Rev Med Int Sindr Down. 2014;**18(2)**:21-8

INTERNATIONAL MEDICAL REVIEW ON DOWN'S SYNDROME

www.elsevier.es/sd

FUNDACIÓ CATALANA SÍNDROME DE DOWN
www.fcsd.org

ORIGINAL ARTICLE

Identification of key genes involved in Down's syndrome pathogenesis by gene therapy

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Received 14 April 2014; Accepted 19 June 2014

KEY WORDS
Down's syndrome;
Dyrk1A;
miRNAs;
Gene therapy

Abstract
Viruses have evolved ways of encapsulating and delivering their genes into human cells. Gene therapy takes advantage of this capability to manipulate the viral genome and convert an infectious agent into an efficient vector that delivers therapeutic genes. In the current work we have applied gene therapy approaches based on adeno-associated virus and lentivirus delivery to identify candidate genes (protein-coding or miRNAs) involved in the cognitive deficits in Down Syndrome. We show that the hippocampal injection of the adeno-associated virus AAV2/1-shDyrk1A normalized Dyrk1A expression in the trisomic Ts65Dn mice. As a consequence the regulation of key molecular players in memory and learning processes was rescued and mice showed an attenuation of synaptic plasticity defects and improved efficacy in learning strategies. All together these results reinforce the role of Dyrk1A in cognition. On the other hand, with the lentiviral strategy developed to specifically inhibit miR-155 and miR-802 (Lv-anti-miR155/802), we were able to show a tight control of the miRNAs target Mecp2 suggesting that the downregulation of MeCP2 in Down syndrome could be a contributing factor to the cognitive defects.

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PALABRAS CLAVE
Síndrome de Down;
Dyrk1A;
miRNA;
Terapia génica

Identificación de genes clave implicados en el síndrome de Down mediante terapia génica

Resumen
La terapia génica nos ofrece la posibilidad de manipular los virus para convertir un agente infeccioso en un vehículo que transporta en su genoma secuencias de DNA con potencial terapéutico. En este trabajo hemos aprovechado la metodología que nos proporciona la terapia génica para desarrollar vectores virales derivados de virus adenoasociados y lentivirus con el objetivo de identificar genes clave (proteínas o microRNA [miRNA]) implicados en las alteraciones cognitivas presentes en el síndrome de Down (SD). Hemos demostrado que en un contexto de trisomía, como es el modelo de ratón Ts65Dn, la normalización de la expresión de Dyrk1A a través de la administración de los virus adenoasociados AAV2/1-shDyrk1A contribuye a restablecer la regulación de moléculas clave en los procesos de memoria y aprendizaje. Ello permite una atenuación de los defectos en plasticidad sináptica y facilita el desarrollo de una estrategia de aprendizaje visuoespacial más eficiente. Estos estudios refuerzan el papel destacado de Dyrk1A en los procesos cognitivos. Por otro lado, la estrategia de control de la expresión de miRNA desarrollada mediante los lentivirus Lv-anti-miR155-802 nos permite proponer a *MeCP2* como un gen cuya desregulación en el síndrome de Down puede tener un papel clave en el deterioro cognitivo.

Chromosome 21

The presence of a complete or partial copy of chromosome 21 (Hsa21) is the cause of Down's syndrome (DS). This excess of genetic material leads to a dysregulated expression of certain genes. The functional impact of these changes could be a direct result of the action of the proteins expressed in excess by the Hsa21 genes, or indirectly, through the proteins that they regulate. In any case the effect will be different according to the protein involved.

In some cases it may be that the excess of a particular protein is innocuous, while small changes in the expression of key proteins may be sufficient to alter fundamental cell processes and functions. In the last few years it has been discovered that the genome contains genes that do not code for proteins, but that they are transcribed to give rise to small RNAs, microRNAs (miRNAs), which exert a negative regulation of gene expression. Each miRNA can act on a large number of proteins, thus, the changes in a single miRNA can control the expression of a large number of proteins and thus, have a very notable impact on certain cell functions. Several miRNAs have been described in chromosome 21, five of which (miR-99a, miR-125b-2, miR-155, miR-802 and let7-c) have been detected in excess in some tissues[1]. The presence of these miRNAs in excess suggests that, in trisomy conditions, some of those proteins regulated by these miRNAs could be under-expressed. The lack of these could cause a change in the physiological processes in which they participate.

The imbalance of the proteins in the trisomic cells, whether mediated by coding or non-coding genes (such as miRNAs) is significantly gene-dependent. While for certain genes the compensatory effects may lessen the impact of the genetic imbalance, for other genes these changes could have a high functional impact. This demonstrates the critical role of certain dose-dependent genes. It is important to identify these dosage-sensitive elements of the genome to be able to better understand their effects and their pathogenic impact.

