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ADDENDUM

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The conditional KO approach: Cre/Lox technology in human neurons

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ABSTRACT

The use of human pluripotent stem cells to model human diseases has become a new standard in biomedical sciences. To this end, patient-derived somatic cells are studied *in vitro* to mimic human pathological conditions. Here, we describe an alternative experimental strategy, the 'conditional KO approach', which allows engineering disease-relevant mutations in pluripotent stem cells from healthy donors. In combination with the Cre/Lox technology, this strategy enables us to study the molecular causes of human diseases independent of the genetic background or of genetic alterations induced by clonal selection. As a proof-of-principle, we generated pluripotent stem cells with conditional loss-of-function mutations in the human *STXBP1* gene that encodes Munc18-1. Using neurons derived from these cells, we show that heterozygous disruption of *STXBP1* produces a specific and selective impairment in synaptic transmission that may account for the severe neurological disease caused by such mutations in human patients.

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Introduction

Revolutionary advances in stem cell biology during the last decade made human somatic cells accessible in a large scale for the study of human diseases. Using induced pluripotent stem cells (iPS) from patients with disease-relevant mutations and subsequent differentiation into the affected cell type has proven to be a powerful tool for modeling diseases and for drug screening.[1,11,14] The current standard of the field is to generate patient-derived iPS cells which are then studied in comparison to either unrelated control iPS cells or, more stringently, to the same iPS cells after isogenic correction of the respective mutation by constitutive genome editing. Comparisons of mutated and corrected cells allow conclusions about the cellular phenotype of a given disease, and open up the possibility for cell replacement therapy. In parallel to the usage of animal model systems, human pluripotent stem cells have thus become a major tool to understand mechanisms of diseases. The main advantage of this 'patient approach' is that the specific gene mutation seen in a patient is analyzed in comparison to the corrected control within the patient's own genomic background (Fig. 1A). This strategy has been used to study a plethora of human diseases, including neurological disorders.[8,12,13] However, this standard approach has two potential limitations. First, it depends on the generation of selected cell clones that may carry new selection-induced mutations and are subject to clonal variation. Second, this approach does not actually test whether a given pathogenic mutation is sufficient to produce a pathological phenotype on the genetic background of a healthy person – it only tests this for a given patient's genetic background. These limitations prompted us to pursue an alternative approach that complements the analysis of patient-derived cells, namely, the analysis of conditional mutations in human cells derived from a healthy person (Fig 1).[6] For this purpose embryonic stem cells or control iPS cells, after genetic screening, can be used.

The conditional knockout (KO) approach

By genetic engineering in combination with the Cre/Lox technology targeting healthy pluripotent stem

