Production of a Human TGF-beta Family Protein with Potential as an anti-Cancer Therapeutic Protein From Plant Chloroplast

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IBE Presentation 30 March 2007

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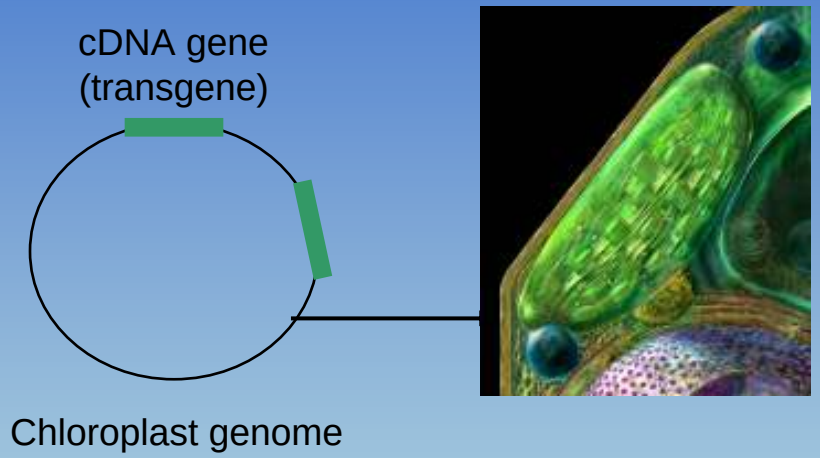
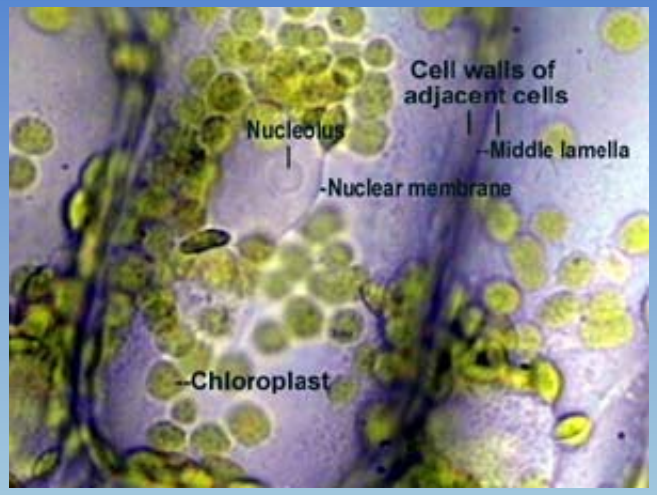
Basis of hyper-expression system - Chloroplast Transformation Technology (CTT™)