Gene therapy as a strategy to study the effects of dosage-sensitive genes

Gene therapy consists of the transfer of genetic material to the cells of an individual, with the aim of correcting genetic defects and to be able to change the course of the disease or relieve some of its symptoms. Its development has arisen mainly, but not exclusively, for the treatment of hereditary diseases in which the causal alteration resides in mutations in a known gene that prevents the correct expression of the protein, and thus its function. There are currently a significant number of gene therapy trials, particularly for the treatment of some rare diseases[2-4]. It should be mentioned that in November 2012, the European Commission approved the first gene therapy drug, and it was for the treatment of a lipoprotein lipase deficiency[5]. Gene therapy clinical trials are also being conducted for the treatment of complex diseases such as Alzheimer's disease (registry number at clinicaltrials.gov: NCT00876863). Thus, in the future it may be possible that individuals with DS could enter gene therapy clinical trials, either for the treatment of the Alzheimer-type anomalies associated with DS, or other clinical manifestations present in individuals with DS. However, at the moment nobody with DS has been included in any clinical trial.

Furthermore, gene therapy has, to a great extent, led to the development of numerous tools and strategies to approach the treatment of diseases, advances that are of interest for many other applications, not necessarily therapeutic. One of the key elements has been the development of viral vectors. The ability of a virus to infect cells is exploited in order to transform them into recombinant, replication-defective, viruses capable of transporting genetic elements that will be expressed within the target cell.

In this work we demonstrate that gene therapy provides a new opportunity to identify key genes in the pathophysiology of Down's syndrome. We have developed a strategy based on the use of viral vectors: *a)* modulating the expression of *Dyrk1A*, a gene for which there is previous

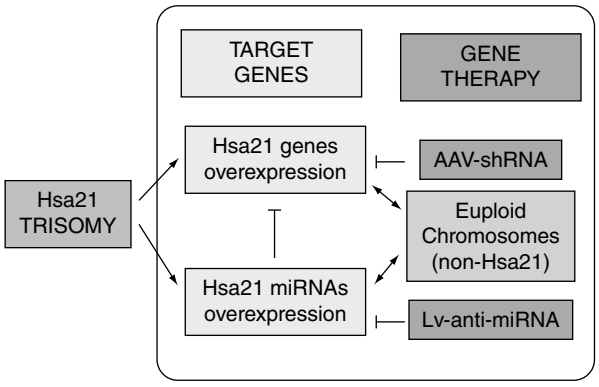

Figure 1 Genetic alterations in Down's syndrome and gene therapy strategies developed in the present work. The presence of an additional copy of Hsa21 causes an excess in the dosage of genes and the miRNAs present in Hsa21. The overexpression of miRNAs can alter gene expression, whether situated in Hsa21 or in other chromosomes. In turn, the altered expression of these genes can have effects on the transcriptome. The diagram illustrates the two gene strategies developed in the present work, based on the modulation of the expression of genes (AAV-shRNA) or miRNAs (Lv-anti-miRNA) of Hsa21 by means of modified viruses.

evidence of its over-expression in DS and being gene-dosage sensitive, and *b)* modulating the content of two candidate miRNAs in trisomic state in DS individuals (fig. 1). We have studied the functional impact that the modulation of these elements exercise on memory and learning processes, which the trisomic Ts65Dn mouse model possesses notable cognitive changes[6,7]. These animals possess a partial trisomy of mouse chromosome 16 (MMU16), syntenic to Hsa21, which comprises, in triple doses, more than 100 genes, among them *Dyrk1A*, and the miRNAs miR-155 and miR-802.

Impact on cognitive aspects of *Dyrk1A* normalization in the hippocampus of Ts65Dn mice

The *Dyrk1A* gene codes for a protein kinase, which has the function of phosphorylating other proteins, among them some important in memory, learning, or brain development processes. Dyrk1A relevance comes from its cellular function in, neurogenesis, neuronal survival, differentiation, as well from notable over-expression in brains of individuals with DS, and its implication in motor and cognitive changes demonstrated in mouse models with different gene doses of *Dyrk1A*[8-11].

However the role of *Dyrk1A* has not been described in the trisomy context. For this reason we have developed the gene therapy strategy to normalize the expression of *Dyrk1A* in Ts65Dn mice and to study its impact on the reversion of the cognitive changes. We use molecular constructions based on RNA interference technology (RNAi). Specifically, we designed *short hairpin* RNA sequences complementary to a region of *Dyrk1A* messenger RNA (mRNA), with the objective that these sequences could be paired, and the degradation of the *Dyrk1A* mRNA would take place through the RNAi mechanisms, preventing its translation to a protein. This would give rise to a lower amount of *Dyrk1A* per cell, which should allow obtaining normal levels of protein contents, thus counteract its over-expression.