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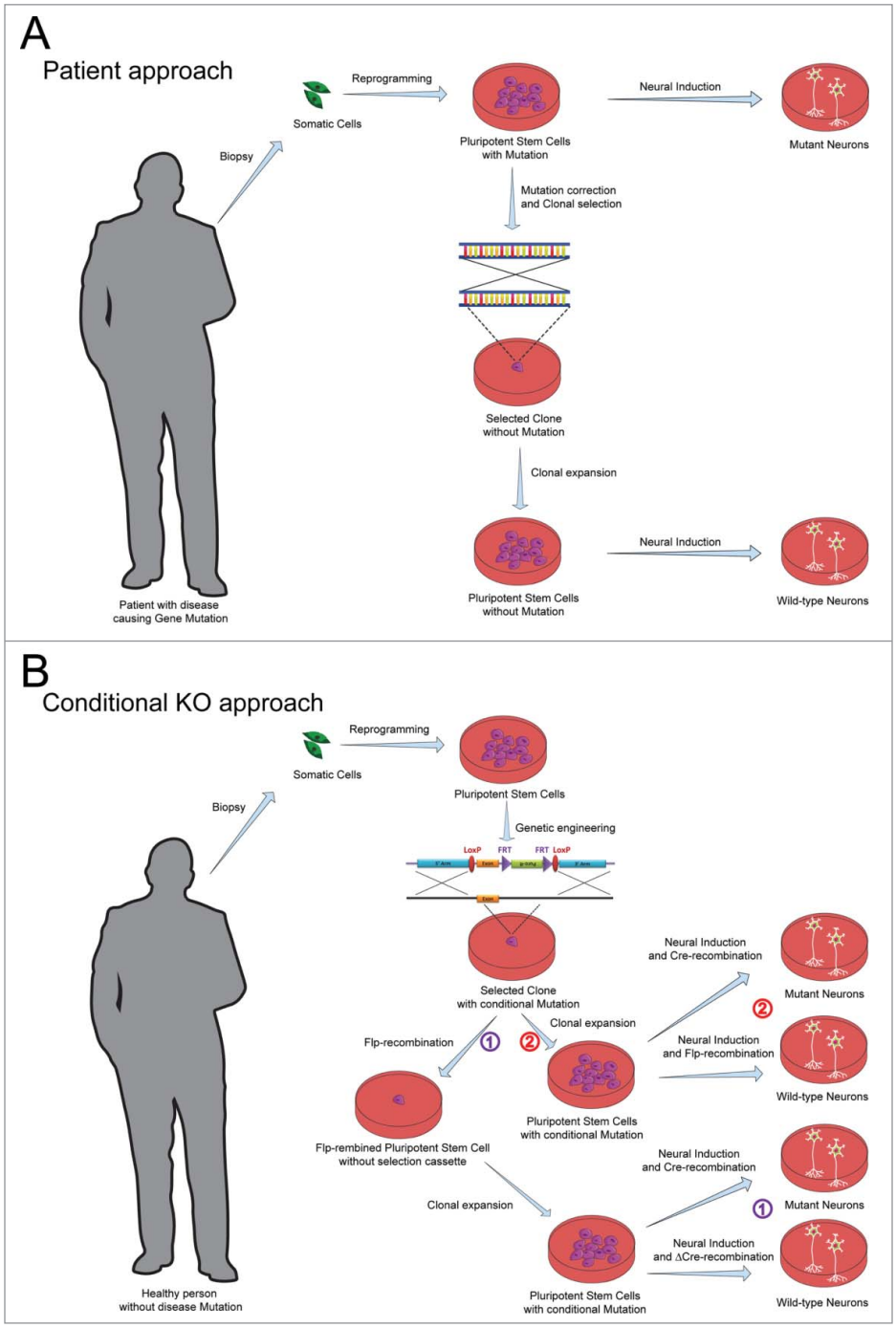

Figure 1. Comparison of 2 different strategies to study human disease using neurons derived from pluripotent stem cells. (A) The patient-approach uses iPS cells from a patient with a pathogenic mutation. iPS cells are differentiated into neurons and analyzed. The matching control neurons are derived from pluripotent stem cells after correction of the mutation by genetic engineering and clonal selection, or are derived from an unrelated healthy control person. (B) The conditional approach starts with pluripotent stem cells from a healthy person without mutations. The disease-relevant mutation is introduced into the stem cells as a conditional allele by genetic engineering and clonal selection, with an intermediate state in which the targeted gene contains a resistance cassette that is then removed by Flp-recombination as shown. The conditionally mutant stem cells are differentiated into neurons with simultaneous expression of mutant Cre-recombinase (as a control) or wild-type Cre-recombinase (to conditionally delete the floxed exon and produce a loss-of-function state). An alternative way is to use Flp-recombined cells as control neurons and Cre-recombined as mutant neurons.

