#30

Controlled, Riboswitch-Regulated Gene and Cell Therapy Targeting HER2+ Tumors

Xuecui Guo, Zhaojing Zhong, George Wang, Jae Gyun Oh, Alexandria J. Forbes

Gene Regulation, MeiraGTx, New York, NY 10016, USA

MEIRAGTx

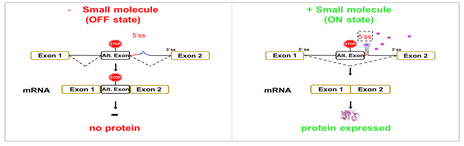
Abstract

Controlled expression of delivered transgene is important for both gene and cell therapies to be efficacious and safe. For this purpose, we have developed a platform for transgene expression control in response to a small molecule inducer. The platform utilizes a mammalian synthetic riboswitch with an aptamer sequence, which binds the small molecule inducer in the context of an alternative exon flanked by 5′ and 3′ introns. This gene regulation cassette is embedded in the coding sequence (CDS) of the transgene, creating an alternative splicing regulated gene expression platform, in which the alternative splicing is modulated by aptamer/small molecule binding. In the absence of the small molecule inducer (aptamer ligand), the splice site sequence at the 3′ end of the alternative exon is accessible, allowing splicing of the alternative exon into the transgene mRNA. The inclusion of the alternative exon generates an in-frame stop codon in the transgene mRNA, leading to mRNA degradation and no protein expression. In the presence of the small molecule inducer, binding of the inducer to the aptamer causes aptamer RNA conformational change that sequesters the splice site, leading to exclusion of the alternative exon from the mRNA, generation of functional mRNA and expression of transgene product. The other component of this gene regulation platform—the small molecule inducers—were designed to specifically bind to the aptamer sequence with high oral bioavailability and a favorable safety profile.

We have applied this novel gene regulation platform to regulation of the expression of various therapeutic genes in response to a small molecule inducer. One of these therapeutic genes is the anti-HER2 antibody. *In vitro*, the expression of anti-HER2 antibody is induced in response to a small molecule inducer in a dose dependent manner. Likewise, *in vivo* (using AAV-mediated transgene delivery), anti-HER2 antibody expression was also dose dependently induced in response to orally administered small molecule inducer. Further, the induced anti-HER2 antibody halted the progression of HER2$^+$ tumors. To further develop treatment targeting the HER2 antigen, we incorporated a riboswitch gene regulation cassette into the coding sequence of a chimeric antigen receptor (CAR) targeting the HER2 antigen. The expression level of CAR on the cell surface is dependent on the presence of the small molecule inducer. During the generation of CAR-T cells, the small molecule inducer is not present in the cultures and the CAR is not expressed—largely preventing tonic signaling and tonic signaling-induced T cell differentiation and exhaustion. We have shown in our preclinical studies that the T cells expressing riboswitch-controlled CAR (RiboCAR-T) are enriched in naïve and stem cell-like memory T cell populations. RiboCAR-T cells' anti-tumor activity can be remotely controlled by orally administered small molecule inducers and is superior to T cells expressing CAR constitutively in eliminating the HER2$^+$ tumor cells.

Aptamer-modulated alternative splicing riboswitch regulates multiple therapeutic genes

Gene expression is controlled by riboswitch in response to small molecule



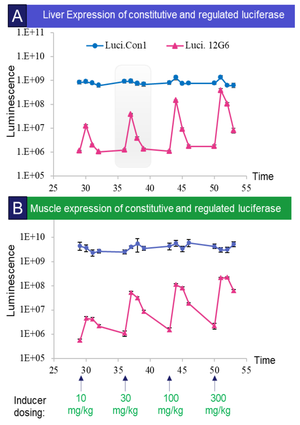
Aptamer is inserted in the downstream intron of an Intron-AltExon-intron cassette within the cDNA of transgene.
In the absence of small molecule (left panel):
Alt. exon 5′ ss is accessible
Alt. exon is included
No protein expressed
In the presence of small molecule (right panel):
Alt. exon 5′ ss is sequestered
Alt. exon is skipped
Protein expressed

Riboswitch-controlled therapeutic genes

Therapeutic Antibodies	Cell Therapy	Therapeutic Hormones / Cytokines / Peptides	Gene/RNA Editing Nucleases