The designed construct also contains a luciferase gene, to allow the in *vivo* expression follow-up, and all this encapsulated within an adeno-associated virus, with a serotype-2 genome and a serotype-1 capsid (AAV2/1) to guarantee the transduction of neuronal cells. Thus, AAV2/1-shDyrk1A (SH) virus is generated, as well as a control virus that should not interfere with Dyrk1A, AAV2/1-scDyrk1A (SC) (fig. 2A). Both viruses were efficient in the transduction of neuronal populations (Tuj positive) and glial populations (GFAP positive) (fig. 2B), and a decrease in the expression of Dyrk1A was only observed in primary cortical neurons from mice treated with the SH virus (fig. 2C). We could also observe an efficient *in vivo* transduction in Ts65Dn mice after the hippocampus injection. The presence of the virus in the hippocampus was selective, and was not detected in other regions of the brain, as observed by the luciferase activity analyzed in different regions at 2 weeks release injection (fig. 2D). At the viral doses injected, we could observe a normalization in the expression of *Dyrk1A* in the Ts65Dn mice treated with the SH virus, while the over-expression was maintained in the SC mice (fig. 2E).

We then studied the implication of *Dyrk1A* in cellular, electrophysiological, and behavioral changes in the Ts65Dn mice using the developed viruses, capable of normalizing *Dyrk1A* in a trisomy context. During memory and learning processes, there is a sustained activation in the liberation of excitatory neurotransmitters that stimulate the activation of postsynaptic neurons. Once activated, the signal is transmitted within the cell through the activation of different intracellular signaling pathways, some of which are altered in the Ts65Dn mice. The phosphorylated form of Erk1 and CREB transcription factor were normalized in the mice treated with the SH virus, where a normalization in protein expression was achieved, indicating that *Dyrk1A* may be associated with the modification of components of the Erk-CREB pathway involved in memory and learning processes (fig. 3A). Furthermore, electrophysiological studies demonstrated that the animals treated with the SH virus showed changes in the electrical activity of the neurons, when measured by means of the Long-term Potentiation (LTP) synaptic plasticity paradigm, a model to explain the underlying changes associated to certain learning and memory processes. A deficit in LTP has previously been described in Ts65Dn mice[12]. Our experiments confirm these changes, and we could observe that the administration of the SH virus gave rise to a partial rescue in the LTP. These data supported the role of Dyrk1A in cognition. In this line, we decided to study the behavior of the animals using a task that evaluates hippocampal function, such as the Morris water maze, a behavioral test in which the mice are individually placed in a swimming pool, and they must learn to find a platform to perch on. Over the days of performing this task, the mice learn to locate the hidden platform using their visual-spatial orientation skills. For this they need the hippocampal function that enables this learning and memory task to be performed. The trisomic mice have a

