Cell-based therapy:
Intelligent drug delivery by engineered encapsulated cells

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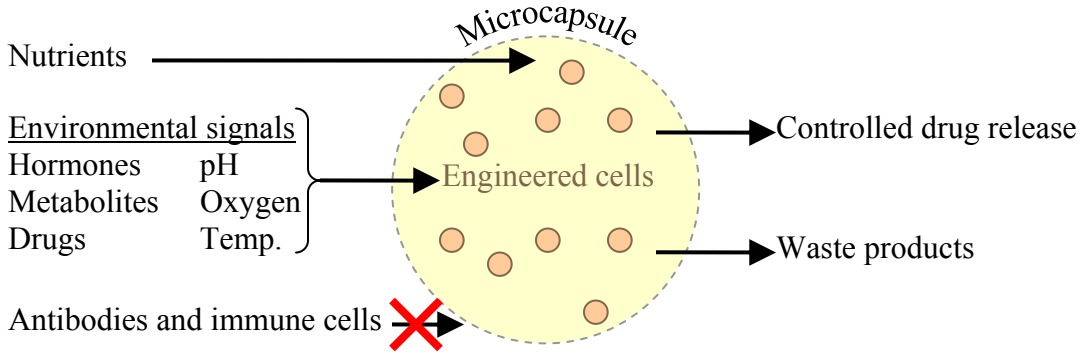
Abstract

Finding drugs to cure diseases is only part of the story: you also have to deliver the right dose to the right place at the right time. To this end, we will engineer cells that manufacture drugs when triggered by the appropriate environmental cues. These cells will be encapsulated in a semipermeable polymer coat to protect them from the immune system, and implanted *in vivo*.

In this proposal, we present encapsulated cell-based strategies for:

- Delivering IL-10 and hydrocortisone to the large intestine for treatment of ulcerative colitis.
- Delivering IL-2 and 5-fluorouracil in a circadian rhythm-dependent fashion for treatment of renal cell carcinoma.



Introduction

Most drugs on the market today are small molecules, which often work by binding to and modulating specific protein targets. This rather crude method of treating diseases — akin to a car mechanic who is only allowed to remove parts from the car — has worked remarkably well over the years. A new generation of therapies, based on engineered macromolecules and even whole cells, promises to give our car mechanic some new skills. Now, he can put in new parts, and reconfigure the existing parts in a more sophisticated way.

We will develop cell-based therapies that deliver small molecule and protein drugs *in situ*, automatically sensing the patient's health status and adjusting the dosing schedule appropriately. To accomplish this, we will capitalize on the convergence of several recent trends.

Cell encapsulation

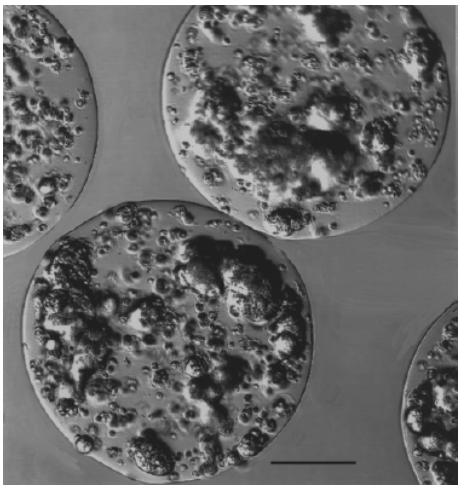


Figure 1. Human embryonic kidney cells immobilized in alginate beads. Scale bar: 145 μm. (Photo from Read 2001)

First, are developments in encapsulated cell technology over the past 20 years. Cells immobilized in polymers have been used to manufacture a wide range of chemicals in bioreactors, including antibiotics, anticancer agents, steroids, proteins, and enzymes (Willaert 1996). Encapsulated cells have also been implanted in various animal disease models (Orive 2002). Endostatin-producing human kidney cells implanted next to rat tumors slowed their growth (Read 2001). *E. coli* engineered to produce urease reduced plasma urea levels in a rat model of kidney failure (Prakash 1996). Encapsulated rat islet cells reversed diabetes in mice (Wang 1997).

The key considerations for an implantable cell encapsulation system are: 1. The capsule should be permeable to nutrients, waste products, and the manufactured drug, while excluding antibodies and immune system cells. 2. The capsule should be biocompatible with both the encapsulated cells and the implantation site. 3. The capsule should be mechanically stable, to prevent release of encapsulated cells.