• Anti-PCSK9
• Anti-VEGFR2 (eye)
• Anti-Amyloid
• Anti-IL-17
• Anti-IL4Ra
• Anti-PD1
• Anti-Myostatin
• Anti-HER2
	• RiboCAR:
	  • Anti-CD19
	  • Anti-PSMA
	  • Anti-mesothelin
	  • Anti-HER2
		• Epo
		• hGH
		• PTH
		• Gut peptide combinations: GLP1, GIP, PYY, Glucagon, Amylin
			• Cas9
			• CasRx

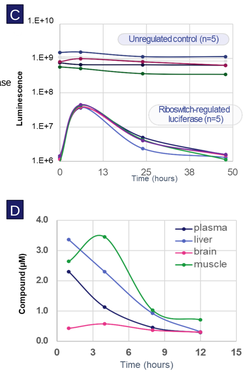
In vivo delivered, riboswitch-regulated transgene is precisely controlled by orally administered small molecule inducer



- **Luci.Con1:** constitutive expression – no regulation cassette
- **Luci.12G6:** identical construct to Luci. Con1 but with regulation cassette

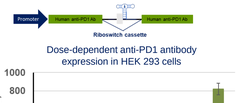
Liver Expression: tail vein injection AAV-Luciferase
Muscle Expression: direct I.M. injection of AAV-Luciferase

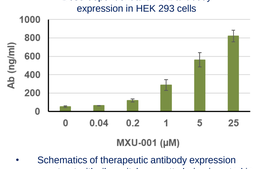
- **Figures A and B** Single oral dose of small molecule results in dose responsive expression of Luciferase from Luci. 12G6 in comparison to constitutive expression with Luci con1 in both tissues.
- **Figure C** is a blow up of the individual mice in each cohort at 30mg/kg dose in Figure A – indicated by the gray box in Figure A. This illustrates the restriction of expression with small molecule dosing between individual mice. On a mouse-by-mouse basis there is about 0.4 log range of expression between the 5 mice from constitutively active unregulated control construct. In contrast, induced expression in response to oral inducer is tightly controlled, expression is limited by the dose of the oral small molecule such that each mouse receiving the same oral dose expresses the same level of luciferase.
- **Figure D** shows the differential tissue distribution of the small molecule inducer when delivered orally. The different shapes of the induced luciferase curves in Fig. A (liver) and Fig. B (muscle) precisely reflect the different tissue biodistribution to liver and muscle. A sharp peak for liver (blue) vs. slow accumulation and then exit from muscle (green). Tissue distribution of orally delivered small molecule inducer shows short term accumulation in muscle, whereas clearance from liver is linear. This is directly reflected in the different profiles of regulated luciferase expression in the liver and muscle (Figures A and B).



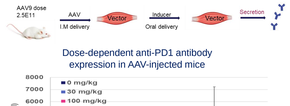
Riboswitch-controlled, small molecule-inducible expression of therapeutic antibodies

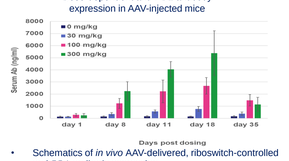
Riboswitch-controlled, small molecule dose-dependent expression of anti-PD1 antibody *in vitro* and *in vivo*





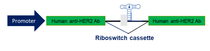
- Schematics of therapeutic antibody expression construct with riboswitch cassette being inserted in the coding sequence of anti-PD1 antibody.
- Transfected HEK 293 cells were treated with small molecule inducer MXU-001 at different concentrations.
- Antibody expression was evaluated by ELISA.
- Antibody expression was induced in response to riboswitch inducer in a dose dependent manner.

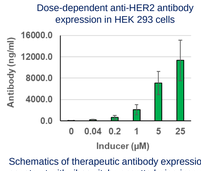


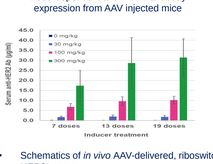


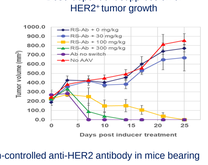
- Schematics of *in vivo* AAV-delivered, riboswitch-controlled anti-PD1 antibody expression.
- Mice were injected I.M. with AAV vector containing anti-PD1 antibody with riboswitch.
- 4 weeks post AAV I.M injection, mice were treated with riboswitch small molecule inducer MXU-001.
- Antibody expression in serum was evaluated by ELISA.
- Antibody expression was induced in response to riboswitch inducer in a dose dependent manner.
- Antibody expression attenuates towards baseline post withdrawal of small molecule inducer.