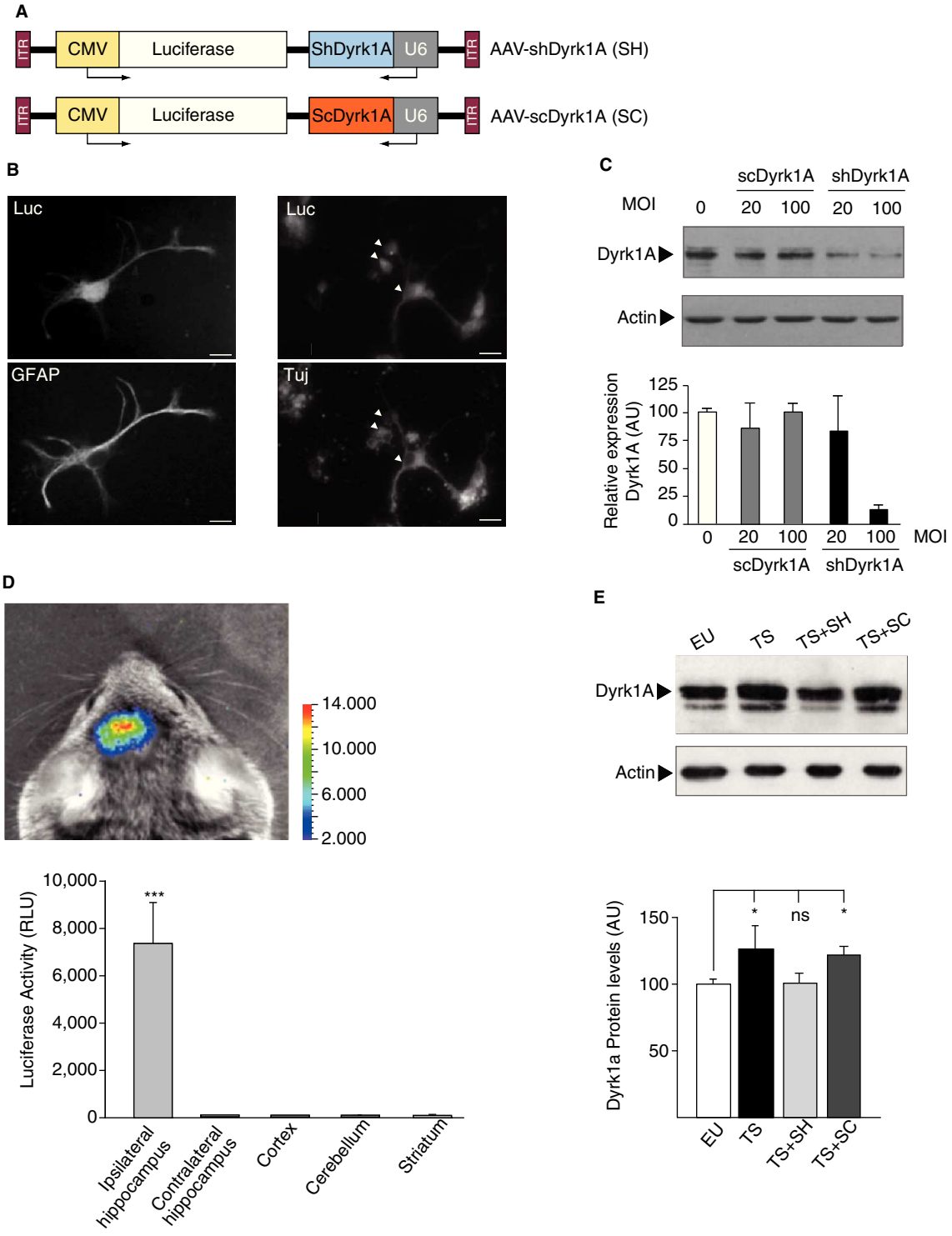

Figure 2 Validation of the capacity of the therapeutic virus, AAV2/1-shDyrk1A to normalize Dyrk1A expression in neuron cultures and in the Ts65Dn mouse hippocampus. A. Gene construction used to reduce Dyrk1A expression in the adeno-associated AAV2/1 virus. The construction also contains a gene marker of luciferase that will enable the viral infection to be traced both *in vitro* and *in vivo*. B. Immunofluorescence analysis of the AAV2/1-shDyrk1A capacity (detected by antibodies against luciferase marking, luc) to infect neurons (expressing the neuronal antigen "Tuj") and glial cells (expressing the glial antigen "GFAP"). C. Dyrk1A expression in primary cultures of neurons transduced with the SH virus or the SC control virus at different doses. D. Luciferase expression in Ts65Dn injected in the hippocampus with AAV2/1-shDyrk1A. The image on the left shows the bioluminescence signal *in vivo* (with the animal anesthetized), corresponding to the injected region. On the right, the histogram shows the luciferase activity in different dissected regions of the treated mouse brain, 15 days post-injection. E. Western blot analysis of Dyrk1A expression in the hippocampus of euploid (EU), trisomic (TS), and trisomic mice injected with the shDyrk1A (SH) or scDyrk1A (SC) virus.

