siRNA-mediated Silencing of Angiotensin Type 1a Receptor ($AT_{1a}R$) Splice Variants Reveals that $AT_{1a}R$ Expression is Translationally Controlled by $AT_{1a}R$ Alternative Splicing in Rat Aortic Smooth Muscle Cells (RASMC)

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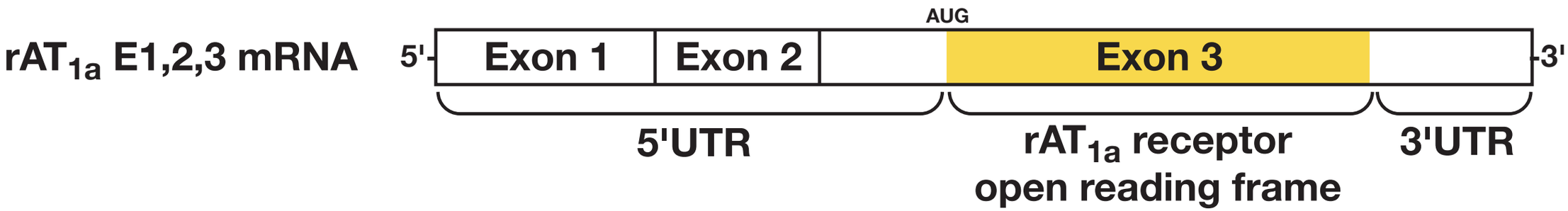
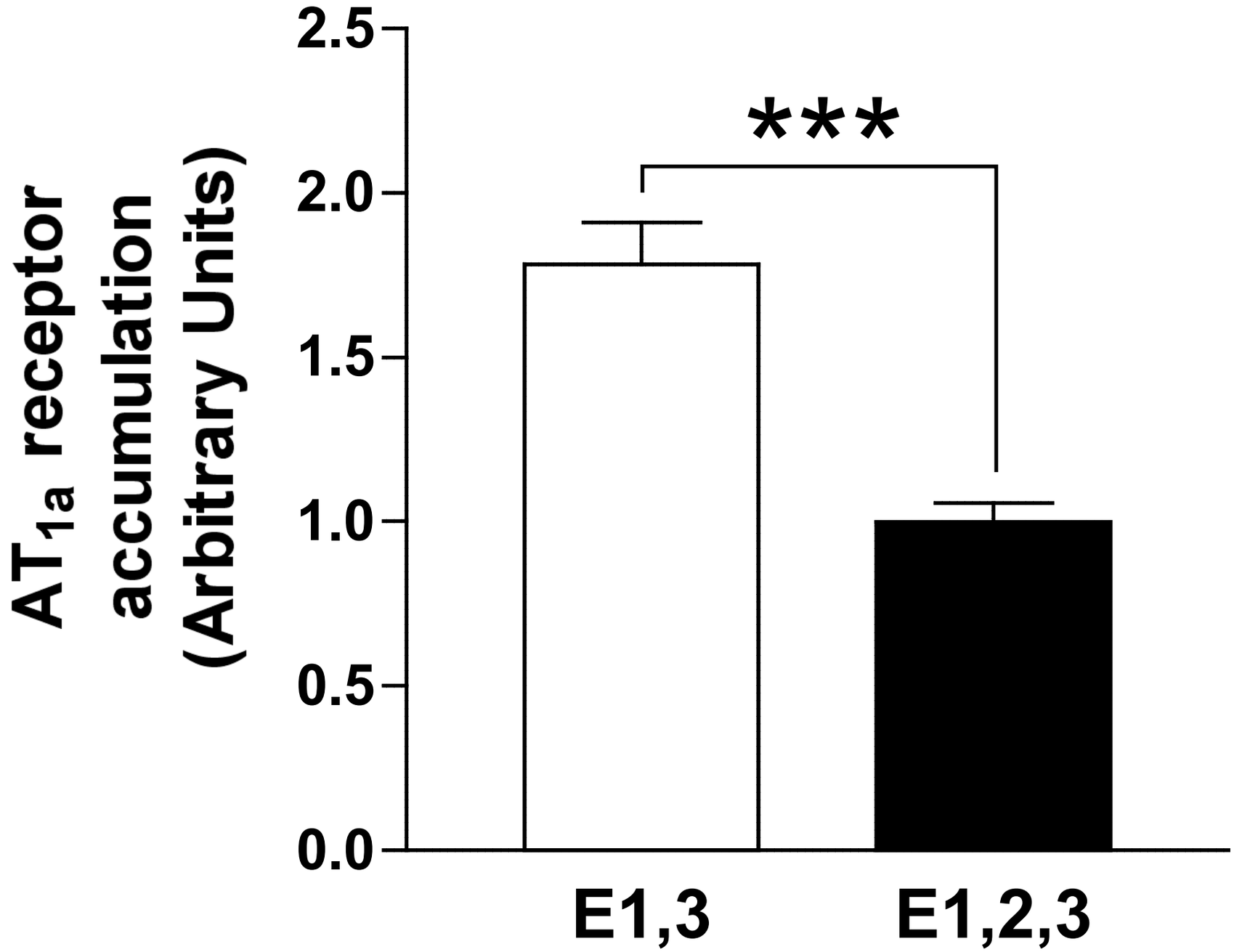