Cells embedded in alginate beads meet all of the above criteria. The embedding procedure involves taking droplets of cells suspended in 1.5% sodium alginate, and inducing gel formation by immersing the droplets in a 0.1 M $CaCl_2$ solution (Figure 1). Human cells encapsulated using this technique were 70% viable after 4 months of implantation in rats (Read 2001). Several other encapsulation methods have also been developed (Wang 1997, Willaert 1996).

Genetic circuit and metabolic engineering

Second, are recent developments in genetic circuit (Hasty 2002, Elowitz 2000, Park 2003) and metabolic (Bailey 1991, Cane 1998, Schmidt-Dannert 2000) engineering.

In a remarkable feat of metabolic engineering, the hydrocortisone biosynthetic pathway was recently reconstructed in *S. cerevisiae* by introducing 8 mammalian steroid synthesis enzymes that convert the yeast's endogenous ergosterol into hydrocortisone (Szczebara 2003, Figure 2). We plan to modify this strain for treatment of ulcerative colitis, as described below.

Dosing schedules

Third, is an increasing literature on the role of circadian rhythms in disease, optimal dosing times for various drugs, and the benefits of complex dosing schedules for some drugs (Lévi 2002). In particular, many diseases follow a daily rhythm: arthritic joint stiffness, asthma attacks, heart attacks, and strokes tend to occur in the morning, and blood pressure increases during the day. Administering drugs at the right time of day can improve the therapeutic effect.

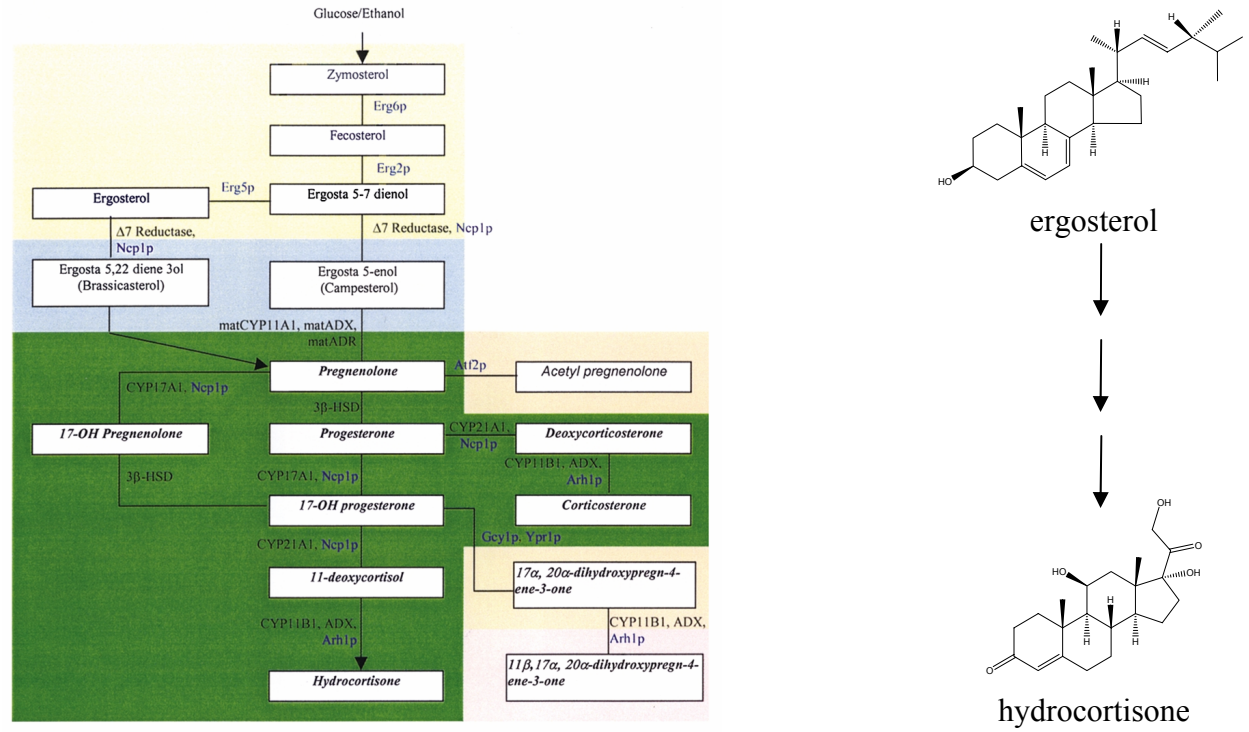


Figure 2. *S. cerevisiae* engineered to produce hydrocortisone. Left panel from Szczebara 2003.

