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The antipsychotropic drug Duloxetine rescues PAX6 haploinsufficiency of mutant limbal stem cells through inhibition of the MEK/ERK signaling pathway

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ABSTRACT

Aniridia is a panocular disease causing progressive severe visual impairment and blindness due to PAX-6 haploinsufficiency. One of the most disabling ocular symptoms is aniridia-related keratopathy (ARK), a progressive corneal opacification due to epithelial impairment, vascular and conjunctival pathologies. There is currently no available treatment to prevent progressive visual loss. For this aim, we have used mutant limbal cells for phenotypic screening using FDA-approved and bio-actives drug library and found Duloxetine, a serotonin and norepinephrine reuptake inhibitor used against severe depression as able to enhance endogenous *PAX6* expression and target genes, which returned fairly to amounts found in normal limbal cells. In addition, Duloxetine could restore cell migration of the mutant cells. Furthermore, we show that Duloxetine activates PAX6 through inhibition of the ERK pathway on limbal mutant cells. This observation fits the recent report that MEK inhibitors enhance PAX6 *in vivo*, partially rescuing aniridia developmental phenotype of $Pax6^{+/-}$ mice.

The discovery of an unique compound able to enhance PAX6 activity and that could be locally administered using eye drops associated with drug repurposing is expected to lead to rapid development of applicable drugs for the topical (eye drops) treatment of aniridia.

Aniridia is a panocular disease causing progressive severe visual impairment and blindness. The disease is characterized by abnormal development of almost all eye structures, caused by dominantly inherited heterozygous mutations in primarily the *PAX6* gene [1]. *PAX6* is expressed in multiple ocular tissues during embryogenesis and in adult including the corneal and lens epithelia, iris, ciliary body and retina. One of the most disabling ocular symptoms is aniridia-related keratopathy (ARK), a progressive corneal opacification due to epithelial impairment, vascular and conjunctival pathologies. ARK is associated with a deficiency of the limbal epithelial stem cells (LSC) that reside at the corneal/conjunctival boundary and normally maintain a healthy corneal epithelium [1]. This loss of corneal LSC function is already apparent in infancy and progressively leads to complete loss of corneal transparency, severe visual impairment and ultimately blindness [1]. Although aniridia is a developmental disorder, corneal opacification begins late in childhood, leaving a large time window for therapy. There is currently no available treatment to prevent progressive visual loss. Stem cell therapies and ocular surface reconstruction only provide a temporary restoration of corneal transparency and limited gain in visual acuity before the disease process recurs and blindness returns. Thus, there is a current need and a potential opportunity to identify drugs to improve corneal transparency or prevent ARK due to *PAX6*

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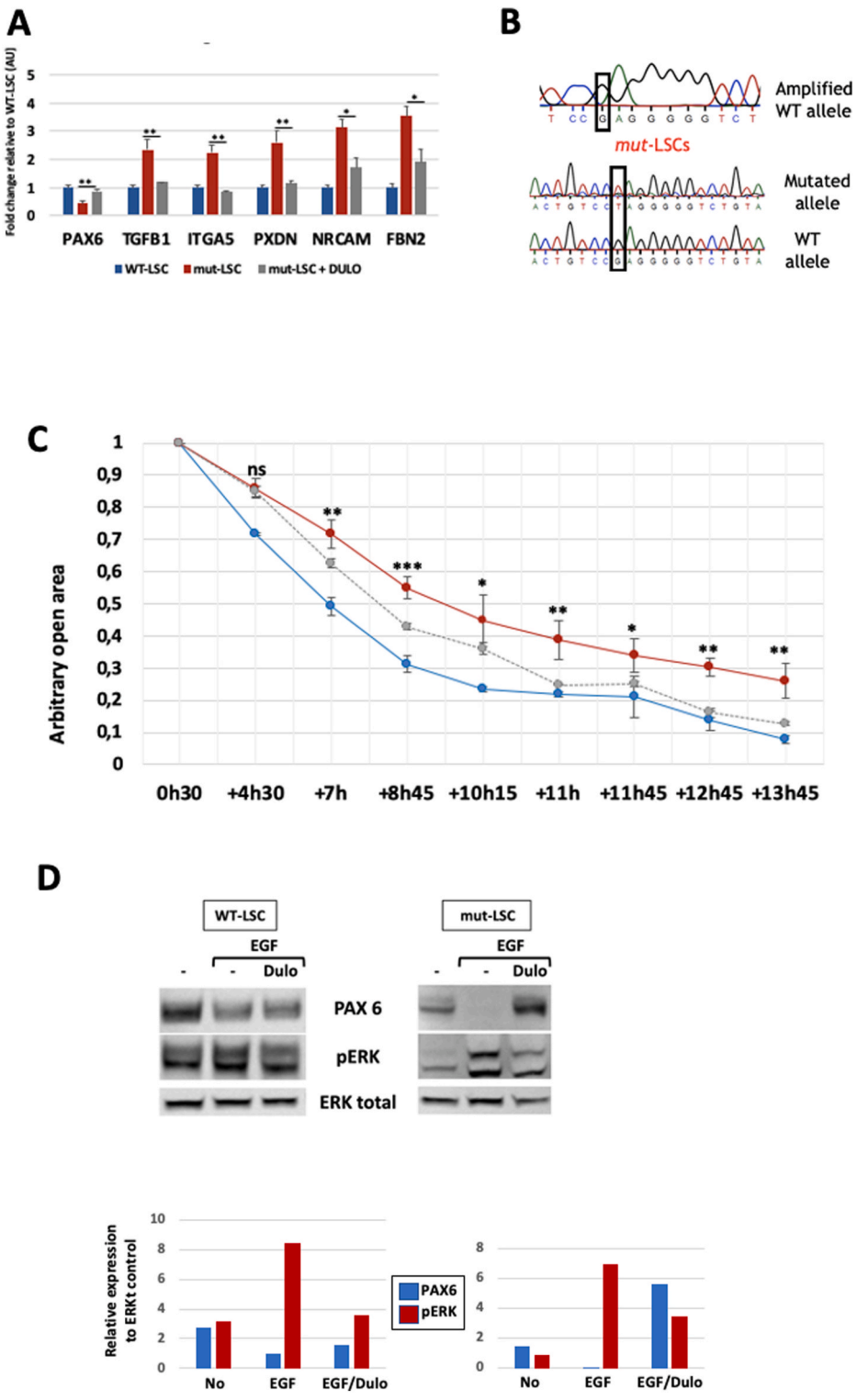