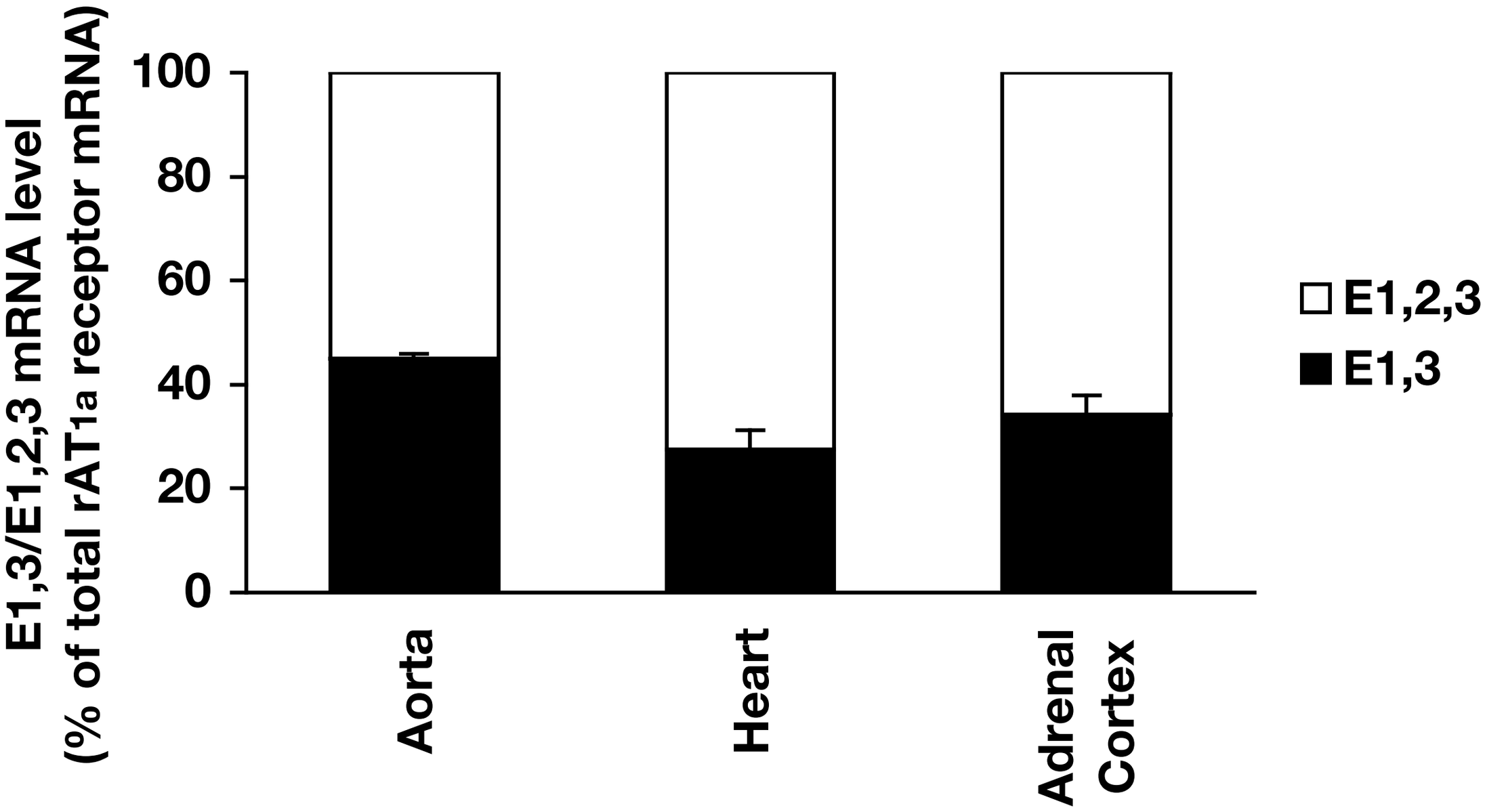
Two rat $AT_{1a}R$ splice variants encode an identical receptor protein

One splice variant (E1,2,3) contains all three exons while the other (E1,3) contains only exons 1 and 3. Since the entire coding region is contained within exon 3 the two splice variants differ only in their 5' untranslated regions (UTRs) and encode identical receptors.



E1,3 mRNA is more efficiently translated than E1,2,3 *in vitro*

Translation of E1,3 mRNA in a wheat germ lysate *in vitro* translation assay results in significantly increased accumulations of $AT_{1a}R$ mRNA than translation of an equal molar quantity of E1,2,3 mRNA.



E1,2,3 mRNA is more abundant than E1,3 mRNA

In the rat, E1,2,3 mRNA is more abundant than E1,3 mRNA in all tissues tested, making up between 55% and 73% of the total $AT_{1a}R$ mRNA population.

These observations raise two questions about the role of splicing in $AT_{1a}R$ expression:

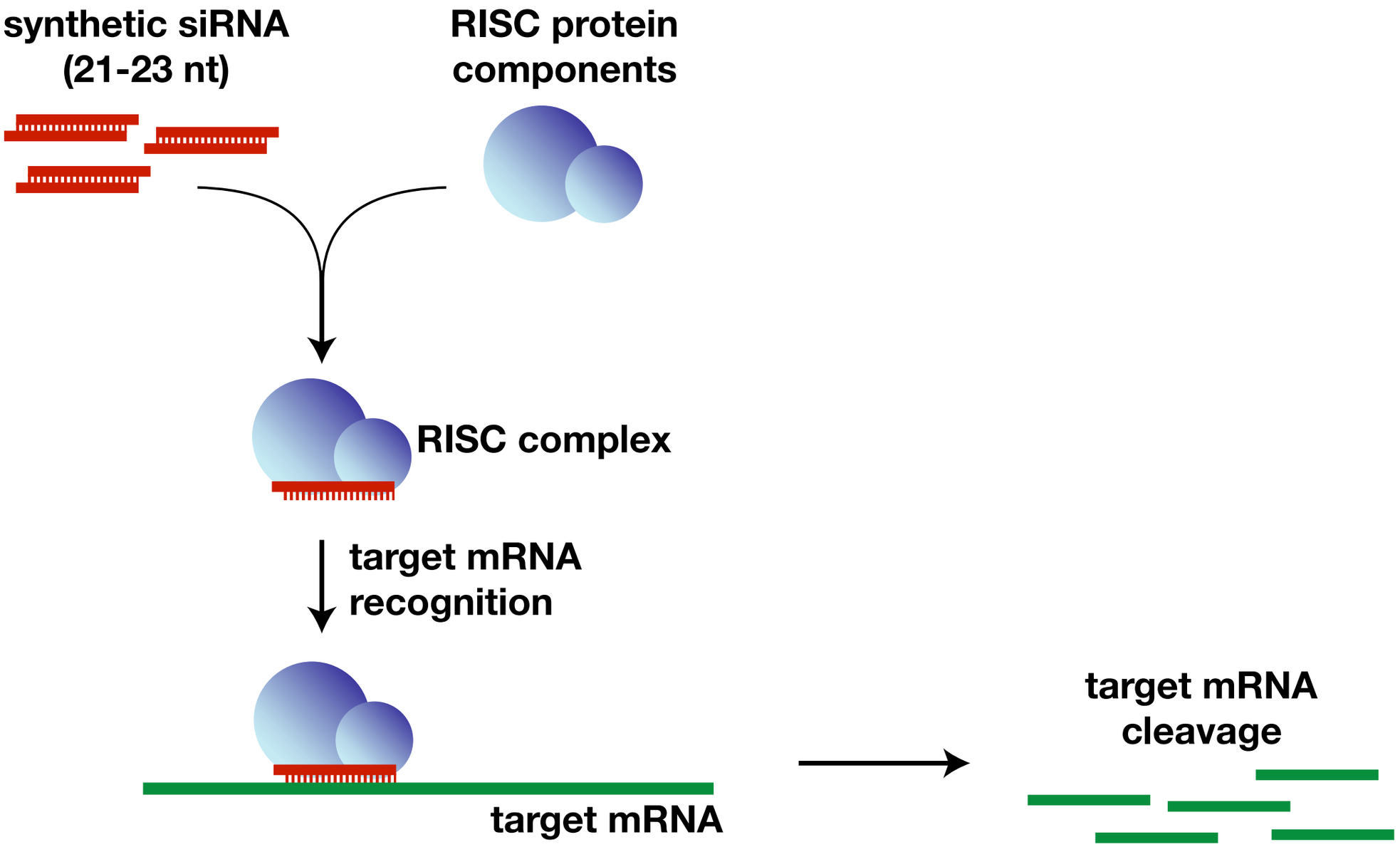
- Is the E1,3 splice variant translated more efficiently than the E1,2,3 splice variant in cells which endogenously express the $AT_{1a}R$?

- If E1,3 mRNA is more efficiently translated but less abundant than E1,2,3 mRNA, what is the relative contribution of the two splice variants to $AT_{1a}R$ expression?

We used RNA interference (RNAi) technology to answer these questions:

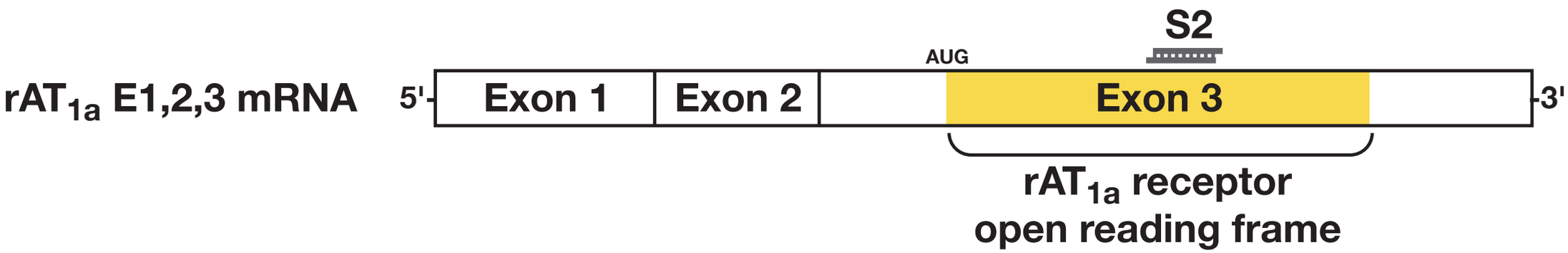
- Use small interfering (si)RNA-mediated RNAi to selectively knockdown the E1,3 splice variant in Rat Aortic Smooth Muscle Cells (RASMC).