Chloroplast

Plant Cell*



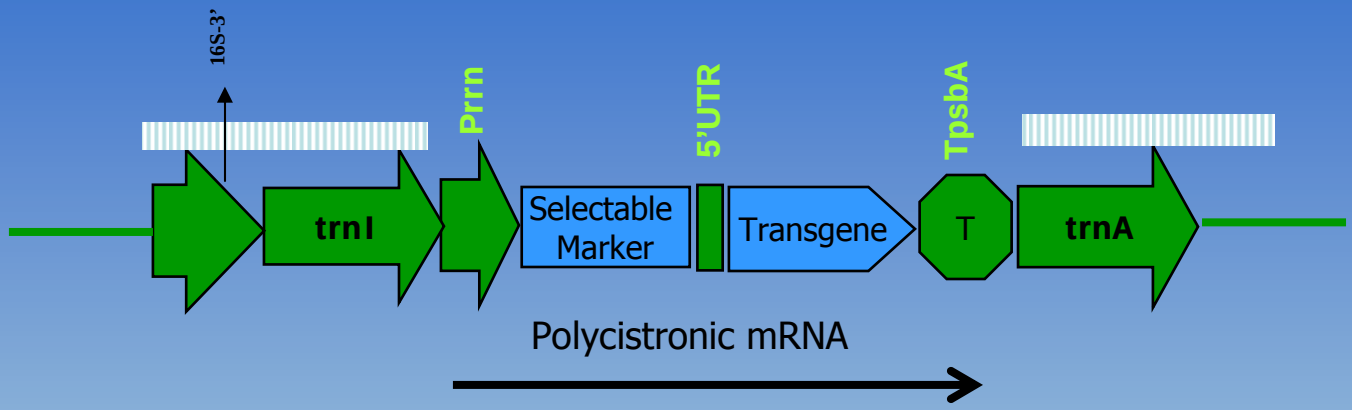


10-100 chloroplast in each cell, therefore the potential to carry 100 to 10,000 copies of the transgene per plant cell

* http://www.progressivegardens.com/knowledge_tree/chloroplast.jpg

Design of the Transformation/Expression Vector to capture the maximum potential of the chloroplast to produce recombinant protein

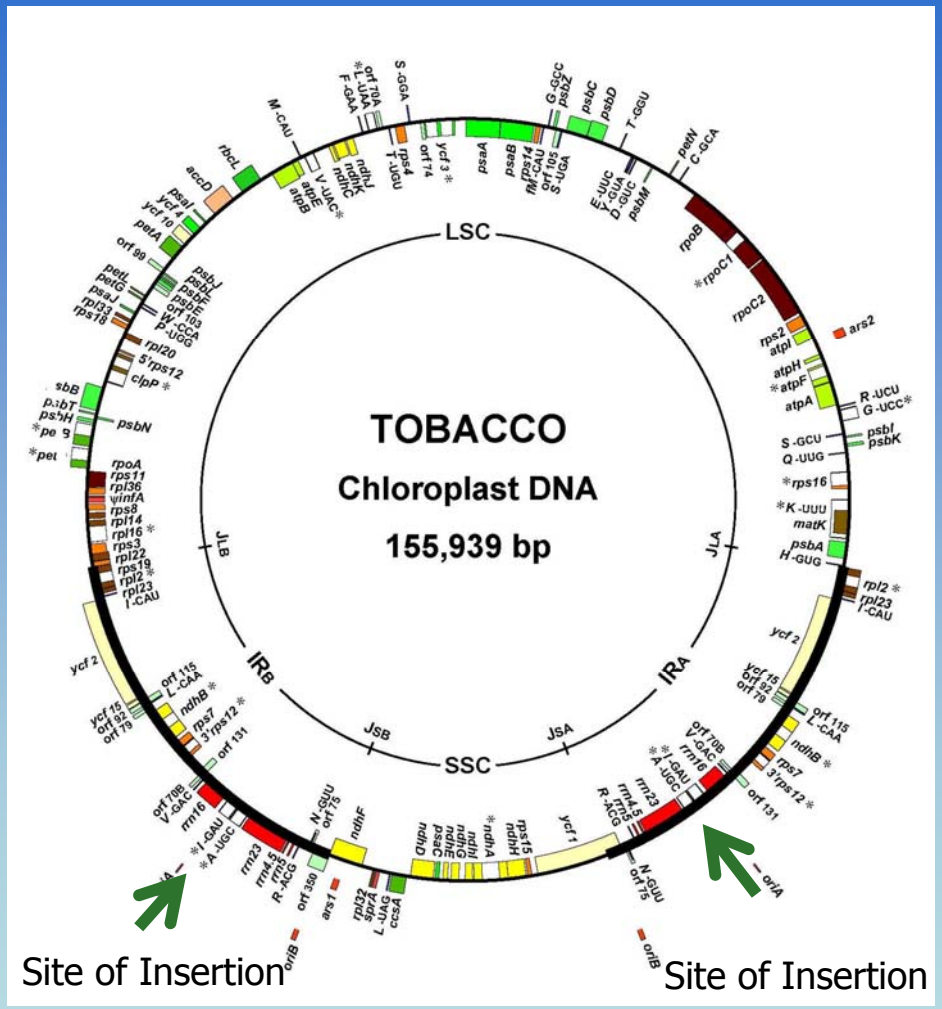
Chloroplast Transformation/Expression Vectors



Transformation/Expression Cassette*:
The perpendicular dotted line shows the vector sequences that are homologous to native chloroplast DNA, resulting in homologous recombination and site specific integration of the gene cassette into the chloroplast genome.

