Inducible and reversible breaching of the blood brain barrier by RNAi

John J. Rossi

Keywords: blood brain barrier; drug delivery; RNAi; shRNA

See related article in EMBO Mol Med (Campbell et al. (2011) EMBO Mol Med 3: 228–238)

Sequence-specific knockdown of gene expression is a goal that has been long sought by both basic and clinical investigators. In this regard, the discovery of RNA interference (RNAi) in Caenorhabditis elegans was immediately recognized as a potential breakthrough for studying gene function (Fire et al., 1998). These findings demonstrated that double-stranded (ds)RNAs are triggers for sequence-specific, post-transcriptional gene silencing via targeted degradation of messenger RNAs harbouring a complementary sequence to one of the two strands. Initially, it was thought that such post-transcriptional regulation of gene expression could not be achieved in mammalian systems due to the strong induction of interferon by dsRNAs. This potential restriction was short lived with the demonstration that endonuclease processed dsRNAs of 21–25 nucleotides in length, designated small interfering RNAs (siRNAs), were able to elicit sequence-specific degradation of mRNAs in mammalian cells without triggering interferon responses (Elbashir et al., 2001). These findings provided a huge impetus to develop RNAi as a therapeutic modality. The dream to selectively block the expression of deleterious proteins and treat formerly non-drugable diseases led to the rapid establishment of new biotech companies and branches of major pharmaceutical companies devoted to RNAi therapeutics.

The use of RNAi as a therapeutic has progressed more slowly than desired though, despite the fact that the design of siRNAs as drugs requires only knowledge of the sequence of the target. To date, there are no approved RNAi-based drugs, although many are in early stages of clinical trial. Part of the restriction in the development of siRNAs into therapeutics has been the difficulty of delivering relatively large, negatively charged macromolecules across cell membranes in the absence of some type of carrier molecule. On the other hand, there have been many successes using RNAi in functional genomics screens, where the power of this mechanism has greatly accelerated our knowledge of gene function and gene pathway interactions.

The most common approach for triggering RNAi in human cells is to utilize chemically synthesized, small dsRNA triggers, which need to be delivered to target cells/tissues with some chemical or physical assistance. An alternative approach is to use promoter expressed short hairpin (sh)RNAs in gene therapy settings. These short hairpins are processed by the RNAi machinery into siRNAs, which trigger sequence-specific degradation of target transcripts. Genes encoding Pol III transcribed shRNAs can easily be incorporated into viral vectors, which in turn can be used for transduction into various cell types. Despite the relative ease designing and expressing shRNAs, the use of promoter expressed shRNAs received somewhat of a setback when it was demonstrated that the high level expression of Pol III expressed shRNAs incorporated into an adeno associated viral (AAV) vector resulted in extreme liver toxicity and death of mice (Grimm et al., 2006). These startling findings put the brakes on the development of all RNAi-based drugs for a time, although the major problem was found to be toxicity related to high levels of expression of the shRNAs resulting in competition for cellular RNAi components and consequent off-target effects. This problem can be controlled by the choice of a promoter system for more moderate levels of shRNA expression. Another issue that had to be addressed was that of continued and often long-term expression of the shRNA, and hence continued knockdown of the therapeutic target. In some instances this is a desired feature, but in others transient target knockdown is preferred. To address this issue, several different versions of inducible Pol III promoters have been developed for the regulated expression of shRNAs. In particular, the Tet-on or Tet-off systems for transcription induction have been very popular. The tetracycline analogue doxycycline is commonly used.
as an inducer because it is relatively non-toxic in human cells.

To date, the major emphases for the applications of RNAi have been functional genomics and therapeutics. For the latter, the ability to knock down deleterious target gene expression has been the primary goal. A conceptually different and novel application of RNAi is described in this issue of EMBO Molecular Medicine (Campbell et al., 2011). These investigators explored the use of doxycycline-inducible shRNA expression for transient knockdown of an mRNA encoding the tight junction protein claudin-5, a component of the microneur-ovascularature that regulates the entry of small molecules through the blood brain barrier (BBB) and inner blood retinal barrier (iBRB) (Fig 1). The objective of these studies was to selectively modulate the levels of tight junction proteins to render these barriers transiently permeable to low molecular weight compounds that are used to treat neural or retinal disorders. Thus, the rationale was not to develop an RNAi therapeutic agent per se, but to use RNAi to allow the application of conventional drugs for treating diseases of the brain, CNS or eye.

