

# Intramuscular Electroporation of an Optimized Rheoswitch®-Regulated Interferon $\alpha$ Plasmid Transgene Shows Long Term Persistence *In Vivo*: Implications for Therapy of Cancer

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## Abstract

**Background:** Pegylated IFN $\alpha$ 2b was approved in early 2011 for malignant melanoma despite significant side effects in clinical trials. Alternate approaches to widen the therapeutic window of IFN $\alpha$  may afford a more pronounced effect as a cancer therapeutic. This study optimized an IFN $\alpha$  plasmid DNA for regulated secretion from skeletal muscle, using Intrexon's novel RheoSwitch® Therapeutic System® (RTS®) inducible expression platform activated by a small molecule activator ligand (AL). The anti-tumor efficacy of an optimized plasmid was assessed in a B16F0 melanoma model *in vivo* following a single intramuscular (IM) administration via electroporation.

**Materials and Methods:** Optimization of RTS controllable DNA vectors for murine or human IFN $\alpha$  was accomplished by evaluating interchangeable modular noncoding regulatory domains and secretion signal peptides, enabled by Intrexon's UltraVector® platform. To determine pharmacodynamics, plasmid DNA was (a) transfected into human HT1080 fibrosarcoma cells or C2C12 myoblasts and (b) plasmids that induced high levels of secreted protein in the presence of AL were electroporated into the skeletal muscle of normal or B16F0 tumor-bearing mice.

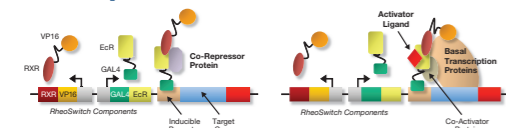
**Results:** Vector optimization increased the *in vitro* secretion of IFN $\alpha$  in response to AL by up to 23-fold, relative to an unoptimized RTS-IFN $\alpha$  plasmid. The single administration of an optimized RTS-mIFN $\alpha$  plasmid by IM electroporation and daily oral dosing with AL, led to persistent plasma expression of IFN $\alpha$  for approximately 4 months (study termination) with no detectable induction of an adverse immune response. This systemic expression was tightly regulated and removal of AL led to a return in expression to basal levels. The regulated expression and therapeutic benefit of this optimized plasmid following IM electroporation was examined in a syngeneic B16F0 melanoma model. IFN $\alpha$  gene therapy in the absence of daily oral AL did not result in inhibition of tumor growth compared to control, untreated tumors. In contrast, the single IM administration of RTS-IFN $\alpha$  and daily oral AL led to significant tumor growth inhibition that was comparable to the therapeutic benefits of DTIC (60 mg/Kg, ip, qdx5) or bolus recombinant mIFN $\alpha$  protein (50,000 IU, sc qod), without overt toxicity as assessed by body weight change. Plasma levels of IFN $\alpha$  obtained with the optimized plasmid were approximately 5000 pg/mL and increased the expression of the anti-angiogenic downstream effector molecule, IP-10.

**Conclusions:** The UltraVector and RTS technologies have been used to create and optimize pDNA for the regulated secretion of IFN $\alpha$  from skeletal muscle. This innovative approach offers the potential advantage of a single administration of optimized RTS-IFN $\alpha$  transgene, in combination with oral AL, for sustained therapeutic anti-tumor efficacy and increased safety by avoidance of fluctuating levels of circulating IFN $\alpha$ .

## Introduction

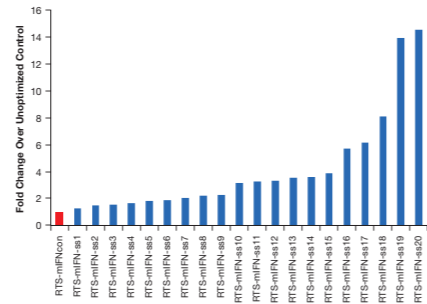
- Recombinant pegylated interferon alpha 2b (Sylatron™) is approved for the adjuvant treatment of melanoma with microscopic or gross nodal involvement within 84 days of definitive surgical resection, including complete lymphadenectomy
- Alternate administration approaches to widen the therapeutic window of interferon (IFN $\alpha$ ) may afford a more pronounced effect as a cancer therapeutic
- This study optimized an IFN $\alpha$  plasmid DNA to allow for the regulated secretion of effective anti-tumor doses from skeletal muscle following administration
- The study utilized Intrexon's novel RheoSwitch Therapeutic System (RTS) inducible expression platform, activated by an orally available small molecule activator ligand (AL)

## RheoSwitch Proteins and Activator Ligand Controls Timing and Level of Target Gene Expression



**Figure 1a.** The RheoSwitch Therapeutic System (RTS) inducible expression platform contains three basic components: (1) an inducible promoter; (2) constitutive transcription factor and a co-activation partner; and (3) a RheoSwitch activator ligand (AL). In the absence of AL, the switch proteins complex to provide an "off" signal. In contrast, in the presence of AL, the complex changes conformation and provides a dose-dependent "on" signal for target gene expression.