* Henry Daniell, PhD, University of Central Florida, Orlando, FL

Chloroplast Transformation/Expression Vectors



Wakasugi, T, Tsudzuki T, Sugiura M (2001) *Photosynthesis Research* 70(1):107-118

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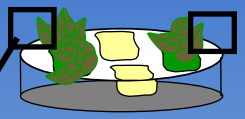
Generation of Transplastomic Plant Line

Single Transformation Events



Spectinomycin Selection

1st Round of Selection

- QPCR Analysis gDNA

Spectinomycin Selection

2nd Roun

- QPC

- QPCR Analysis gDNA
- Elisa – Protein Analysis
- SDS-PAGE Western Analysis
- Enzyme Assay

0.5 – 1.0 Million seeds/plant

Production Seed Line (T_0)

Production Seed Line (T_1)

Gene to Seed ➡ 7-8 months

Easy scale-up to biomass production (48 metric tons/acre) – Kentucky, S Carolina and Missouri

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Transforming Growth Factors

Mullerian Inhibiting Substance (MIS)

Mammalian Cell Cycle Modulators

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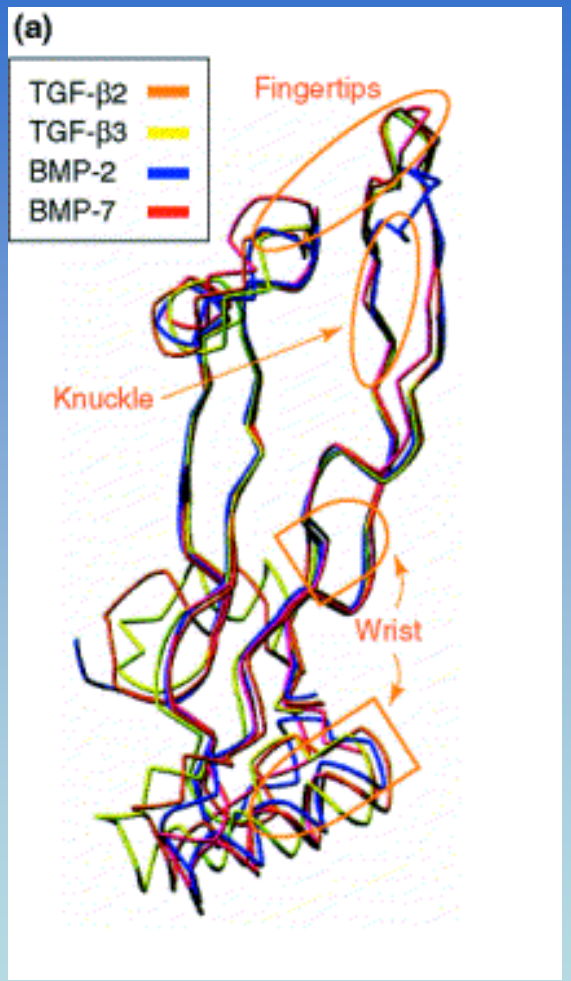
TGF-β Superfamily

- The TGF-β (Transforming Growth Factor) superfamily encompasses a large group of soluble extracellular proteins that are potent regulators of embryonic and adult tissues.

- TGF-β superfamily members can direct a wide range of cellular responses including proliferation, cellular morphology, apoptosis, cellular maturation, organogenesis, and tissue homostasis

- Members of the family are generally classified as:
  - TGF-β (Transforming Growth Factors)
  - BMP (Bone Morphogenetic Proteins)
  - GDF (Growth and Differentiation factors)
  - Activin