Fig. 1. Duloxetine rescues PAX6 haploinsufficiency *in vitro* through ERK inhibition. (**A**) Rescue of PAX6 haploinsufficiency as revealed by qRT-PCR analysis of endogenous *PAX6* and *PAX6*-target gene expression in the absence (in red) or the presence of 1 μM of Duloxetine (in grey) during 24 h on mut-LSCs and compared to WT LSC (in blue). $n = 5$. The genes tested were: PAX6, TGF-β1, ITGA5, PXDN, NRCAM, FBN2. Abbreviation: mut-LSC: mutated limbal stem cells. (**B**) Sequencing analysis of PCR amplicon obtained after qRT-PCR analysis of mut-LSC treated with Duloxetine. *Upper*: the amplicon sequence; *lower*: sequences of the WT and mutant alleles. Only the WT allele transcript has been systematically amplified. (**C**) Mut-LSC and WT cells were seeded into IBIDI chambers for cell migration test. Mut-LSC were treated with 0.5 μM of Duloxetine (grey) or untreated (red) and compared to untreated WT-LSC (blue). Closure was measured at different time points. $n = 3$. One-way ANOVA followed by Dunnett's test was performed: *, $p < .05$; **, $p < .01$; ***, $p < .001$, ns $p > .05$. (**D**) Western blot analysis of WT-LSC and mut-LSC. Cells were depleted of EGF and BPE overnight, treated or not 15 min with 5 ng/ml of EGF ± 0.5 μM of Duloxetine. Untreated cells were cultivated with methanol, as Duloxetine is resuspended in methanol. Blots were tested for PAX6 and phospho-ERK1/2 (pERK). Total ERK (ERKt) antibody was used as loading control. Histograms represent the relative quantification of PAX-6 levels and phospho-ERK normalized to total ERK by densitometry and imageJ. The relative intensity are represented in Suppl. Fig. 2. $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

haploinsufficiency. For this aim, we have generated a TRE-tomato-HEK293 reporter cell line, in which multiple copies of PAX6 transcriptional responsive elements (TRE) have been inserted upstream to tdTomato gene in HEK293 cell line, which expresses a low level of *PAX6* gene. These TRE-tomato-HEK cells were used for phenotypic screening using FDA-approved and bio-actives drug library (Microsource, Spectrum Collection) in 384 format. Most promising hits were further evaluated both experimentally and computationally for their EC50 and toxicity profiles (Suppl. Fig. 1A). Duloxetine (EC50 = 22.7 μM), an antidepressant drug was selected for further validations for its lack of cell toxicity and efficient enhanced tomato red fluorescence (Suppl. Fig. 1B). Duloxetine is a serotonin and norepinephrine reuptake inhibitor used (as Cymbalta) against severe depression [3]. It is indicated for the treatment of neuropathic pain, generalized anxiety disorder, osteoarthritis, and stress incontinence. Although we do not know yet the link between PAX6 and Duloxetine, the effect of this compound on PAX6 expression is conceivable, as it is known that PAX6 is expressed in neural progenitor cells in different regions of the developing CNS and