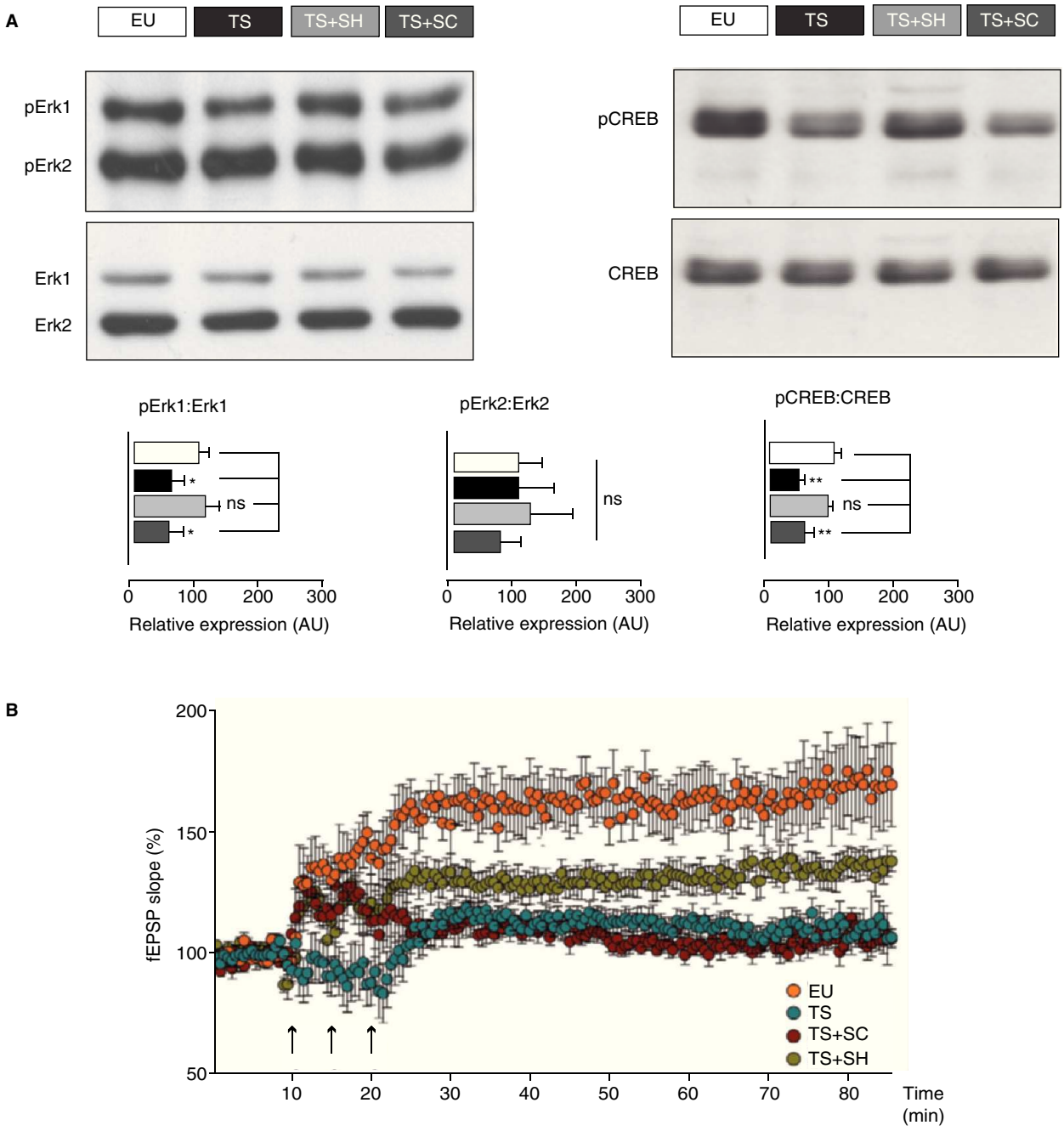

Figure 3 Effects of the administration of AAV2/1-shDyrk1A on the molecular and electrophysiological aspects of hippocampal function. A. Western blot analysis of ERK1,2 and CREB expression, as well as their phosphorylated forms (pERK1,2 and pCREB) in the hippocampus of euploid (EU), trisomic (TS), and trisomic mice injected with the shDyrk1A (SH) or scDyrk1A (SC) virus. The histogram represents the relationship between the expression of the active form (phosphorylated) and the non-phosphorylated form, of a mean of 3 independent experiments; **P*<.05; ***P*<.01. B. Electrophysiological study of the field excitatory postsynaptic potentials (fEPSP) in baseline conditions and after LTP induction (3 tetanization pulses, indicated with arrows, at 5 minute intervals).

defect in the execution of the task, which can be assessed by the distance that they cover until the platform is reached. On performing this experiment, it was also observed that the swimming pattern of the trisomic mice was thigmotactic, with a trajectory concentrated on the periphery of the pool. This was not a very efficient strategy to find the platform situated in the centre of a circular pool. In trisomic mice treated with the therapeutic virus, although they continued to show significant alterations in performing the task, a reduction in the thigmotactic navigation pattern was observed, indicative of a more efficient navigation strategy, probably as a result of an improved hippocampal function (fig. 4). Altogether, the strategy developed in the present study enables us to propose Dyrk1A as a key protein in cognition, and suggests its potential as a therapeutic target.