Specific aims

1. Using existing technologies and "off-the-shelf" components, design cell-based treatments for:
    a. **Ulcerative colitis**. Engineer yeast cells to produce IL-10 and hydrocortisone in the large intestine, starting with a recently developed yeast-strain that contains the mammalian hydrocortisone biosynthetic pathway.
    b. **Renal cell carcinoma**. Engineer encapsulated mammalian cells to produce IL-2 and 5-fluorouracil in a circadian rhythm-dependent fashion, using melatonin-driven gene expression.
2. Test these engineered encapsulated cells *in vivo*, in collaboration with labs experienced with the relevant animal models.

Experimental approach

Hydrocortisone and IL-10 for ulcerative colitis

Ulcerative colitis is a chronic inflammatory disease of the large intestine, with an incidence of about 1 in 10,000. Symptoms include abdominal pain and bloody diarrhea, and 30% of patients require surgical removal of the large intestine. Distal ulcerative colitis is treated with rectal corticosteroids (such as hydrocortisone) administered as a liquid enema, rectal foam, or rectal

suppository. Rectal corticosteroids can only reach the descending colon, so to treat the rest of the colon, the patient must take oral or intravenous corticosteroids. Unfortunately, systemic corticosteroids have a higher incidence of side effects, including weight gain, mood changes, and osteoporosis. (Ardizzone 1998)

To address this problem, we will deliver topical hydrocortisone throughout the length of the large intestine using genetically engineered *S. cerevisiae*. This will allow the use of higher local doses without systemic side effects.

There is a precendent for using genetically engineered organisms to treat ulcerative colitis. *Lactococcus lactis*, which is used in yogurt cultures, was engineered to secrete IL-10, and caused a 50% reduction of colitis in a mouse model system (Steidler 2000). Based on this success, we plan on tranfecting the hydrocortisone-producing yeast with an additional vector encoding murine IL-10 production.

Optimizing persistence in the gut

Yeasts have been used in food and beverages for centuries, and *S. cerevisiae* and *S. boulardii* are classified by the Food and Drug Administration as "generally recognized as safe." Both *Saccharomyces* species are probiotics that promote the natural balance of intestinal flora. In fact, wild type *S. boulardii* taken orally reduces relapses in Crohn's disease, the other major type of inflammatory bowel disease (Guslandi 2000). This indicates that *Saccharomyces* can survive passing through the acidic conditions in the stomach in order to have an effect on the intestines. Survival in the stomach could be improved further by using an enteric coating, such as phenyl salicylate or keratin, which remains intact under acidic conditions in the stomach, but dissolves under basic conditions in the intestines. The enteric coating will be applied by dip coating.

In order to be effective, the engineered *S. cerevisiae* should remain in the large intestine for long enough to deliver the hydrocortisone and IL-10, and should not remain in the small intestine. We will try two different strategies for optimizing persistence times in the large intestine.

Strategy 1: The yeast will be transfected with adhesins from other species, such as *ALS1* from *Candida albicans* (Fu 1998), or *EPA1* from *Candida glabrata* (Cormack 1999). *Candida* colonizes the stomach and colon when fed to antibiotic-treated mice (Wiesner 2001), so it seems reasonable that adhesion genes from *Candida* species would help *S. cerevisiae* persist in the gut as well.

Strategy 2: The yeast will be encapsulated in mucoadhesive polymers such as polyacrylic acid, or encapsulated in a polymer coat and then coated with lectins, which adhere to the intestinal epithelium (Lehr 2000, Willaert 1996). Lectins can be covalently attached to the surface of an alginate bead using N-hydroxysuccinimide / carbodiimide coupling.

To measure adherence *in vitro*, the yeast will be incubated with human adenocarcinoma cell line HT-29, and the number of adherent cells counted under a microscope (Adlerberth 1996). To measure persistence times *in vivo*, mice will be orally innoculated with the engineered yeast, sacrificed at various timepoints, and the fecal matter at different positions along the colon

examined for the presence of engineered yeast cells. This will be done by quantitative PCR using appropriately chosen primers. To ensure that the yeast are still actively producing the desired products, IL-10 will be measured in an ELISA assay (Amersham Biosciences), and hydrocortisone will be measured in an HPLC or GCMS assay.