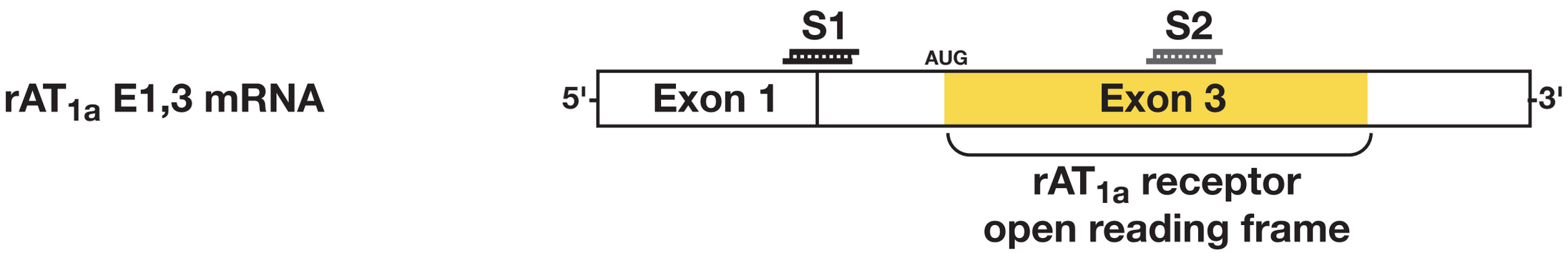
- Measure the effect of E1,3 knockdown on AT_1 receptor binding.



RNAi can markedly reduce the expression of a target mRNA and protein

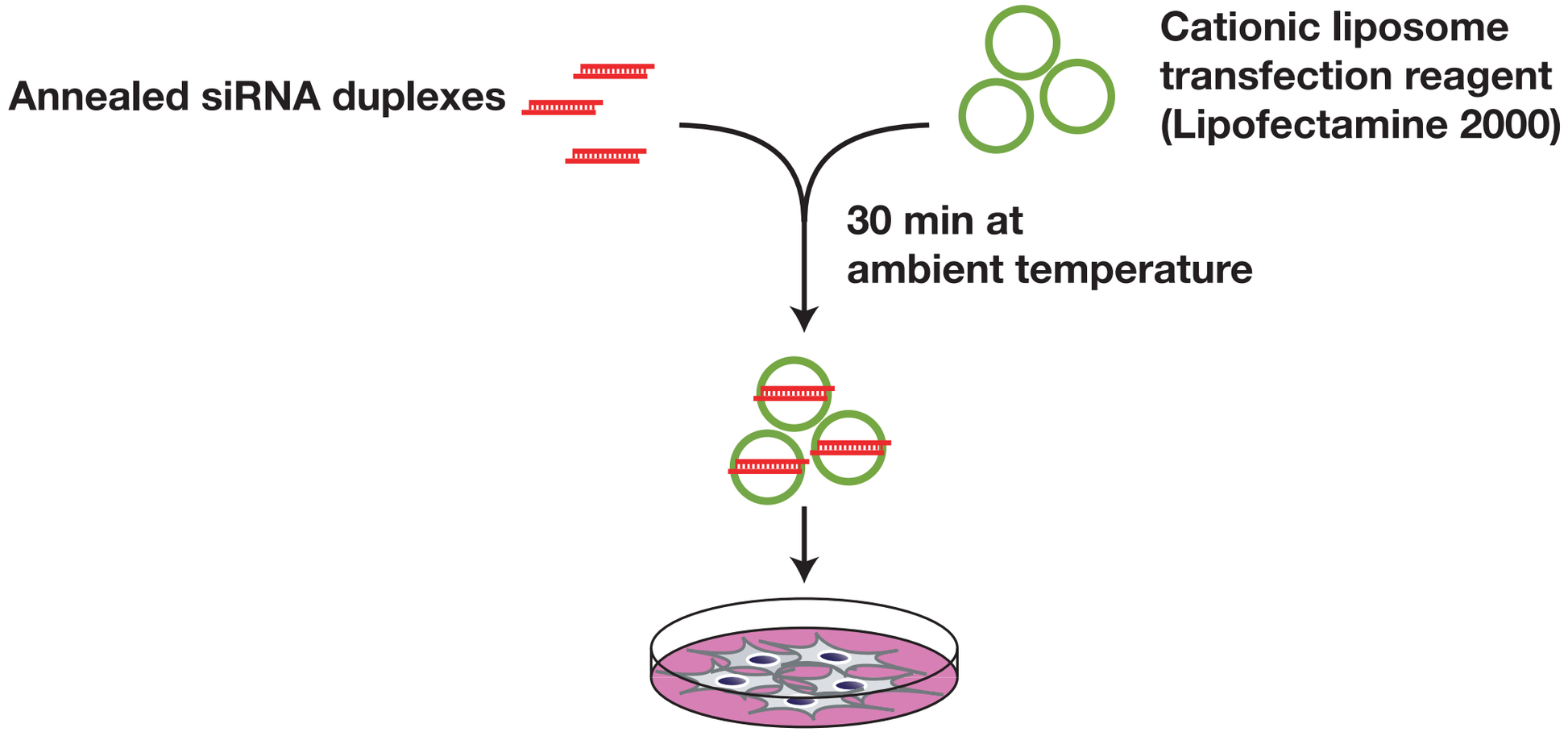
Recent studies have shown that transfection of mammalian cells with short double-stranded RNA results in the selective degradation of mRNA containing homologous sequences by an RNA-induced silencing complex (RISC complex). Subsequently, expression of the target protein is reduced.





Design of siRNA duplexes targeting $AT_{1a}R$ splice variants

Two siRNAs targeting either the E1,3 splice variant only (S1) or both the E1,3 and E1,2,3 splice variants (S2) were selected. BLAST searching showed these sequences matched no other mammalian genes. Additionally, two control sequences were chosen for use in CHO cells and RASMC.

