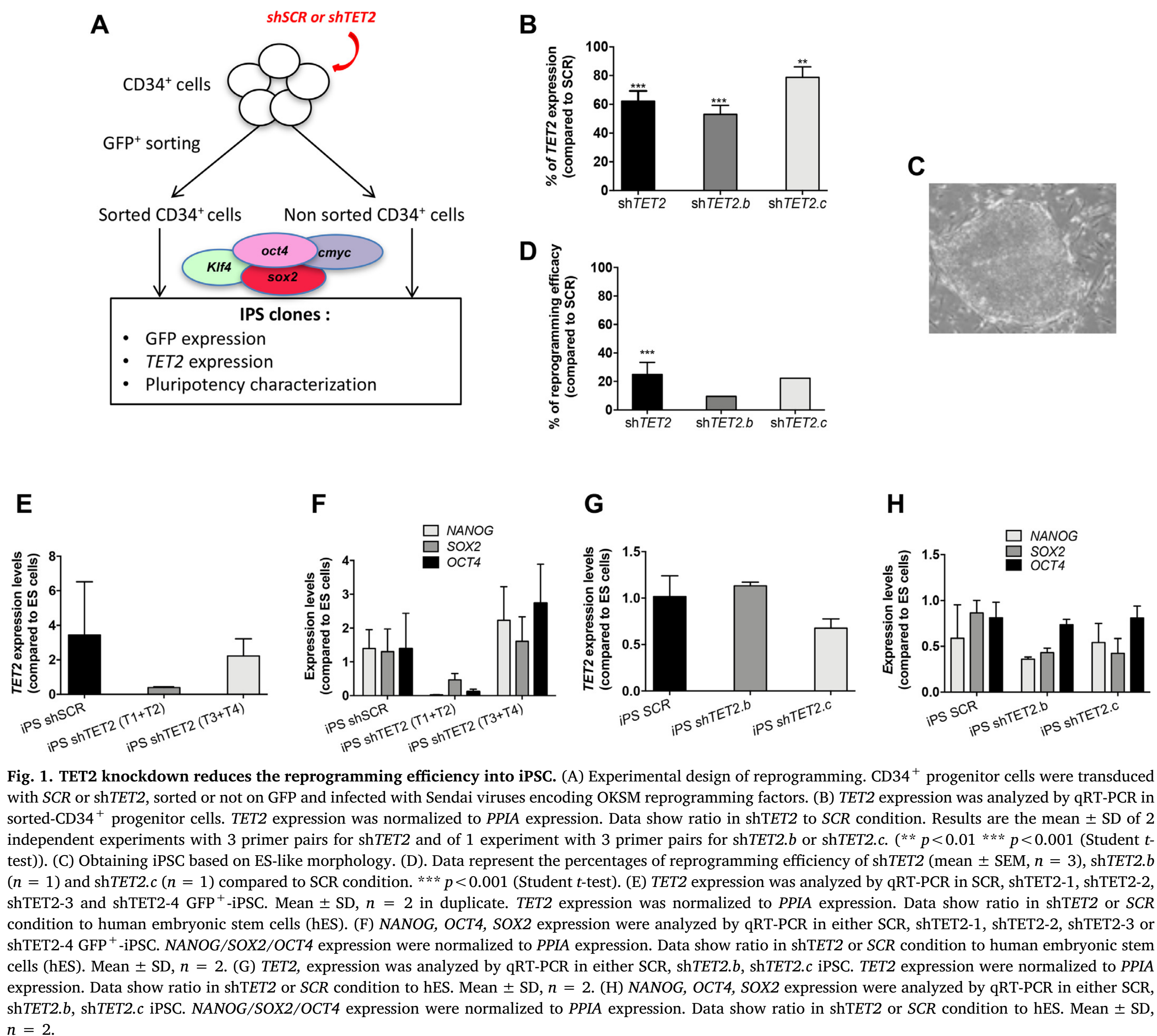

Fig. 1. TET2 knockdown reduces the reprogramming efficiency into iPSC. (A) Experimental design of reprogramming. CD34$^+$ progenitor cells were transduced with *SCR* or sh*TET2*, sorted or not on GFP and infected with Sendai viruses encoding OKSM reprogramming factors. (B) *TET2* expression was analyzed by qRT-PCR in sorted-CD34$^+$ progenitor cells. *TET2* expression was normalized to *PPIA* expression. Data show ratio in sh*TET2* to *SCR* condition. Results are the mean ± SD of 2 independent experiments with 3 primer pairs for sh*TET2* and of 1 experiment with 3 primer pairs for sh*TET2.b* or sh*TET2.c*. (** $p<0.01$ *** $p<0.001$ (Student *t*-test)). (C) Obtaining iPSC based on ES-like morphology. (D). Data represent the percentages of reprogramming efficiency of sh*TET2* (mean ± SEM, $n = 3$), sh*TET2.b* ($n = 1$) and sh*TET2.c* ($n = 1$) compared to SCR condition. *** $p<0.001$ (Student *t*-test). (E) *TET2* expression was analyzed by qRT-PCR in SCR, shTET2-1, shTET2-2, shTET2-3 and shTET2-4 GFP$^+$-iPSC. Mean ± SD, $n = 2$ in duplicate. *TET2* expression was normalized to *PPIA* expression. Data show ratio in sh*TET2* or *SCR* condition to human embryonic stem cells (hES). (F) *NANOG, OCT4, SOX2* expression were analyzed by qRT-PCR in either SCR, shTET2-1, shTET2-2, shTET2-3 or shTET2-4 GFP$^+$-iPSC. *NANOG/SOX2/OCT4* expression were normalized to *PPIA* expression. Data show ratio in sh*TET2* or *SCR* condition to human embryonic stem cells (hES). Mean ± SD, $n = 2$. (G) *TET2,* expression was analyzed by qRT-PCR in either SCR, sh*TET2.b*, sh*TET2.c* iPSC. *TET2* expression were normalized to *PPIA* expression. Data show ratio in sh*TET2* or *SCR* condition to hES. Mean ± SD, $n = 2$. (H) *NANOG, OCT4, SOX2* expression were analyzed by qRT-PCR in either SCR, sh*TET2.b*, sh*TET2.c* iPSC. *NANOG/SOX2/OCT4* expression were normalized to *PPIA* expression. Data show ratio in sh*TET2* or *SCR* condition to hES. Mean ± SD, $n = 2$.