- Highly Conserved Structure in Vertebrates and Invertebrates



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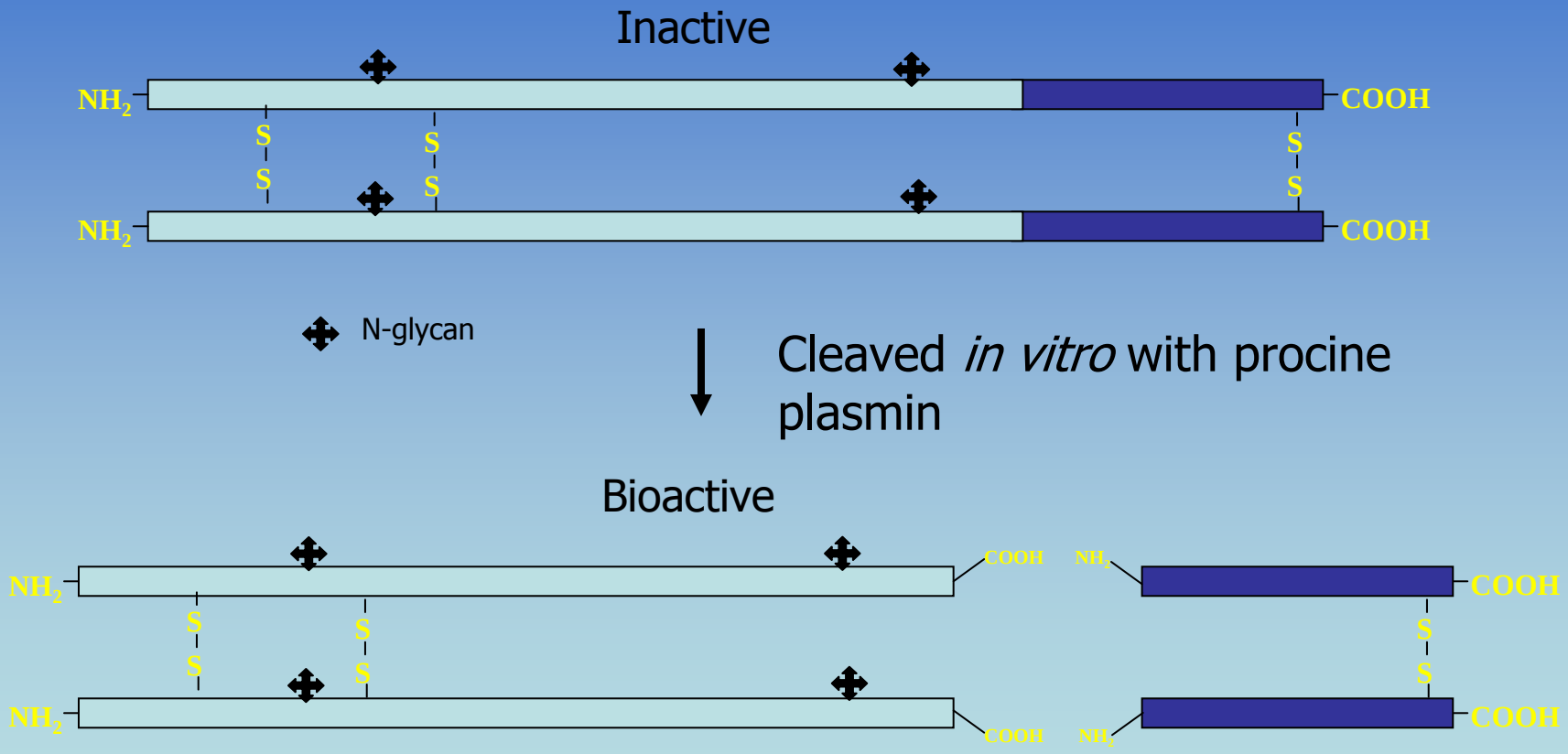
Müllerian Inhibiting Substance (MIS)

MIS is a member of the TGF-β family involved with the regression of the Müllerian duct in male fetuses during embryonic development.