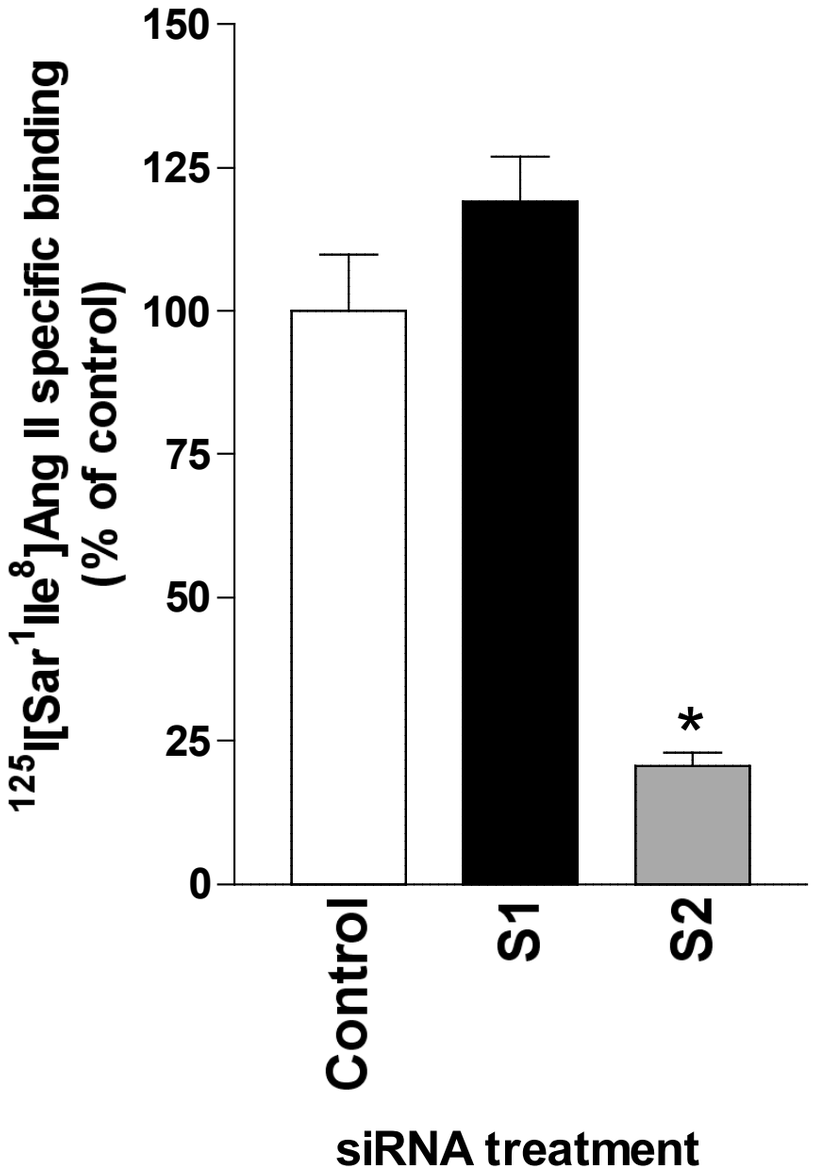


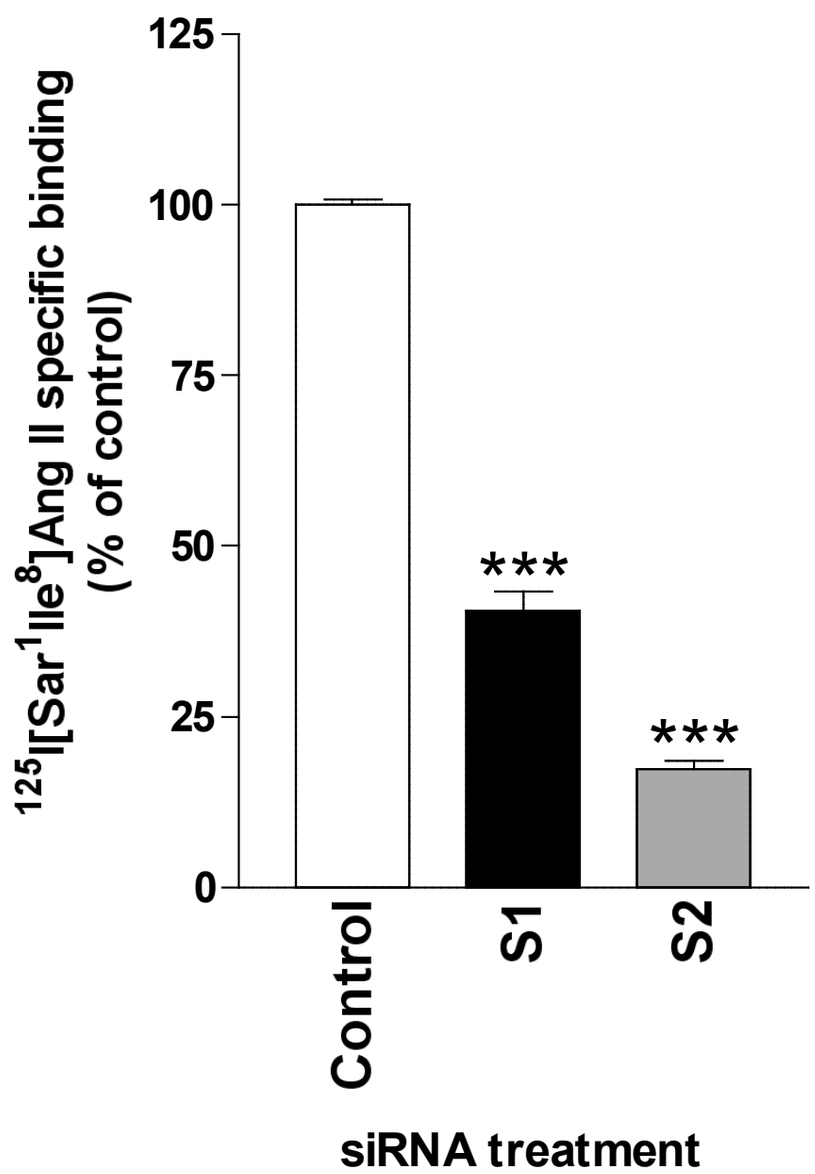
48 h at 37°C;
95% O_2, 5% CO_2

AT_1 receptor Binding Assay
or
RNA extraction for rAT_{1a} receptor mRNA quantification by real-time PCR