Riboswitch-controlled, AAV-vectorized anti-HER2 antibody halts tumor progression *in vivo*









- Schematics of therapeutic antibody expression construct with riboswitch cassette being inserted in the coding sequence of anti-HER2 antibody.
- Transfected HEK 293 cells were treated with small molecule inducer MXU-001 at indicated concentrations.
- Antibody expression was evaluated by ELISA.
- Antibody expression was induced in response to riboswitch inducer in a dose dependent manner.

- Schematics of *in vivo* AAV-delivered, riboswitch-controlled anti-HER2 antibody in mice bearing HER2$^+$ tumor.
- Mice were injected I.M. with AAV vector containing anti-HER2 antibody with or without riboswitch.
- 4 weeks post AAV I.M injection, mice were inoculated with HER2$^+$ tumor cells subcutaneously.
- 7 days post tumor inoculation, mice were treated with riboswitch small molecule inducer MXU-001 at the indicated doses.
- Antibody expression in serum was evaluated by ELISA.
- Antibody expression was induced in response to riboswitch inducer in a dose dependent manner.
- Induced anti-HER2 antibody inhibited tumor growth and halted tumor progression in response to small molecule inducer treatment.

Highly dynamic inducible and reversible expression of Riboswitch CAR

CAR construct with or without riboswitch

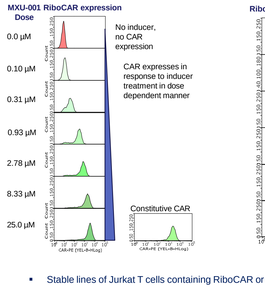
Constitutive anti-CD19 CAR (ConstCAR)

Riboswitch anti-CD19 CAR (RiboCAR)

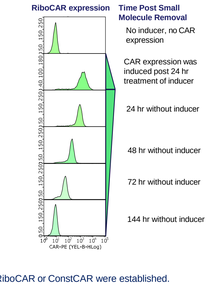
RiboCAR integrated into TRAC locus

- Schematics of CAR constructs. Riboswitch gene regulation cassette is inserted in the coding sequence of the CAR molecule.
- Schematics of RiboCAR expression cassette was targeted to TRAC locus by CRISPR/Cas9-mediated knock-in.

CAR expression activated in response to small molecule dose



CAR expression switches off when small molecule removed



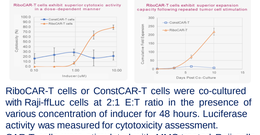
- Stable lines of Jurkat T cells containing RiboCAR or ConstCAR were established.
- Jurkat T cells were treated with MXU-001 at different doses.
- Jurkat T cells were stained with anti-FMC63 antibody 48 hours after MXU-001 treatment
- CAR expression was measured by flow cytometry.
- CAR expression was induced by MXU-001 in dose-dependent manner.
- CAR expression declined to undetectable levels post small molecule removal.

RiboCAR-T cells targeting CD19 are more potent than ConstCAR-T cells in anti-tumor activity

RiboCAR-T cells are enriched in naive/stem cell-like memory phenotype and display reduced exhaustion marker

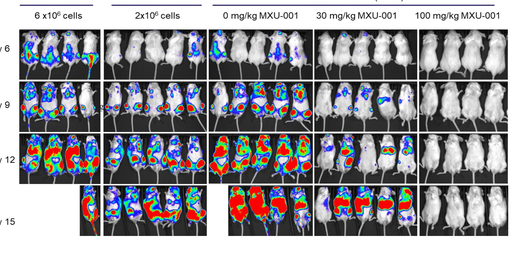
- Expression of CD62L and CD45RA (left) and CD39 (right) on RiboCAR-T and ConstCAR-T cells was measured by FACS 6 days post CRISPR/Cas9 transfection and AAV transduction.
- RiboCAR-T cells have a more naive/stem cell memory T cell phenotype in culture compared to ConstCAR-T cells.
- RiboCAR-T cells have reduced exhaustion compared to ConstCAR-T cells.