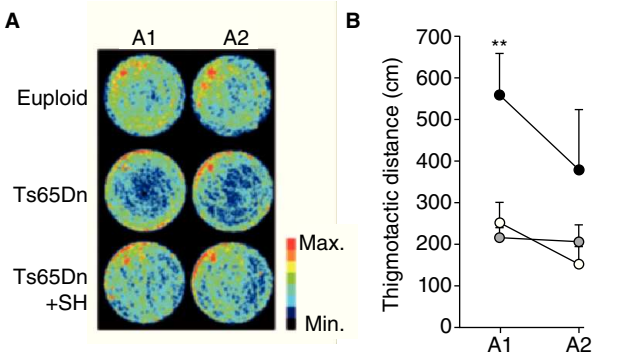

Figure 4 Analysis of hippocampus-dependent learning and memory capacity in mice treated with AAV2/1-shDyrk1A. A. Density map representing the occupation of the spaces in the Morris water maze, in the first acquisition of the task sessions. B. Analysis of thigmotactic behavior during the task, during the first two sessions of the water maze. The data represents the mean value ± standard error of the mean **P<.01.

Impact of the modulation of miRNAs in Ts65Dn mice

The Ts65Dn mice possess the miRNAs, miR-155 and miR-802 in trisomy, and their overexpression is observed in the hippocampus (fig. 5A). As a result of this, there may be genes regulated by these miRNAs, which are found to be under-expressed in the trisomic cell and may act, in part, as mediators of some of the cell alterations. In the present project, we designed a gene therapy strategy aimed at normalizing the miRNAs content in order to be able to assess their role in the cognitive defects of Ts65Dn mice. We generated lentiviral vectors, carriers of the d2EGFP gene marker (a variant of the green fluorescent protein) with adjacent sequences capable of modulating the miRNAs content (Lv-anti-miR155-802) (fig. 5B). These constructs are designed as such that they could bind the cellular miRNAs miR-155 and miR-802 to these sequences, thus reducing their contents, and giving rise to fluctuating d2EGFP protein levels depending on the miR-155 and miR-802 content. Thus, in the presence of the miRNAs there would be a low expression of d2EGFP, while this would be high in its absence. We observed that the transduction of cells with low miRNAs content using Lv-anti-miR155-802 gave rise to an elevated expression of the d2EGFP transgene, while the expression was much reduced in cells with a high content of miR-155 and miR-802 (fig. 5C). The intra-hippocampal injection of the generated virus in the hippocampus of Ts65Dn mice allows us to evaluate changes in the expression of the *Mecp2* gene, a target gene with recognition sequences for both miRNAs (fig. 6A). We observed that, it has been described,