Optimizing production and delivery of hydrocortisone and IL-10

Current hydrocortisone rectal enema preparations contain 1.7 mg hydrocortisone / ml. The hydrocortisone-producing yeast unfortunately only produces 10 μg hydrocortisone / ml after 172 hours. On the other hand, plasma concentrations of hydrocortisone cycle between 20 and 200 ng / ml during the day, indicating that even small amounts of hydrocortisone may have a physiological effect. Thus, the existing hydrocortisone-producing yeast may already produce enough hydrocortisone, but it may need to be improved by 2 orders of magnitude. This potential problem, which only recently came to our attention, could be very difficult to solve. However, we can briefly outline, in a general way, how to attack the problem. It's worth noting that the enzymes involved in hydrocortisone synthesis from different species have a wide range of kinetic constants. For example, the K_m of adrenodoxin reductase ranges from 0.94 μM to 60 μM, depending on the species. Furthermore, it is likely that feedback inhibition may work differently in the different varieties of each enzyme. Therefore, trying different varieties of each enzyme may improve the yield. To determine which enzymes to optimize, we will grow the engineered yeast under the pH and nutrient mix present in the intestines, and measure the concentrations of the intermediate steroids in the pathway by HPLC. Enzymes downstream of any buildup of intermediates, or upstream of any depletion of intermediates, should be optimized.

If the yeast needs to be encapsulated to improve persistence times in the gut, then it will be important to measure the diffusion coefficients of hydrocortisone and IL-10 in the encapsulating polymer. The diffusion coefficient will be measured by making alginate beads containing IL-10 or hydrocortisone, then placing the beads in a buffer and measuring the hydrocortisone or IL-10 concentration in the buffer over time. These diffusion coefficients may be increased by varying the polymer properties (such as alginate concentration), and the encapsulation conditions (Willaert 1996, Martinsen 1992).

It's unclear how IL-10 (a 160 amino acid homodimer) produced by genetically engineered *Lactococcus lactis* cells in the intestine (Steidler 2000) crosses the intestinal epithelium to reach its target cells (macrophages). One hypothesis is passive diffusion, and the other hypothesis is that it's due to Lactococcus's ability to actively translocate across the intestinal epithelium. If IL-10 delivered by *L. lactis* reduces inflammation, but IL-10 delivered by *S. cerevisiae* doesn't reduce inflammation, then that supports the Lactococcus translocation hypothesis. To improve delivery of IL-10 to target cells, we will try fusing it to various peptide tags (such as an N-terminal YGRKKRRQRRR) that have been shown to substantially increase passive diffusion of macromolecules across lipid bilayers (Schwarse 1999, Lindgren 2000).

Suicide mechanism

It might be useful to kill the genetically engineered yeast when the colitis goes into remission. This could be done using 5-fluoroorotic acid, which is toxic to *URA3*$^+$ yeast.

Animal model

We will test our engineered yeast in two different mouse models of ulcerative colitis: colitis induced by 5% dextran sulfate sodium and colitis that spontaneously develops in IL-$10^{-/-}$ mice (Steidler 2000).

Melatonin-driven chemotherapy

The toxicity and efficacy of many chemotherapy drugs follows a daily rhythm (Table 1). We will engineer encapsulated cells to produce chemotherapy drugs at the correct time of day. To do this, we take advantage of circadian fluctuations in hormone concentrations (Figure 3). We adapt the pathways that normal cells use to respond to these hormones, to drive expression of genes involved in drug synthesis. Specifically, we will engineer mammalian cells to produce IL-2 at night and 5-fluorouracil during the day in response to daily fluctuations in melatonin concentration.

Drug	Optimal time for delivery (hours after light onset)	Biosynthesis
daunorubicin	3	Produced by encapsulated *Streptomyces peucetius*.
epirubicin	3	S. peucetius engineered to convert anthracyclinone precursor to epirubicin. Epirubicin O-glucuronide prodrug converted to epirubicin by β-glucuronidase.
5-fluorouracil (5-FU)	4	Aldehyde oxidase converts 5-fluoropyrimidine into 5-FU. Cytosine deaminase converts prodrug 5-fluorocytosine to 5-FU.
etoposide	9	Arylsulfatase removes sulfate from etoposide 4'-sulfate prodrug.
melphalan	9	Prodrug converted to melphalan by β-lactamase.
cyclophosphamide	12	Cytochrome P450 activates cyclophosphamide (activation normally takes place in the liver).
doxorubicin	14	Produced by *Streptomyces peucetius* (41 kb gene cluster). Glu-SP-doxorubicin converted to doxorubicin by β-glucuronidase. Cephem-derivative prodrug converted to doxorubicin by β-lactamase.
methotrexate	18	Prodrug converted to methotrexate by carboxypeptidase A.
mitomycin C	20	Prodrug converted to mitomycin C by alkaline phosphatase.
actinomycin D	20	Produced by encapsulated *Streptomyces parvullus*.
interleukin 2 (IL-2)	20	Protein, 133 a.a. monomer, 1 disulfide bond, glycosylated; produced in encapsulated Gibbon lymphoblastoid cells; IL-2 linked to HER-2 antibody (for breast cancer) produced in encapsulated mouse myoblasts.
tumor necrosis factor (TNF)	20	Protein.