RiboCAR-T cells exhibited superior cytotoxicity and expansion capacity following repeated tumor cell stimulation



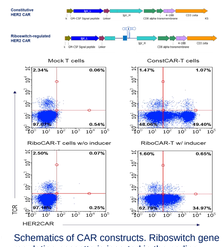
- RiboCAR-T cells or ConstCAR-T cells were co-cultured with Raji-ffLuc cells at 2:1 E:T ratio in the presence of various concentration of inducer for 48 hours. Luciferase activity was measured for cytotoxicity assessment.
- CAR-T cells were stimulated with MMC-treated Raji cells at 1:1 ratio in the presence of inducer. Stimulation was repeated every 3 days under the same conditions.

RiboCAR-T cells are more potent in anti-cancer activity *in vivo*



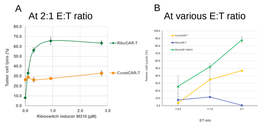
- Raji-ffLuc cells were injected I.V. into NSG mice and small molecule inducer was dosed orally and daily at the indicated doses, starting the day before CAR-T cells were injected.
- RiboCAR-T cells' anti-tumor activity was remotely controlled by oral inducer.
- RiboCAR-T cells outperformed ConstCAR-T cells expressing constitutive CAR.

RiboCAR-T cells targeting HER2 are more potent than ConstCAR-T cells in anti-tumor activity



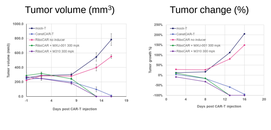
- Schematics of CAR constructs. Riboswitch gene regulation cassette is inserted in the coding sequence of the CAR molecule.
- Schematics of RiboCAR expression cassette was targeted to TRAC locus by CRISPR/Cas9.
- HER2 CAR expression was induced in human primary T cells in response to MXU-001 treatment.

RiboCAR-T cells targeting HER2 exhibited superior cytotoxicity against HER2$^+$ tumor cells



A. RiboCAR-T cells or ConstCAR-T cells were co-cultured with HER2$^+$ Calu-3 cells at 2:1 E:T ratio in the presence of various concentration of inducer for 48 hours. CellTiter-Glo was used to measure cell viability and cytotoxicity. RiboCAR-T cells are more cytotoxic against HER2$^+$ tumor cells and their cytotoxicity is dose-dependent.
B. RiboCAR-T cells were co-cultured with Calu-3 cells at various E:T ratio in the presence or absence of riboswitch inducer M310 for 48 hours. Luciferase activity was measured for cytotoxicity assessment. RiboCAR-T cells exhibit superior cytotoxic effect against tumor cells at low E/T ratio.

RiboCAR-T cells targeting HER2 are more potent than ConstCAR-T cells *in vivo*



NOD/SCID/IL2Rγ$^{-/-}$ (NSG) mice (Jackson Laboratory) were injected with 2.5x10^6 Calu-3 cells subcutaneously. 7 days post Calu-3 cell engraftment, mice were dosed once daily via oral gavage with or without 300 mg/kg MXU-001 or M310. 6 hours post first dosing of inducers, 2x10^6 CAR$^+$ RiboCAR-T cells or ConstCAR-T cells were infused intravenously into the Calu-3 tumor-bearing mice. Tumor progression or regression was monitored.
RiboCAR-T cells exhibited more potent antitumor activity against HER2$^+$ tumors, outperforming CAR-T cells expressing CAR constantly.

Summary

- ✓ Our riboswitch gene regulation system has uniquely high dynamic range for regulating gene expression.
- ✓ Small molecule inducers are safe and orally bioavailable for *in vivo* use.
- ✓ Our riboswitch enables precise control of expression of therapeutic antibodies *in vivo*.
- ✓ Riboswitch-controlled, small molecule-induced anti-HER2 antibody delivery eradicates HER2+ tumor *in vivo*.
- ✓ Our gene regulation technology enables precise control of expression of chimeric antigen receptor molecule (CAR).
- ✓ Riboswitch-controlled CAR-T cells are more potent in CAR-mediated cytotoxicity.
- ✓ Riboswitch-controlled CAR-T cells are more potent in anti-tumor activity *in vivo.*
- ✓ Our gene regulation technology enables more efficacious and safe gene and cell therapy treating cancer.