Cells were transfected with siRNA using lipofectamine 2000

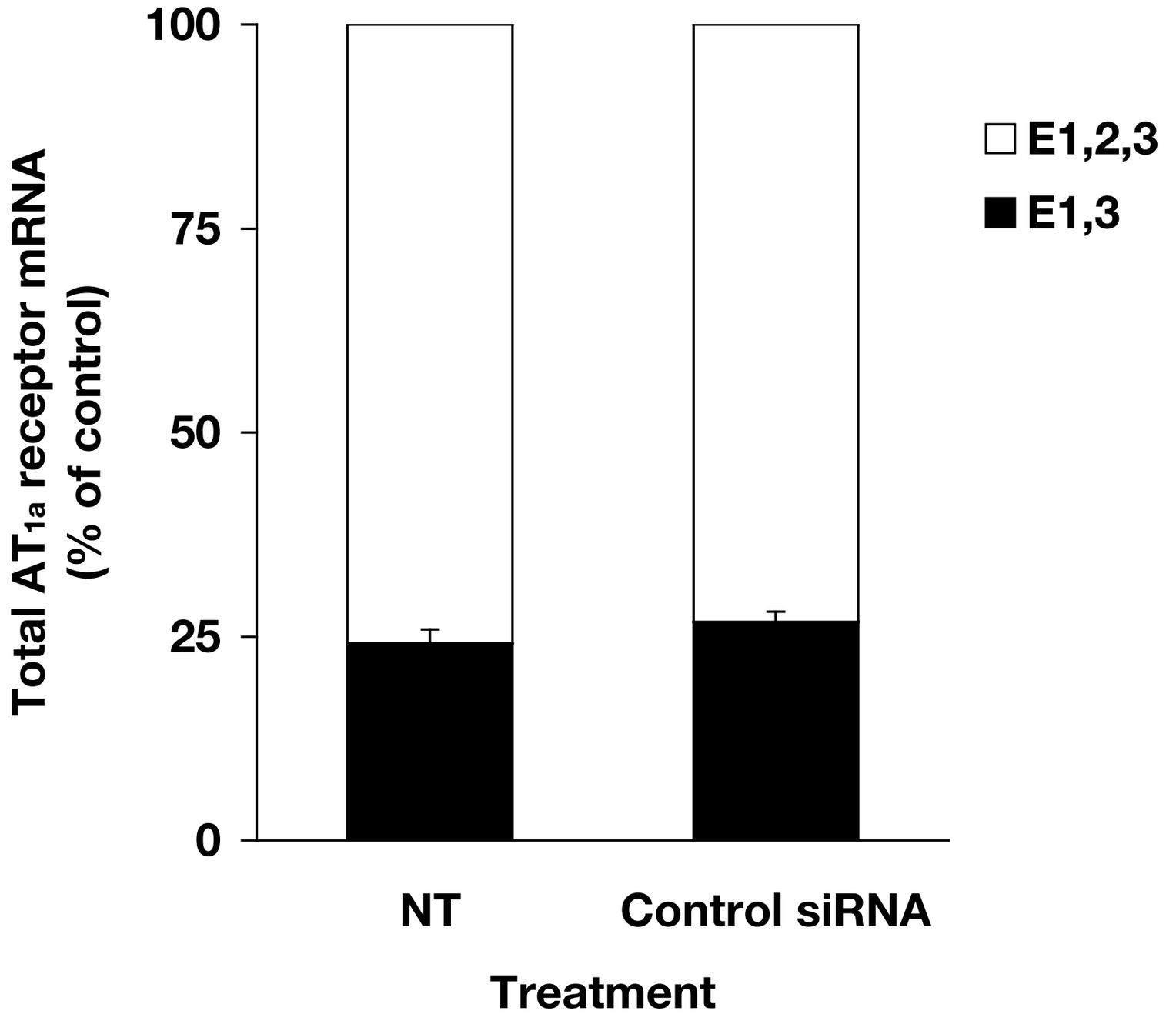
siRNA was chemically synthesized by Qiagen Inc and transfected into both CHO cells and RASMC using the liposomal transfection reagent lipofectamine 2000 (Invitrogen).