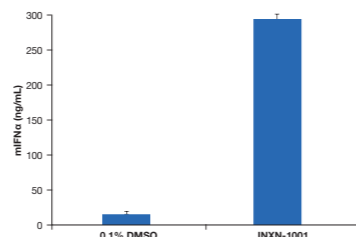
## Vector Optimization Enabled by the Ultravector Platform Increases Regulated Mouse IFN $\alpha$ Expression *In Vitro*



**Figure 1b.** A matrix of RTS platform-regulated mouse IFN $\alpha$  expression vectors, with modifications in the non-coding regulatory elements (Inducible Promoter, 5'REG, 3'REG) was generated to optimize mouse IFN $\alpha$  expression. The RTS-mIFN $\alpha$  plasmids were screened by transient transfection in C2C12 myoblast cells, and IFN $\alpha$  expression was induced by treatment with 100 nM of activator ligand (INXN-1001, AL). Supernatants were harvested 48 hours after transfection, and screened for mouse IFN $\alpha$  expression by ELISA. Expression levels are depicted as fold change over unoptimized pRTS-mouse IFN $\alpha$  (red bar). n = 3, mean  $\pm$  s.e.m.

**Summary:** Intrexon's UltraVector and RTS platforms can facilitate vector optimization to achieve high levels of INXN-1001-regulated IFN $\alpha$  expression *in vitro*.

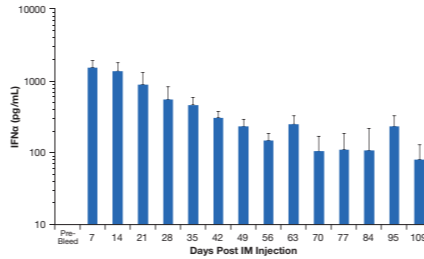
## *In Vitro* Regulated Expression of pRTS-mIFN $\alpha$ in C2C12 Mouse Myoblast Cells



**Figure 1c:** pRTS-mIFN $\alpha$ -ss17 was selected from the matrix described above (Figure 1b) because it showed high INXN-1001-induced expression *in vitro*, and the lowest expression relative to the other high-expressing plasmids in the absence of ligand (data not shown). Here, C2C12 cells were transiently transfected with pRTS-mIFN $\alpha$ -ss17. Activator ligand (INXN-1001, AL) was added to the culture medium at a concentration of 100 nM, or cells were treated with 0.1% DMSO as a negative control. Supernatants were collected at 48h and assayed for mouse IFN $\alpha$  expression. n = 3, mean  $\pm$  s.d.

**Summary:** pRTS-mIFN $\alpha$ -ss17, shows robust INXN-1001-regulated expression *in vitro*, and was selected for subsequent *in vivo* studies.

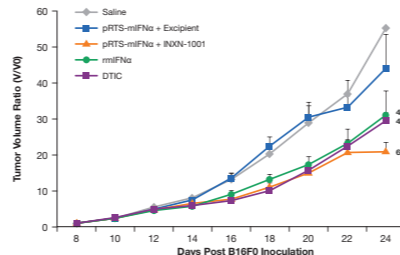
## Single Intramuscular Administration of pRTS-mIFN $\alpha$ Leads to Sustained Regulated Plasma Expression *In Vivo*



**Figure 2:** pRTS-mIFN $\alpha$  (250  $\mu$ g) was administered to Balb/C female mice by intramuscular (IM) electroporation. AL (INXN-1001) was provided in the feed *ad libitum* (oral equivalent dose of approximately 50 mg/kg/day) starting on the day of plasmid injection. IFN $\alpha$  levels in the mouse plasma were assessed by ELISA (PBL Interferon Source). A control group injected with pRTS-mIFN $\alpha$ , received normal feed without AL (excipient). IFN $\alpha$  plasma levels in this control group were below the level of detection of the assay (LOD: 12.5 pg/mL) [Data not shown]. This study was terminated 110 days post IM electroporation. In a repeat of this study, removal of AL on Day 95 led to a return in serum expression to basal levels, and re-induction with AL on Day 109 led to a detectable increase in IFN $\alpha$  plasma levels (data not shown). n = 5 mice/group, mean  $\pm$  s.e.m.