Table 1
Number of IPSC and reprogramming efficiency obtained from CD34$^+$ cells transduced with shSCR or shTET2.

	Number of pluripotent clones	Reprogramming efficiency rate
	1st experiment	
Unsorted CD34$^+$ + shSCR (80% GFP$^+$ and 20% GFP$^-$)	60	0.060%
Unsorted CD34$^+$ + shTET2 (80% GFP$^+$ and 20% GFP$^-$)	19	0.019%
	2nd experiment	
Sorted CD34$^+$ + shSCR	50	0.050%
Sorted CD34$^+$ + shTET2	4	0.004%
	3rd experiment	
Sorted CD34$^+$ + shSCR	739	0.739%
Sorted CD34$^+$ + shTET2	258	0.258%
Sorted CD34$^+$ + shTET2.b	165	0.165%
Sorted CD34$^+$ + shTET2.c	70	0.07%

observed after analysis of teratoma after injection of HO iPSC into immunodeficient mice (Fig. 2E/F).

Second, we successfully reprogrammed patient (P2) cells carrying *CALR*ins5 and a *TET2* nonsense mutation (c.269G > *T*; p.G898X) that presumably removes the catalytic domain, but leads to stable truncated protein expression detected by immunofluorescence in the iPSC (supplemental Fig. S4). We obtained 32 *CALR*ins5 iPSC of which 12 clones were homozygous for *TET2* mutation. Two out of the 12 *CALR*ins5/*TET2*$^{MUT/MUT}$ clones were expanded and characterized. By immunofluorescence, both clones showed the expression of pluripotency surface markers and transcription factors (Fig. 3A, B). We also verified the extinction of the SV identified by RT-PCR (Fig. 3C). After EB formation assay and spontaneous differentiation, cells of all 3 embryonic germ layers were observed by immunofluorescence (AFP-endoderm, SMA-mesoderm, TuJ1-ectoderm) (Fig. 3D and E). Therefore, we confirmed the pluripotent state of iPSCs homozygously mutated for *TET2*.