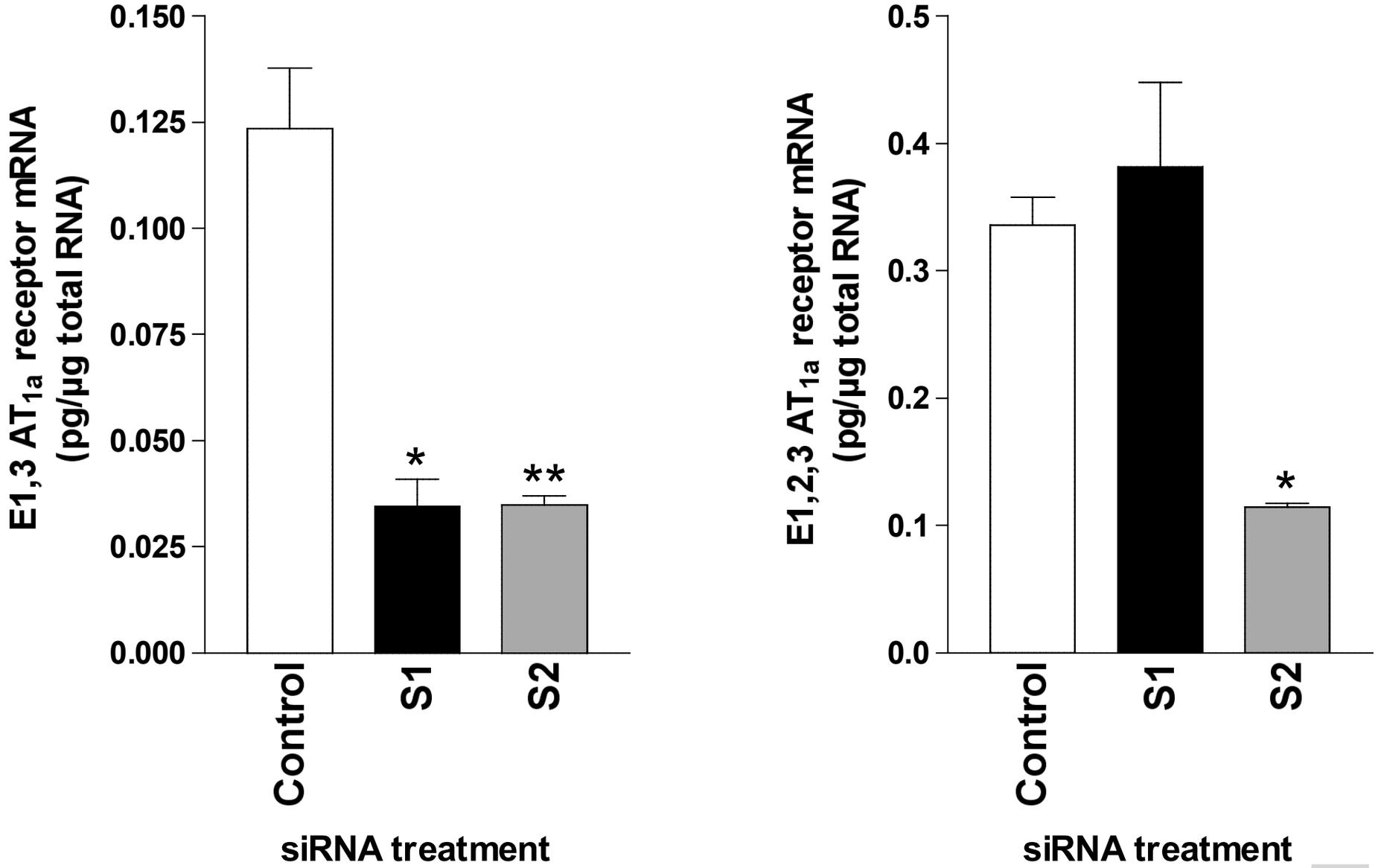
Validation of siRNA designs in CHO cells expressing E1,3 and E1,2,3 mRNA

The selectivity of S1 and S2 siRNA was tested in Chinese Hamster Ovary cells stably expressing either the E1,3 or E1,2,3 splice variant. S1 siRNA was found to selectively target E1,3 mRNA, while S2 targeted both E1,3 and E1,2,3 mRNA.



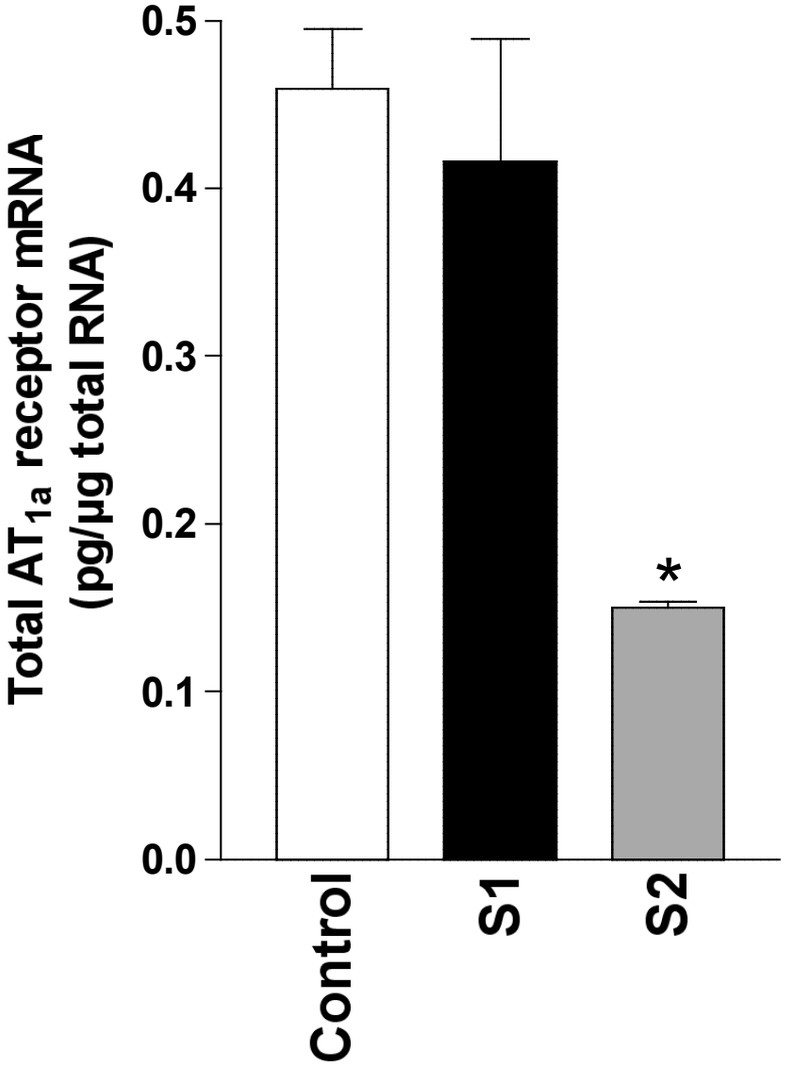
E1,2,3 mRNA is more abundant than E1,3 mRNA in RASMC