Table 1. Circadian rhythm dependence of selected anticancer drugs in mice (Lévi 2002) and their biosynthesis (see, for example, Syrigos 1999). Toxicity typically varies 2- to 8-fold as a function of time of delivery. Mice are mostly inactive during 12 hours of light, and active during 12 hours of dark.

Creation of melatonin-responsive cell lines

As far as we know, no one has ever engineered a cell to produce a protein in response to melatonin. Here, we present two strategies for doing that:

Strategy 1: cAMP response element-driven expression. Much of the melatonin signaling pathway was worked out in mammalian cells (human embryonic kidney, or Chinese hamster ovary) expressing a recombinant melatonin receptor. These cells exhibit decreased intracellular cAMP in response to melatonin (Masana 2001, Conway 1997, Witt-Enderby 1996). cAMP levels, in turn, can be used to drive gene expression by placing 6 tandem cAMP response elements (CRE, TGACGTCA) and a minimal RSV promoter before the gene of interest (Obrietan 2002). See Figure 5. One potential problem is cross-talk from other pathways that also use cAMP as a second messenger. This problem might be minimized by overexpression of the melatonin receptor, crowding out other cell surface receptors. However, we also present a backup strategy:

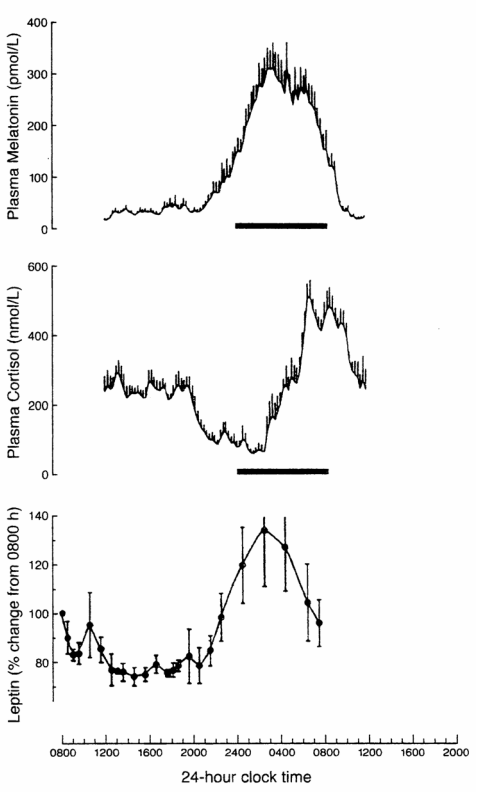


Figure 3. Melatonin, cortisol, and leptin follow a circadian rhythm. (Graphs from Larson 2003, p. 94.)

Strategy 2: Enhancer trap followed by flow cytometry selection. We start with a mammalian cell line expressing a recombinant melatonin receptor, and also expressing β-galactosidase under a GAL4 promoter. Next, a GAL4 enhancer trap construct (Xavier-Neto 1998) will be integrated randomly into the genome. We will then find the clones that exhibit melatonin-responsive gene expression. This is done by incubating melatonin- or buffer-treated cells with a fluorogenic β-galactosidase substrate (di-β-D-galactopyranoside) that accumulates inside the cell. Cells expressing β-galactosidase can then be sorted by flow cytometry (Nolan 1988). See Figure 4.

Melatonin-driven synthesis of IL-2 and 5-fluorouracil

In both strategies, the tetracycline repressor is used to drive IL-2 synthesis ≈ 180° out of phase with cytosine deaminase synthesis. Cytosine deaminase converts 5-fluorocytosine, an inactive prodrug, into 5-fluorouracil (Syrigos 1999). The prodrug will be integrated into biodegradable controlled-release microspheres (Okada 1995) implanted near the encapsulated cells. As a precedent for this prodrug approach, human colorectal carcinoma cells expressing cytosine deaminase kill neighboring cancer cells, *in vivo* and *in vitro*, at non-toxic concentrations of 5-fluorocytosine (Huber 1994). 5-fluorouracil should not kill the engineered cells, because it is primarily toxic to dividing cells, and the polymer capsule prevents the engineered cells from dividing.

Human IL-2 is glycosylated; however, this is not critical for its function. The clinically used IL-2 derivative, aldesleukin, has two point mutants and no glycosylation.

IL-2 (17 kDa) is much smaller than an antibody (100 kDa), so it should be possible to create a pollymer capsule that's permeable to IL-2 but not antibodies. This would be done as described in the section on ulcerative colitis.