**Summary:** The RTS platform allows for the *in vivo* regulation of gene expression. No systemic expression is detected in pRTS-mIFN $\alpha$  injected animals in the absence of INXN-1001. In contrast, intramuscular delivery of pRTS-mIFN $\alpha$ , and induction with INXN-1001 results in robust and sustained plasma expression of IFN $\alpha$ .

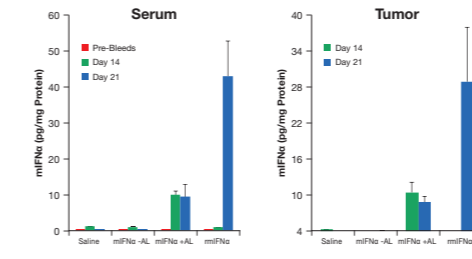
## Antitumor Activity of pRTS-mIFN $\alpha$ in the B16F0 Murine Melanoma



**Figure 3a:** C57/BL6 mice were injected subcutaneous with  $1 \times 10^5$  B16F0 cells. On Day 8 post B16F0 inoculation, when tumors reached  $\sim 80 - 100$  mm<sup>3</sup>, all cohorts received either pRTS-mIFN $\alpha$  (250  $\mu$ g in 40  $\mu$ L) or saline (40  $\mu$ L) by intramuscular (IM) electroporation. Activator ligand (AL) or excipient was administered daily by oral gavage to all groups, starting on Day 9 post cell inoculation. The mIFN $\alpha$  + INXN-1001 (orange line), saline (blue line), mIFN $\alpha$  (grey line), and DTIC (purple line) cohorts received AL at a dose of 50 mg/Kg/day. The mIFN $\alpha$  - INXN-1001 control group (black line) received excipient only. Recombinant mIFN $\alpha$  (50,000 IU sc qod) and DTIC (60 mg/Kg ip qx5) treatments were initiated on Day 9. The data is plotted as a ratio of tumor volume on the indicated day (V) relative to tumor volume on Day 8. Percent tumor growth inhibition on Day 24 for mIFN $\alpha$ , DTIC, and pRTS-mIFN $\alpha$  + INXN-1001 cohorts, relative to saline control, are indicated. (\*p = 0.05, mIFN $\alpha$  + INXN-1001 vs. saline).

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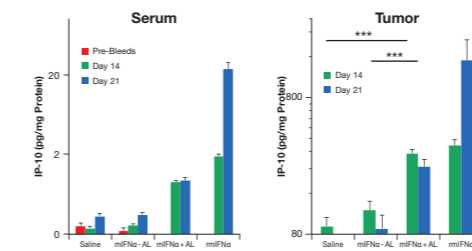
## Generation of Systemic and Intratumoral mIFN $\alpha$ Following IM Administration with pRTS-mIFN $\alpha$



**Figure 3b:** Serum and tumor samples were collected 14 (D14) and 21 (D21) days post B16F0 inoculation. Since mIFN $\alpha$  protein was administered every other day, tissue and serum collection on D21 but not D14 coincided with the day of mIFN $\alpha$  treatment. Mouse IFN $\alpha$  levels were assessed using a MSD assay (Meso Scale Discovery). n = 7, mean  $\pm$  s.e.m.

**Summary:** Transcription activation following the single IM administration of pRTS-mIFN $\alpha$  shows significant antitumor efficacy which is comparable to repeated bolus mIFN $\alpha$  protein injection or treatment with the alkylating chemotherapeutic agent, decarbazine. In the absence of the transcription activator, INXN-1001, no antitumor activity is observed. Transcription activation of pRTS-mIFN $\alpha$  results in sustained, continuous levels of IFN $\alpha$ , in contrast to the excursion levels of mIFN $\alpha$  observed with bolus recombinant mIFN $\alpha$  treatment.