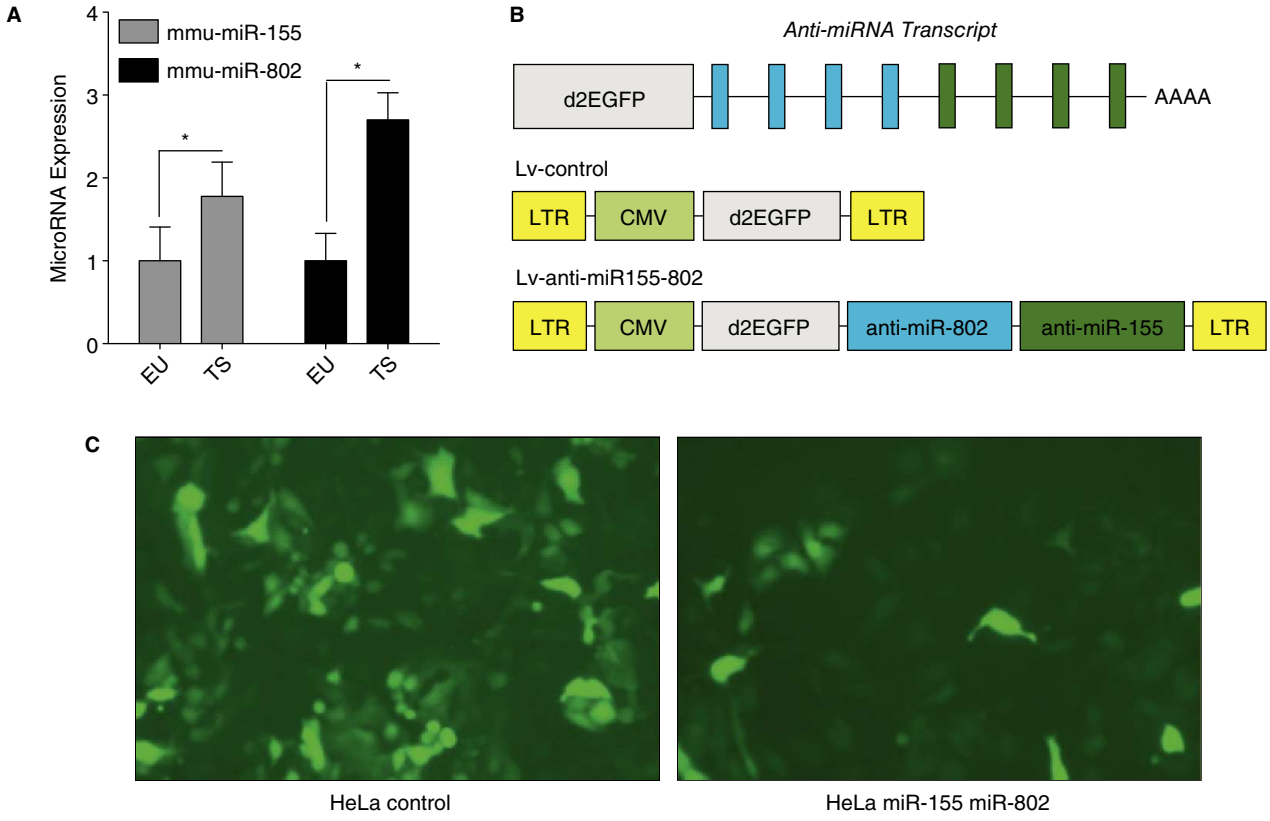

Figure 5 Validation of the capacity of Lv-anti-miR155-802 to act as a modulator of miR-155 and miR-802. A. Analysis of miR-155 and miR-802 expression in the hippocampus of euploid (EU) and Ts65Dn (TS) mice. The data represents the mean value ± standard error of the mean *P<.05. B. Scheme of the Lv-anti-miR155-802 lentivirus and Lv-control. C. Comparative analysis of d2GFP expression in control HeLa cells (low content of miRNAs) and HeLa miR-155 miR-802 (high content of both miRNAs) transduced with Lv-anti-miR155-802 at 10 MOI.