Adjusting kinetic parameters of the genetic circuit

The genetically engineered cells will be tested *in vitro* by cycling the melatonin concentration (by spinning down cells periodically and resuspending in a different buffer) and measuring the response. IL-2 production can be measured by ELISA (Amersham Biosciences), and 5-fluorocytosine conversion to 5-fluorouracil by cytosine deaminase can be followed by HPLC.

If the basal activity of the cytosine deaminase activity is too high, this could be fixed by adding a protein degradation tag to the enzyme. For example, the half life of β-galactosidase in mammalian reticulocytes varies from 0.8 hours to 20 hours, depending on the N-terminal residue

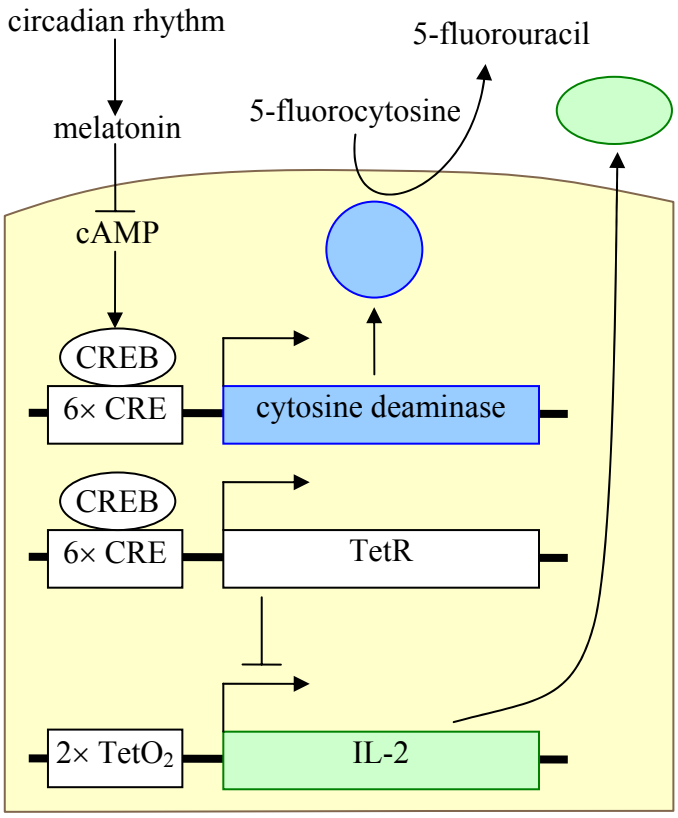


Figure 5. Mammalian cell engineered to produce IL-2 at night and 5-fluorouracil during the day (CRE-driven expression strategy). CRE = cAMP response element. CREB = CRE binding protein. TetR = tetracycline repressor. $TetO_2$ = tetracycline operator.

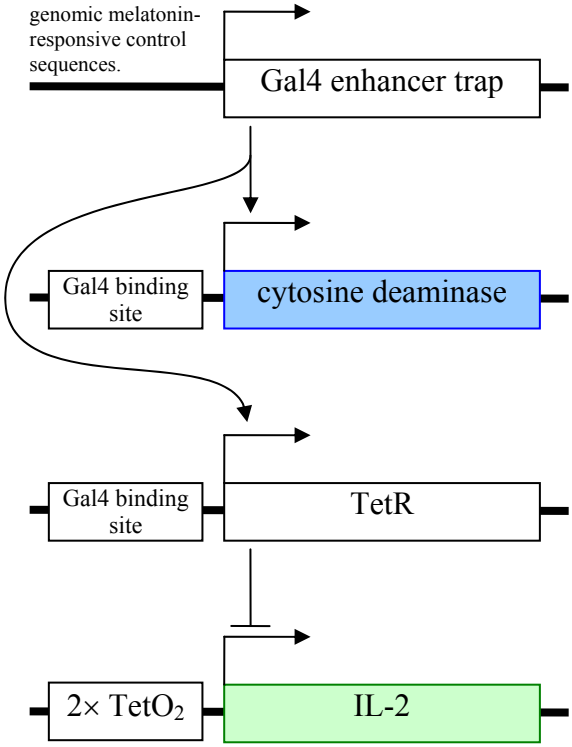


Figure 4. Mammalian cell engineered to produce IL-2 at night and 5-fluorouracil during the day (enhancer trap strategy).

(Gonda 1989). The stability of cytosine deaminase's mRNA can also be reduced, by adding AUUUA repeats in the 3'-UTR (Guhaniyogi 2001).

The same considerations apply to the tetracycline repressor: if IL-2 production is constitutively repressed, then the stability of the tetracycline repressor protein or mRNA needs to be reduced.