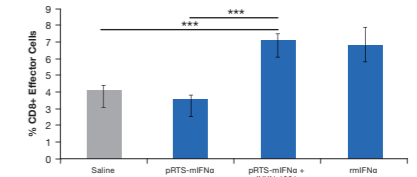
## Production of Systemic and Intratumoral IP-10 Following IM Administration with pRTS-mIFN $\alpha$



**Figure 4:** Serum and tumor samples were collected 14 and 21 days post B16F0 inoculation, corresponding to 5 and 12 days post treatment initiation. Tissue and serum collection on D21 but not D14 coincided with the day of mIFN $\alpha$  treatment. Mouse IP-10 levels were assessed by ELISA. \*\* p < 0.05, \*\*\* p < 0.001, n = 7, mean  $\pm$  s.e.m.

**Summary:** Treatment with pRTS-mIFN $\alpha$  and daily INXN-1001 results in sustained high levels of IP-10 in the serum and tumor, unlike the intermittent levels seen with every other day recombinant mIFN $\alpha$  treatment. In the absence of INXN-1001, IP-10 levels are unchanged relative to untreated controls.

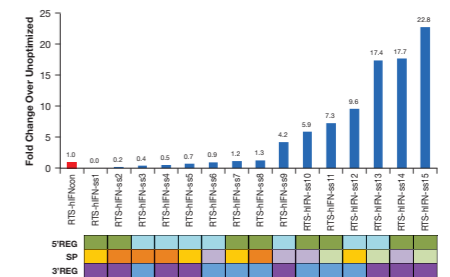
## pRTS-IFN $\alpha$ + INXN-1001 Treatment Increases CD8+ Effector Cell Population



**Figure 5:** Single cell suspensions from mouse spleens, collected 14 days after B16F0 inoculation, were analyzed by flow cytometry using markers for CD4, CD8, NK, macrophages, and dendritic cells. pRTS-mIFN $\alpha$  treatment, in the presence of INXN-1001, resulted in a significant increase in CD8+ effector cells (CD44 high, CD62L low). As expected based on previous published reports, pRTS-mIFN $\alpha$  treatment also enhanced activation of CD4 T cells, CD8 T cells, and NK cells, and increased maturation of macrophages and dendritic cells, but only in the presence of INXN-1001 (not shown). \*\*\* p < 0.001, n = 7, mean  $\pm$  s.e.m.

**Summary:** pRTS-IFN $\alpha$  systemic expression, when induced with INXN-1001, results in robust immune cell activation, comparable to repeated bolus recombinant IFN $\alpha$  treatment.

## Vector Optimization Increases *In Vitro* Expression Of Human IFN $\alpha$



**Figure 6:** A matrix of RTS platform-regulated human IFN $\alpha$  expression vectors, with modifications in the non-coding regulatory elements (5'REG, 3'REG) and secretion signal peptides (SP), was generated to optimize human IFN $\alpha$  expression. The RTS-hIFN $\alpha$  plasmids were screened by transient transfection in HT1080 cells, and IFN $\alpha$  expression was induced by treatment with 100 nM of activator ligand. Supernatants were harvested 48 hours after transfection, and screened for human IFN $\alpha$  expression by ELISA. Expression levels are depicted as fold change over unoptimized pRTS-human IFN $\alpha$  (red bar). n = 3, mean  $\pm$  s.e.m.

**Summary:** Vector optimization, enabled by Intrexon's UltraVector platform, can increase human IFN $\alpha$  expression by up to 23 fold over wild-type vector, affording a significant benefit for translation research.

## Conclusions

- Using the Rheoswitch Therapeutic System inducible expression platform and the Ultravector platform a DNA vector encoding a regulated, secretory optimized, IFN $\alpha$  can be created
- The single IM electroporation of pRTS-IFN $\alpha$  combined with daily oral activator ligand treatment led to significant tumor growth inhibition, comparable to chemotherapy or repeated bolus injection with recombinant mIFN $\alpha$  protein
- No toxicity, as assessed by survival and changes in body weight, was observed in pRTS-IFN $\alpha$  treated groups
- Treatment with pRTS-IFN $\alpha$  resulted in sustained serum and tumor expression of mouse IFN $\alpha$ , the angiogenic biomarker IP-10, and activation of T cells (CD4 and CD8), NK cells, and dendritic cells
- Overall, our approach offers the potential advantage of a single administration of any optimized RTS-inducible transgene, in combination with oral AL, for sustained therapeutic anti-tumor efficacy and increased therapeutic window