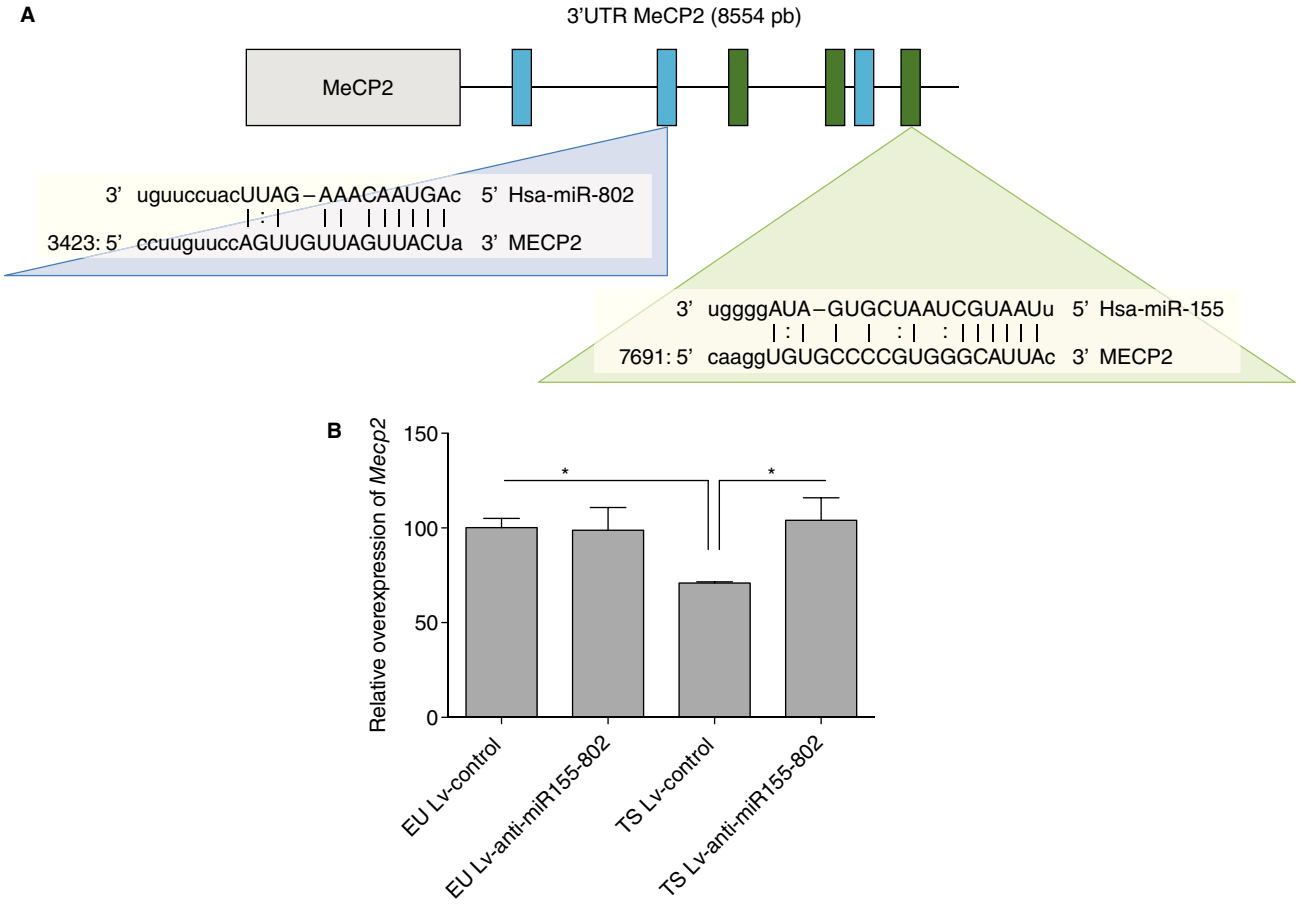

Figure 6 Effects of the administration of Lv-anti-miR155-802 on *Mecp2* expression in the injected hippocampus of Ts65Dn mice. A. Scheme of the distal region of the *MeCP2* gene (3'UTR region) where the potential recognition sequences for miR-155 y miR-802 are indicated. B. Analysis of *Mecp2* expression by quantitative PCR (RT-qPCR) in the hippocampus of euploid (EU), trisomic (TS), and trisomic mice injected with the Lv-anti-miR155-802 virus or Lv-control.

Mecp2 is decreased in the hippocampus of Ts65Dn mice[13]. The injection of Lv-anti-miR155-802 into the hippocampus of trisomic mice rescued the *Mecp2* expression, reaching values similar to those of a control mouse (fig. 6B). These data suggest that the reduced MeCP2 levels in the brain of the fetuses with DS could be due to the overexpression of miR-155 and miR-802. Defects generated by the loss of MeCP2 functions give rise to Rett syndrome, a disease associated to intellectual disability. Mouse models carrying Mecp2 mutations exhibit synaptic plasticity alterations and learning and memory impairments[14]. MeCP2 is a transcription factor of the family of proteins that bind to methylated cytosines in CpG islands, and its activity controls the expression of numerous genes. All these data suggest that either through the control of the expression of the miRNAs (miR-155 and miR-802) or through direct MeCP2 modulation, cognitive disabilities associated with these syndromic diseases could be attenuated. In this sense, it has been reported that a nutritional supplement of choline in the perinatal phase of mice deficient in *Mecp2* leads to neurochemical changes that preserve neuronal integrity[15]. It has recently been shown that a diet with a choline supplement given to Ts65Dn females improves the learning of Ts65Dn mice[16]. Taken together, these data suggest that the cognitive amelioration induced by choline supplement, could be associated with a recovery of the functions linked to the genes regulated by *Mecp2*. The gene therapy strategy developed in the present work shows MeCP2 as an important player in the cognitive alterations associated to DS and opens new intervention routes to improve intellectual disability.

Conclusion

The quality of life of individuals with DS has increased spectacularly in the last few years, thanks to the efforts of professionals in very distinct fields, and also to the support and involvement of the families. However, intellectual disability continues to be one of the main limitations, and for this reason, advances in the improvement of the cognitive aspects is one of the principal challenges in order to obtain the optimum integration of these individuals into society. In this sense, the identification of key genes (coding for proteins or miRNAs), in which deregulation has a negative impact on cognition is an important step towards the identification of potential therapies. In this context, our work proposes gene therapy as a research strategy to dissect target molecules with significative relevance in intellectual disability.

Acknowledgements

Fundació Catalana Síndrome de Down, for the granting of the XIII Ramon Trias Fargas award to this work. The funding of the work has been thanks to grants from the Fundación Jérôme Lejeune; Instituto de Salud Carlos III: FIS PI041559, PI 082038, CIBERER, IIS10/00014; CP10/00548; Generalitat de Catalunya: SGR091527, SGR091313; EU FP6-2005-LIFESCIHEALTH-6 ANEUPLOIDY no. 037627 y CureFXS ERare-EU/FIS PS09102673; y PEII10-0095-8727 de la Consejería de Educación Ciencia y Cultura (JCCM), BFU2011-26339/BFI Ministerio de Ciencia e Innovación (MICINN) and INCRECyT (JCCM and PCYTA).

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