Animal model

We will test our engineered cells in a mouse model of renal cell carcinoma (Yamazaki 2002). In this model system, 5-fluorouracil treatment reduced the rate of metastasis. In humans, IL-2, 5-fluorouracil, and IFNα-2b combination therapy achieves some anti-tumor activity in metastatic renal cell carcinoma (Ellenhorst 1997). The encapsulated cells and prodrug microspheres can be implanted either next to the tumor, or at a distal site.

The polymer capsule prevents immune cells and antibodies from reaching the engineered cells, thus reducing the immune response. However, if the immune system still manages to destroy the encapsulated cells, then we will try injecting a single dose of anti-CD4 antibodies at the same time the encapsulated cells are implanted. This procedure induces tolerance to transplanted tissues in mice and primates (Graca 2003).

References

1. Adlerberth I, Ahrné S, Johansson ML, Molin G, Hanson LÅ, Wold AE. (1996) "A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29." *Applied and environmental microbiology*. 62(7): 2244-51.
2. Ardizonne A and Porro GB. (1998) "A practical guide to the management of distal ulcerative colitis." *Drugs*. 55(4): 519-42.
3. Bailey JE. (1991) "Toward a science of metabolic engineering." *Science*. 252: 1668-75.
4. Cane DE, Walsh CT, and Khosla C. (1998) "Harnessing the biosynthetic code: Combinations, permutations, and mutations." *Science*. 282: 63-8.
5. Conway S, Drew JE, Canning SJ, Barrett P, Jockers R, Strosberg AD, et al. (1997) "Identification of Mel_{1a} melatonin recpetors in the human embryonic kidney cell line HEK293: Evidence of G protein-couples melatonin receptors which do not mediate the inhibition of stimulated cyclic AMP levels." *FEBS letters*. 407: 121-6.
6. Cormack BP, Ghori N, and Falkow S. (1999) "An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells." *Science*. 285: 578-82.
7. Ellerhorst JA, Sella A, Amato RJ, Tu SM, Millikan RE, Finn LD, et al. (1997) "Phase II trial of 5-fluorouracil, interferon-α and continuous infusion interleukin-2 for patients with metastatic renal cell carcinoma." *Cancer*. 80: 2128-32.
8. Elowitz MB and Leibler S. (2000) "A synthetic oscillatory network of transcriptional regulators." *Nature*. 403: 335-8.
9. Fu Y, Rieg G, Fonzi WA, Belanger PH, Edwards JE, Filler SG. (1998) "Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells." *Infection and immunity*. 66(4): 1783-6.

10. Gonda DK, Bachmair A, Wünning I, Tobias TW, Lane WS, Varshavsky A. (1989) "Universiality and structure of the N-end rule." *Journal of biological chemistry*. 264(28): 16700-12.
11. Graca L, Le Moine A, Cobbold SP, Waldmann H. (2003) "Dominant transplantation tolerance." *Current opinion in immunology*. 15: 499-506.
12. Guhaniyogi J, Brewer G. (2001) "Regulation of mRNA stability in mammalian cells." *Gene*. 265: 11-23.
13. Guslandi M, Mezzi G, Sorghi M, Testoni PA. (2000) "*Saccharomyces boulardii* in maintenance treatment of Crohn's disease." *Digestive diseases and sciences*. 45(7): 1462-4.
14. Hasty J, McMillen D, and Collins JJ. (2002) "Engineered gene circuits." *Nature*. 420: 224-30.
15. Huber BE, Austin EA, Richards CA, Davis ST, Good SS. (1994) "Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase." *Proc. Natl. Acad. Sci. USA*. 91: 8302.
16. Kokkola T, Watson MA, White J, Dowell S, Foord SM, Laitinen JT. (1998) "Mutagenesis of human Mel_{1a} melatonin receptor expressed in yeast reveals domains important for receptor function." *Biochemical and biophysical research communications*. 249: 531-6.
17. Larson PR et al. (2003) Williams textbook of endocrinology, 10^{th} ed.
18. Lehr CM. (2000) "Lectin-mediated drug delivery: The second generation of bioadhesives." *Journal of controlled release.* 65: 19-29.
19. Lévi F. (2002) "From circadian rhythms to cancer chronotherapeutics." *Chronobiology international.* 19(1): 1-19.
20. Lindgren M, Hällbrink M, Prochiantz A, Langel Ü. (2000) "Cell-penetrating peptides" *Trends in pharmacological sciences*. 21(3): 99-103.
21. Looger LL, Dwyer MA, Smith JJ, Hellinga HW. (2003) "Computational design of receptor and sensor proteins with novel functions." 423: 185-90.
22. Martinsen A, Storrø I, Skjåk-Bræk G. (1992) "Alginate as immobilization material: III. Diffusional properties." *Biotechnology and bioengineering*. 39: 186-94.
23. Masana MI, Dubocovich ML. (2001) "Melatonin receptor signaling: Finding the path through the dark." *Science's STKE*. 107: pe39.
24. Nolan GP, Fiering S, Nicolas JF, Herzenberg LA. (1988) "Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*." *Proc. Natl. Acad. Sci. USA*. 85: 2603-7.
25. Obrietan K, Impey S, Storm DR. (2002) "cAMP response element-mediated gene expression in transgenic reporter gene mouse strain." *Methods in enzymology*. 345: 570-84.
26. Okada H, Toguchi H. (1995) "Biodegradable microspheres in drug delivery." *Critical reviews in therapeutic drug carrier systems*. 12(1): 1.
27. Orive G, Hernández RM, Gascón AR, Igartua M, Pedraz JL. (2002) "Encapsulated cell technology: from research to market." *Trends in biotech.* 20(9): 382-7.
28. Oshitani N, Kamata M, Ooiso R, Kawashima D, Inagawa M, Sogawa M, et al. (2003) "Outpatient treatment of moderately severe active ulcerative colitis with pulsed steroid