These results strongly suggest that disruption of the TET2-catalytic domain still allows the reprogramming of *JAK2*V617F or *CALR*-mutated CD34$^+$progenitors. Accordingly, we have also previously reprogrammed another *JAK2*V617F patient with a *TET2* mutation in a splice

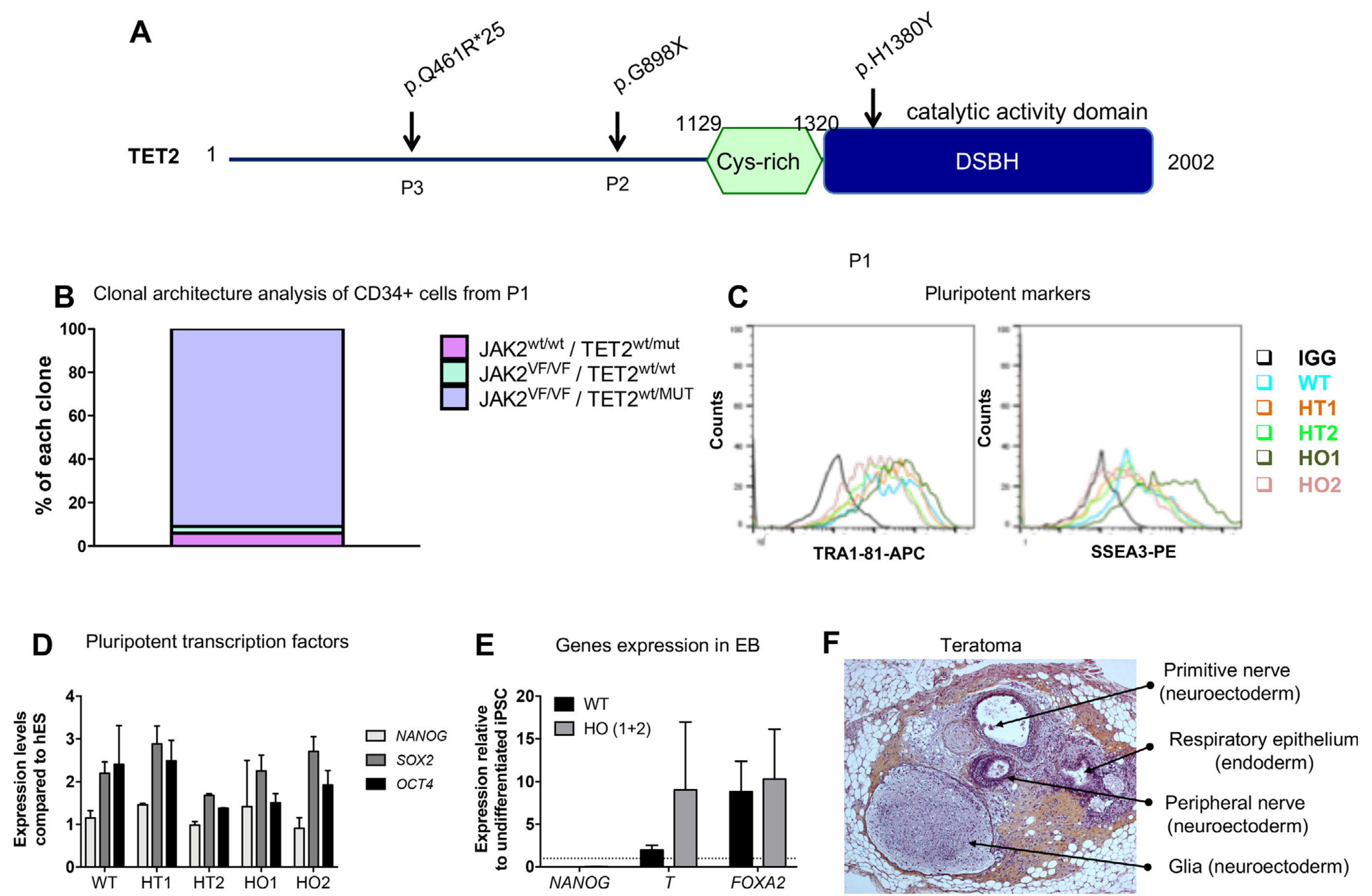

Fig. 2. Generation of iPSC from patient P1 mutated for the catalytic domain of *TET2*. (A) Scheme of TET2 protein with different mutants identified in patient cells. (B) Clonal architecture in CD34$^+$ progenitors of patient P1. CD34$^+$ progenitors were purified and sorted at 1 cell/well and cultured in serum-free medium with hematopoietic cytokines for 14 days. Each colony (53) was lysed, *JAK2*V617F genotyping was done by allele-specific qPCR and *TET2* was sequenced by Sanger method. (C) iPSC derived from patient P1 were analyzed for pluripotency markers TRA1-81 and SSEA3 by flow cytometry. (D) iPSC derived from patient P1 were quantified for *NANOG, OCT4, SOX2* expression by qRT-PCR. *NANOG/SOX2/OCT4* expression were normalized to *PPIA* expression. Data show ratio in the different iPSC to hES. Data are mean ± SD of 2 technical replicates. WT is the JAK2$^{VF/VF}$ / TET2$^{wt/wt}$ iPSC, HT1 and HT2 are two different iPSC with JAK2$^{VF/VF}$ / TET2$^{wt/MUT}$ and HO1 and HO2 are two different iPSC with JAK2$^{VF/VF}$ / TET2$^{MUT/MUT}$. (ESC: embryonic stem cells) (E) iPSC derived from patient P1 were quantified for *NANOG* (for undifferentiated state), *T* (for mesoderm), *FOXA2* (for endoderm) expression by qRT-PCR after spontaneous differentiation using EB formation. *mRNA* expression were normalized to *PPIA* expression. Data show ratio in the differentiated iPSC to undifferentiated iPSC. (F) iPSC derived from patient P1 were injected in immunodeficient mice and teratoma was examined for HO1 with evident neuroectoderm and endoderm formation *in vivo*.

site (c.3500 + 3A > *C*), which result in lower TET2 enzymatic activity. With this patient, we obtained 10 *JAK2*$^{wt/VF}$ *TET2*$^{wt/MUT}$ iPSC (Saliba et al., 2015). iPSC from CMML patient could also been generated with *TET2* homozygous mutations associated with *KRAS* and *KDM6A* mutations (Beke et al., 2020). In MPN, *JAK2* and *CALR* mutations are