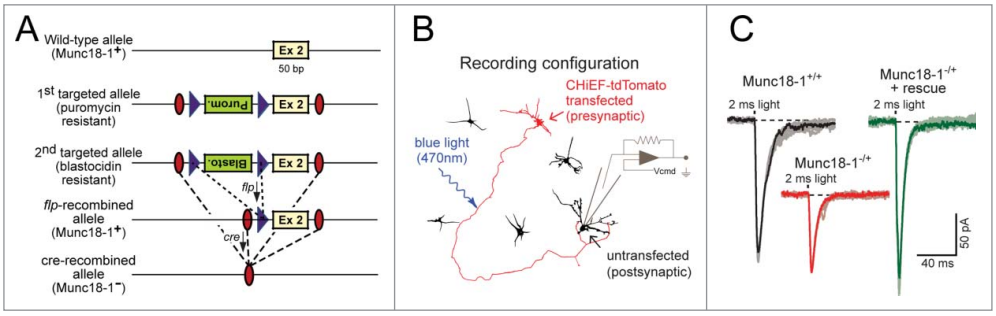

Figure 2. Gene-targeting of the *STXBP1* gene encoding Munc18-1, and reduced synaptic transmission in heterozygous *STXBP1* mutant neurons. (A) Using homologous recombination, Exon 2 (encompassing 50 bp of coding sequence 3′ of the translational start codon) is flanked by loxP sites and a resistance cassette for Puromycin or Blastocidin surrounded by frt sites is inserted into the intron 5′ of exon 2. Excision of the resistance cassette by flp recombinase restores the wild-type gene containing loxP sites 3′ and 5′ of exon 2. However, Cre-recombination excises exon 2, causing a frameshift that leads to a loss-of-function mutation of *STXBP1*. [Taken from [6], Fig. 1A]. (B) Paired-recordings from synaptically coupled neurons using optogenetics. Human neurons are sparsely transfected with tdTomato-tagged CHIEF (a channelrhodopsin-2 variant), and stimulated by blue light. Synaptic responses are measured from an untransfected adjacent post-synaptic neuron. (**C**)Decreased light-evoked synaptic transmission in heterozygous *STXBP1* mutants. Co-transfection of wild-type rat Munc18-1 with channelrhodopsin into the heterozygous *STXBP1*-mutant neurons rescues the heterozygous phenotype. [B and C are taken from [6], Fig. 6E-F].

cells, we created pathogenic mutations in a conditional manner. This allowed us to investigate whether a specific molecular change in a defined genetic background is inducing a phenotypic alteration. Specifically, in pluripotent stem cells without disease-causing mutations (H1 human embryonic stem cells), we flanked exon 2 of the human *STXBP1* gene with loxP sites, encompassing a drug-resistance cassette which itself is flanked by frt sites (Fig. 2A). For every experiment, the targeted cells were split into two populations: the control population was treated with Flp-recombinase in order to restore the wild-type locus, and the second group was converted into *STXBP1*-mutant cells by Cre-recombination and excision of exon 2 (Fig. 2A). At the same time, both populations of pluripotent stem cells were converted into glutamatergic human neurons by lentiviral overexpression of the transcription factor Neurogenin-2.[16]

The advantage of this conditional approach is that mutant and control neurons carry the identical genetic background, and are not derived from different cell clones. No clonal selection is necessary to produce isogenic wild-type and mutant cells. The single difference consists in the expression of the recombinases during neuronal differentiation (Fig. 1B) A derivative of this method is to first remove the selection cassette by Flp-recombination and then apply Cre-recombinase or inactive Cre-recombinase (ΔCre) to produce mutant and control cells (Fig. 1B). In a second, independent project, we recently validated this approach by targeting neurexin-1 mutations.[5] So far, every version of the 'patient approach' using mutation correction of patient iPS cells has been carried out by clonal selection, so that it cannot be excluded that additional mutations have occurred during the selection process. The conditional KO approach enables gene function study in human cells independent of their genetic background.

Moreover, in contrast to the 'patient approach', this method allows for homozygous loss-of-function mutations of genes that are expressed in pluripotent stem cells because the mutations are only activated upon cre-recombination. For this purpose, heterozygous mutant pluripotent stem cells are used to target the second allele. In terms of this objective, the relation of 'patient approach' to 'conditional KO approach' is similar to the relation of constitutive vs. conditional mouse model.

Conclusions

Using the conditional mutation approach described above, we studied in human neurons heterozygous and homozygous mutations of *STXBP1*, which encodes Munc18-1, a protein that is essential for synaptic vesicle fusion.[15] We used this gene as a proof-of-principle study because hundreds of independent heterozygous mutations in *STXBP1* have been associated with extremely severe forms of early infantile epileptic encephalopathy (often referred to as Ohtahara or as West syndrome) that can exhibit a broad spectrum of clinical manifestations.[2,3,4,7,9,10] The pertinent

question was: What is the molecular and cellular cause of *STXBP1* mutation-mediated Ohtahara syndrome phenotype in human neurons? The main finding of our study is that a partial reduction of the Munc18-1 expression caused by a heterozygous loss-of-function mutation in the *STXBP1* locus is sufficient to reduce synaptic transmission between neurons by around 50% (Fig. 2B–C).

This novel 'conditional KO approach' on human cells complements the 'patient approach' and may be an important step forward on the way of understanding the molecular causes of human diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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