therapy and conventional steroid therapy." *Digestive diseases and sciences*. 48(5): 1002-5.
29. Park SH, Zarrinpar A, and Lim WA. (2003) "Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms." *Science*. 299: 1061-4.
30. Prakash S and Chang TMS. (1996) "Microencapsulated genetically engineered live *E. coli* DH5 cells administered orally to maintain normal plasma urea level in uremic rats." *Nature medicine.* 2: 883-7.
31. Read TA, Sorensen DR, Mahesparan R, Enger PØ, Timpl R, Olsen BR, et al. (2001) "Local endostatin treatment of gliomas administered by microencapsulated producer cells." *Nature biotech.* 19: 29-34.
32. Sabir S and Werth VP. (2000) "Pulse glucocorticoids." *Dermatologic clinics*. 18(3): 437.
33. Schmidt-Dannert C, Umeno D, and Arnold FH. (2000) "Molecular breeding of carotenoid pathways." *Nature biotech.* 18: 750-3.
34. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. (1999) "In vivo protein transduction: Delivery of a biologically active protein into the mouse." *Science*. 285: 1569-72.
35. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. (2000) "Treatment of murine colitis by *Lactococcus lactis* secreting IL-10." *Science*. 289: 1352-5.
36. Syrigos KN, Epenetos AA. (1999) "Antibody directed enzyme prodrug therapy (ADEPT): A review of the experimental and clinical considerations." *Anticancer research*. 19: 605-14.
37. Szczebara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duman B, et al. (2003) "Total biosynthesis of hydrocortisone from a simple carbon source in yeast." *Nature biotech.* 21: 143-9.
38. Wang T, Lacík I, Brissová M, Anilkumar AV, Prokop A, Hunkeler D, et al. (1997) "An encapsulation system for the immunoisolation of pancreatic islets." *Nature biotech.* 15: 358.
39. Werstuck G and Green MR. (1998) "Controling gene expression in living cells through small molecule-RNA interactions." *Science*. 282: 296-8.
40. Wiesner SM, Jechorek RP, Garni RM, Bendel CM, Wells CL. (2001) "Gastrointestinal colonization by *Candida albicans* mutant strains in antibiotic-treated mice." *Clinical and diagnostic laboratory immunology*. 8(1): 192-5.
41. Willaert RG and Baron GV. (1996) "Gel entrapment and microencapsulation: Methods, applications, and engineering principles." *Reviews in chemical engineering.* 12(1-2): 1-205.
42. Witt-Enderby PA, Dubocovich ML. (1996) "Characterization and regulation of the human ML_{1A} melatonin receptor stably expressed in Chinese hamster ovary cells." *Molecular pharmacology*. 50: 166-74.
43. Xavier-Neto J, Pereira AC, Motoyama AH, Kreiger JE. (1998) "A luciferase-engineered cell line for study of cAMP regulation in endothelial cells." *American Joural of Physiology: Cell physiology*. 275: C75-81.
44. Yamazaki S, Morita T, Endo H, Hamamoto T, Baba M, Joichi Y, et al. (2002) "Isoliquiritigenin suppresses pulmonary metastasis of mouse renal cell carcinoma." *Cancer letters*. 183: 23-30.
45. Yokobayashi Y, Weiss R, and Arnold FH. (2002) "Directed evolution of a genetic circuit." *Proc. natl. acad. sci. USA*. 99(26): 16587-91.