proliferative hits and it has been shown that higher proliferation enhances reprogramming efficiency (Kumano et al., 2013; Schlaeger et al., 2015). We cannot rule out that these mutations could counteract the intrinsic defect in the TET machinery even if it seems unlikely since it has never been described. Accordingly, in a *JAK2*WT *CALR*WT background, iPSC carrying a *TET2*Q729X nonsense mutation could be reprogrammed from a 95-years-old subject (Lo Sardo et al., 2017). The observed reprogramming activity might potentially rely on the expression the N-terminal part of TET2 protein that is still present in patients P1 and P2.

It has been previously shown that reprogramming activity is

Table 2
Number of IPSC in four *TET2*-mutated patients harboring PV and MF.

Patients	Disease	*TET* mutations	Others mutations	IPSC clones genotype
Patient 1	MPN PV	*TET2* c.4138 *C* > *T* p.H1380Y	*JAK2V617F*	1 clone - JAK2$^{VF/VF}$ / TET2$^{wt/wt}$ 23 clones - JAK2$^{VF/VF}$ / TET2$^{wt/MUT}$ 3 clones - JAK2$^{VF/VF}$ / TET2$^{MUT/MUT}$
Patient 2	MPN MF	*TET2* c.2692G > *T*;p.G898X	*CALRins5*	20 clones – CALR^{ins5}/TET2$^{wt/wt}$ 12 clones - CALR^{ins5}/TET2$^{MUT/MUT}$
Patient 3	MPN PV	*TET2* c.1380delC;p.Q461R*25 *TET3* c.G5237T; p.W1746L	*JAK2V617F*	8 clones - JAK2$^{VF/VF}$/TET2$^{wt/wt}$/TET3$^{wt/MUT}$

* PV: polycythemia vera, MF: myelofibrosis.

Fig. 3. Generation of iPSC from patient P2 mutated for the catalytic domain of *TET2*. (A) Morphology of undifferentiated iPS colony (*CALR*ins5, *TET2*^{MUT}/^{MUT}) derived from patient P2. Scale bars, 1000 µm (B) Expression of pluripotent markers SOX2, SSEA-4, TRA-1–81 and OCT-4 (red/green) were detected by immunofluorescence analysis. Scale bars, 100 µm. (C) Detection of virus (SV)-free status was performed by RT-PCR for amplification of transgenes: c-MYC, KLF4, KOS and SeV. *C*+: positive control (transduced cell pool at passage 0), C-: negative control (non-template control). (D) Image of EB formation. Scale bars, 100 µm (E) Detection of three germ layers markers after spontaneous differentiation from EBs by immunofluorescence analysis using AFP, SMA and TuJ1 specific markers. (red/green). Nuclei staining with DAPI. Scale bars, 400 µm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