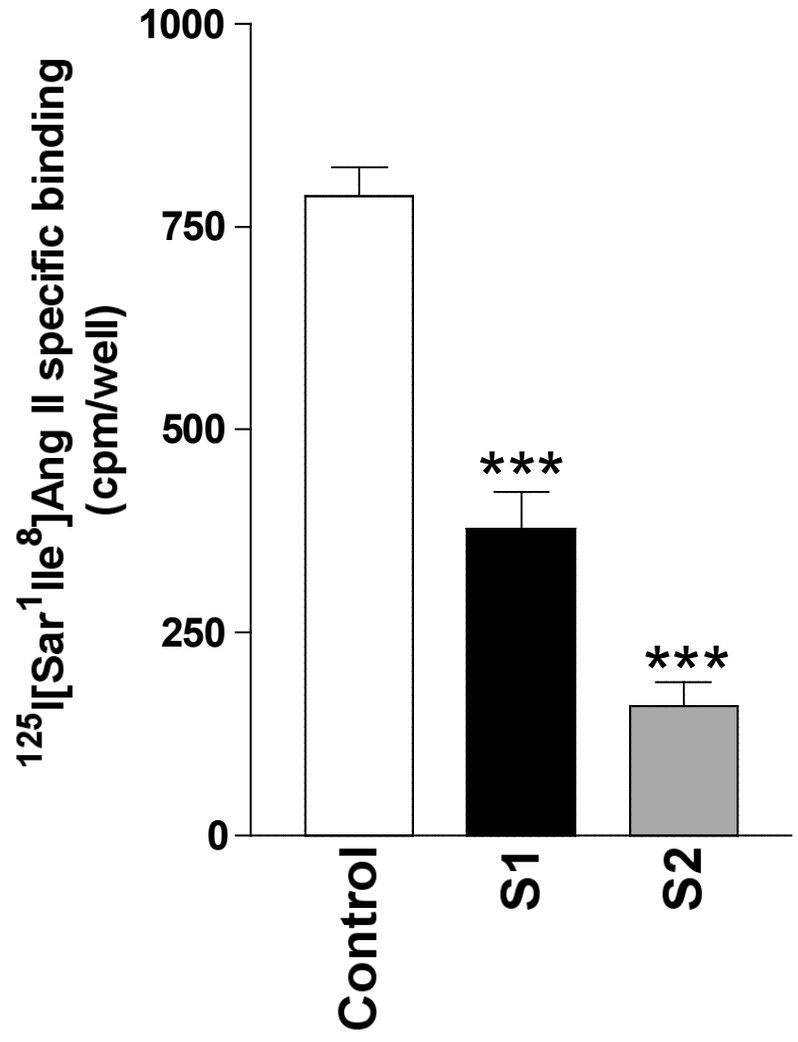
The E1,2,3 splice variant was found to make up three quarters of the total $AT_{1a}R$ mRNA population. The relative proportion of the two splice variants was unaffected by treatment with control siRNA.



S1 siRNA treatment reduced E1,3 but not E1,2,3 levels in RASMC

48 h following siRNA treatment cells treated with S1 siRNA showed marked reductions in E1,3 mRNA but not E1,2,3 mRNA. Treatment with S2 siRNA caused significant reductions in levels of both E1,3 and E1,2,3 mRNA.





S1 siRNA induces a disproportionately large reduction in AT_1R binding

Because E1,3 mRNA makes up only 24% of AT_{1a}R mRNA S1 siRNA treatment induced only a small reduction in total AT_{1a}R mRNA. In contrast the same treatment induced a marked reduction in binding. S2 siRNA treatment caused similar reductions in AT_{1a}R mRNA and binding.

Summary (1)

These results demonstrate that it is possible to use siRNA-mediated RNAi to specifically knock down the E1,3 $AT_{1a}R$ mRNA splice variant while leaving the E1,2,3 splice variant intact.

The data show that E1,3 mRNA is more efficiently translated than E1,2,3 mRNA in cells endogenously expressing the $AT_{1a}R$.

Furthermore, the results suggest that at least half of the $AT_{1a}R$ population is translated from a splice variant which makes up less than a quarter of total $AT_{1a}R$ mRNA.

Summary (2)

These data suggest that alternative splicing could play a role in the regulation of $AT_{1a}R$ expression. There are two potential mechanisms by which splicing might contribute to $AT_{1a}R$ expression:

- Alterations in the relative proportions of E1,3 and E1,2,3 mRNA could alter the level of expression of the receptor
- E1,3 and E1,2,3 mRNA may be differently regulated by cytosolic binding proteins as a consequence of differences in the structure of their 5'UTRs.

Potentially, aberrant regulation of $AT_{1a}R$ splicing could lead to pathophysiological states.