in adult neural stem cells, and a strong relationship between PAX6, neural and sleep disorders has been reported [4]. Moreover, the cornea is the most innervated tissue of the body, producing most neurotransmitters like serotonin and norepinephrine. Duloxetine is also able to enhance dopamine levels by inhibiting norepinephrine transporters. This increase in dopamine specifically takes place in the prefrontal cortex where dopamine transporters are scarce, and reuptake relies more heavily on norepinephrine transporters. Of interest, inactivation of dopamine in the retina induces keratoconus and Pax6 is an important determinant of the dopaminergic phenotype in the olfactory bulb [5]. Moreover, olfactory bulb dopaminergic neurons are severely reduced in $Pax6^{sey/+}$ mice that express reduced amounts of Pax6 [5], suggesting that Pax6's function in dopaminergic specification is dose-dependent.

Recently, we have generated in vitro PAX6 haploinsufficiency cells by genome editing on normal LSC, in which a nonsense mutation has been introduced in one allele of the PAX6 gene [2]. The mutated cells (mut-LSC) recapitulated the ARK phenotype in vitro with reduced cell migration and enhanced cell adhesion [2].

We used this original cellular model to validate the effect of Duloxetine on endogenous PAX6 expression. Treatment of mut-LSC with 1 μM Duloxetine for 48 h was sufficient to enhance endogenous *PAX6* expression, which returned fairly to amounts found in LSC parental cells (Fig. 1A). Of interest, Duloxetine activated only the WT allele, as shown by sequencing of the PCR amplicon (Fig. 1B), strongly suggesting that this small compound could be efficient for any mutation without the risk of producing a mutant truncated protein that could induce a dominant negative deleterious effect. This is in contrast with the various read-through drugs that suppresses premature termination codons (PTCs), like Ataluren, restoring functional but truncated protein production from genes disrupted by nonsense mutations. Next, we tested the effect of Duloxetine treatment on *PAX6* target genes. We have previously shown that, in LSC, PAX6 acts mainly as a repressor [2]. Accordingly, the *PAX6*-target genes which are activated in mutant cells returned to normal following treatment with Duloxetine (Fig. 1A).

We have shown previously that mut-LSC displayed delayed cell migration as compared to normal LSC [2]. We tested whether Duloxetine could restore cell migration of the mutant cells. Using IBIDI migration chambers, gap closure of mut-LSC was monitored following drug treatment. As illustrated in Fig. 1C, gaps closed at similar kinetics to WT LSC following treatment of mut-LSC with Duloxetine. These data strongly suggest that Duloxetine restores defective limbal function.

PAX6 is repressed by EGF to allow corneal epithelial cell proliferation [6] and Duloxetine has been shown to negatively regulate ERK on murine amygdala *in vivo* [7]. When mut-LSC were treated with 5 ng/ml EGF to activate transiently the MEK/ERK pathway, PAX6 production was drastically reduced but restaured by the addition of 0.5 μM of Duloxetine, even to a higher extent (Fig. 1D, right). A similar behavior was observed on mut-LSC, to lower extent. Duloxetine reduced significantly the phosphorylation of ERK in mut-LSC, suggesting that the activation of *PAX6* by the drug occurred partially through inhibition of the ERK pathway on limbal cells. In WT-LSC, the effect of Duloxetine on pERK was weaker. It remains to be demonstrated that this modulation is direct. Nevertheless, this observation fits the recent reports that both MEK inhibitors enhance PAX6 *in vivo*, partially rescuing aniridia developmental phenotype of $Pax6^{+/-}$ mice [8] while the serotonin receptor antagonist ritanserin rescued PAX6 in vitro through MEK inhibition [9].

More than 7000 patients with congenital aniridia in EU need to be treated. As the cornea is easy to target with topical formulation, the discovery of an unique compound able to enhance PAX6 activity and that could be locally administered using eye drops associated with drug repurposing is expected to lead to rapid development of applicable drugs for the topical (eye drops) treatment of aniridia. Its potential action on cornea transparency should be more restricted and specific than global MEK inhibitor treatment. Moreover, the anti-psychotic drug is administrated orally as medication without major eye side effect, which is not the case of the anti-tumoral MEK inhibitors [10].

Declaration of competing interest

The authors indicated no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtos.2021.12.003.

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