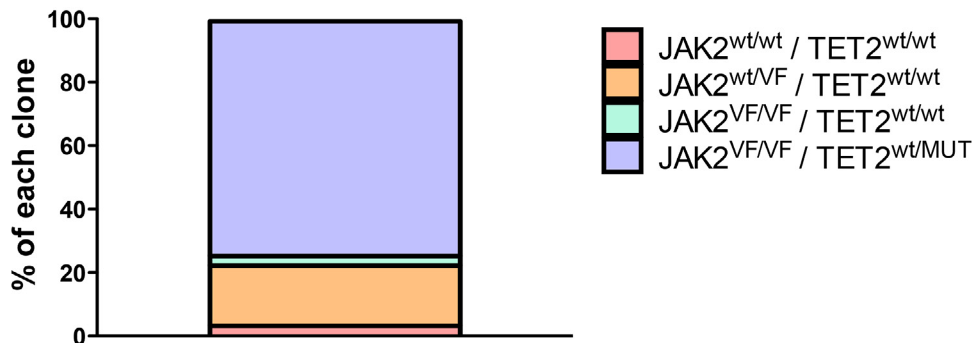

Fig. 4. Clonal architecture in CD34^{+} hematopoietic progenitors from patient P3. CD34^{+} progenitors were purified and sorted at 1 cell/well and cultures in serum-free medium with hematopoietic cytokines for 14 days. Each colony (33) was lysed, *JAK2*V617F genotyping was done by allele-specific qPCR and *TET2* was sequenced by Sanger method.

controlled by a 5-hmC-dependent mechanism and TET proteins could be redundant in this context. Indeed, it has been observed that in the presence of mutated Tet1, Tet2 activation can compensate for a defect in catalytic activity during reprogramming with Nanog (Costa et al., 2013; Wang et al., 2013; Sardina et al., 2018). In addition, in *tet2* KO cells, overexpression of Tet1, Tet2 or Tet3, but not a catalytic dead Tet2 was able to rescue iPSC colony formation (Sardina et al., 2018). These results suggest that other TET proteins can compensate for the lack of catalytic activity in TET2-mutated $CD34^+$ progenitors.

Interestingly, we reprogrammed patient P3 cells harboring the *JAK2*V617F mutation, a *TET2* frameshift mutation (c.1380delC; p.Q461R*25) and a *TET3* catalytic missense mutation (c.G5237T; p.W1746L) (Table 2). By RNA-seq in platelets, *TET2* VAF was 30% and *TET3* VAF was 75% indicating that cells might exhibit heterozygous, homozygous or wild-type *TET3* mutation. Moreover, clonal architecture performed in $CD34^+$ progenitors showed 75% of cells with *JAK2*V617F and *TET2* heterozygous mutation (Fig. 4). With these patients' cells, we observed a complete inhibition of the reprogramming into iPSC of *TET2* or *TET2/TET3*-mutated cells but we only obtained 8 *TET3* heterozygous mutated iPSC. These results suggest that TET2 requires a cooperation with TET3 to reprogram $CD34^+$ cells to pluripotent stem cells. It remains to be determined if this phenomenon is dependent or not of TET3-mediated generation of 5-hmC. In contrast, the KO of all Tet members is required for abolishing MEF reprogramming. This difference with our results could be due to the abundant expression of TET2 and 3 and low expression of TET1 in the hematopoietic system, in contrast to MEF in which Tet1 is highly expressed (Langlois et al., 2014; Moran-Crusio et al., 2011).

4. Conclusion

Altogether, these results highlight that TET2 haploinsufficiency plays a role in reprogramming into iPSC. Although TET2 expression influences the reprogramming of $CD34^+$progenitors into iPSC, if a TET2 catalytic mutant is expressed, other TET such as TET3 might compensate the default. It remains to be determined what the minimal region of the N-terminal part of TET2 involved in the reprogramming is and with which partners it cooperates.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Lise Secardin: Data curation, Writing - original draft. **Cintia Elisabeth Gomez Limia:** Data curation, Writing - original draft. **Antonio di Stefano:** Data curation, Writing - original draft. **Martin Hernan Bonamino:** Data curation, Funding acquisition. **Joseph Saliba:** Data curation. **Keisuke Kataoka:** Data curation, Formal analysis. **Stevens K. Rehen:** Data curation. **Hana Raslova:** Data curation, Writing - original draft, Funding acquisition. **Caroline Marty:** Data curation, Writing - original draft. **Seishi Ogawa:** Conceptualization, Data curation. **William Vainchenker:** Data curation, Writing - original draft. **Barbara da Costa Reis Monte-Mor:** Writing - review & editing, Investigation, Data curation, Funding acquisition. **Isabelle Plo:** Data curation, Investigation, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101755.

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