Campbell et al first induced expression of an anti-claudin-5 shRNA under control of a doxycycline-inducible promoter in an AAV vector (AAV 2/9 serotype), which was sub-retinally injected in the eyes of C57/BL16 mice, using doxycycline. Analyses of the levels of claudin-5 mRNA in the retinal layers of the treated animals showed a statistically significant reduction of claudin-5 expression in the vascularized retinal layers. Using a GFP-expressing vector, they qualitatively evaluated the extent of transduction and the types of cells that were transduced, which demonstrated that trans-
duction by this vector was confined solely to the retinal tissue and did not affect the optic nerve.

Having established the patterns of AAV 2/9 transduction and the efficient doxycycline-induced knockdown of claudin-5, Campbell et al. next sought to test the size limitations for small molecules crossing these barriers. For this, they compared the traverse of two different size agents, Gd-DTPA (742 Da) and microperoxidase (1881 Da) across the iBRB. After retinal injection of the AAV construct and doxycycline administration, MRI showed that the smaller compound could cross the selectively opened iBRB while the larger one was excluded.

To demonstrate that similar effects could be achieved at the BBB, they systemically administered the AAV 2/9-anti-claudin-5 shRNA followed by doxycycline induction. This procedure generated the same phenotype in the iBRB as direct retinal injection but in addition resulted in claudin-5 reduction in brain capillaries. In addition, direct injection of the vector into the right hippocampus followed by doxycycline treatment resulted in localized knockdown of claudin-5 along with increased permeability of Gd-DTPA into this area of the brain. In all cases, when doxycycline was removed from the drinking water, the BBB and iBRB permeability barriers were restored, demonstrating the reversibility of the claudin-5 knockdown with this system of shRNA expression.

To demonstrate the therapeutic value of their approach, Campbell et al used the light-induced retinal degeneration model, in which they previously investigated the therapeutic effect of opening the iBRB to small therapeutic drugs (Campbell et al., 2009). Since calpain is central to light-induced photoreceptor cell degeneration, ALLM (401 Da), a potent inhibitor of calpain I, II, cathepsins and the proteasome, can be used to prevent the degeneration once it is able to reach the damaged site. Albino mice were treated with either the doxycycline-inducible AAV 2/9 anti-claudin-5 shRNA or a control AAV-shRNA, fed doxycycline and then subjected to light-induced photoreceptor cell degeneration and treatment with intra-peritoneal injected ALLM. Using this model, a 70% protection in the retinas of mice treated with the inducible anti-claudin-5 shRNA when compared to the control shRNA-treated retinas was obtained. Similarly, choroidal neovascularization caused by an intense laser burn to the back of the eye and triggered through localized increases in VEGF could be alleviated by two drugs (17-AAG [585 Da] and Sunitinib malate [532 Da]), which were only able to cross the iBRB because of the claudin-5 down-regulation. Importantly, being able to readily target drugs that block neovascularization to the macula could be a major breakthrough in the treatment of wet age-related macular degeneration. Since AAV can stably transduce non-dividing cells for long periods of time, it is conceivable that only a single injection of the AAV–shRNA vector would be necessary for long-term treatment of this debilitating disease.

...clearly highlights a novel use of the RNAi pathway for therapeutic application in humans.

The exciting aspect of this study is that a localized reduction in just one of the tight junction proteins of the BBB or iBRB can permeabilize the retina, CNS or brain to small molecular weight drugs that normally do not readily cross these barriers. There are many potential disease applications for which small molecule drugs can be efficacious if delivered in sufficient quantities to the CNS or retina, including AMD, Alzheimer’s, other neurodegenerative diseases and even brain cancer. The caveat to this approach is the absolute requirement to make the breaching of the BBB or iBRB short lived and reversible. Prolonged or too extensive opening of the barrier could subject the CNS to lethal penetration of normally blocked macromolecules. In that regard, the use of an inducible shRNA is attractive, but the use of doxycycline or other tetracycline derivatives in humans may have limitations due to the widespread presence of this antibiotic in poultry and the allergic reactions many people develop towards tetracycline-based antibiotics. To this end, there are other possible inducible systems that could be adapted for the transient knockdown of BBB or iBRB tight junction proteins. In the final analysis, this study has great merit because it demonstrated both the feasibility and efficacy of such a therapeutic approach, and clearly highlights a novel use of the RNAi pathway for therapeutic application in humans.

The authors declare that they have no conflict of interest.

References
Campbell M et al (2009) Proc Natl Acad Sci USA 106: 17817-17822
Campbell M et al (2011) EMBO Mol Med DOI: eemmm201100126
Elbashir SM et al (2001) Nature 411: 494-498
Fire A et al (1998) Nature 391: 806-811
Grimm D et al (2006) Nature 441: 537-541