- Extensive animal studies consistently reveal that MIS is effective against ovarian, uterine, breast, endometrial, and prostate cancers
- CHO cells have not been capable of generating adequate supplies for clinical trials or commercial operations due to interference with cellular metabolism in the production cells.
- Chlorogen has succeeded in producing bioactive MIS in tobacco and has positioned the company as the singular producer of MIS in plants, and by extension, other TGF-β proteins

Human pro-Mullerian Inhibiting Substance from CHO (Chinese Hamster Ovary Cells)



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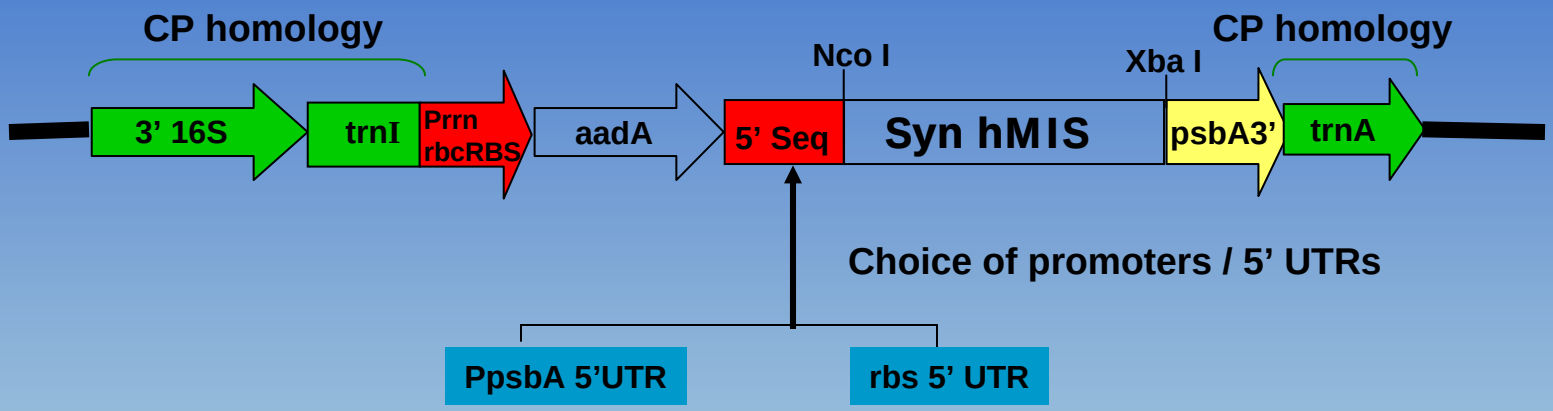
CTT Transformation Vectors holoMIS



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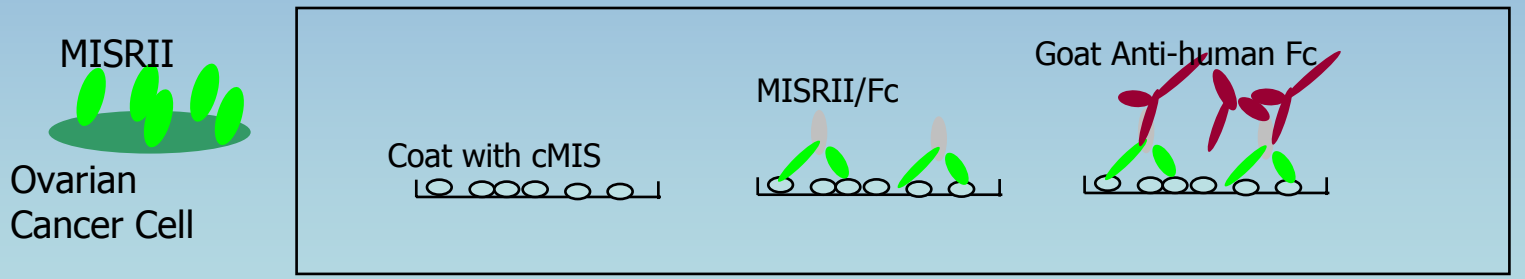
Detection Tools for hMIS

Chicken antibody to cMIS Ag002

- Antibody detects monomer & cMIS dimer

MIS-Receptor II binding Assay: Elisa-based Assay

- Soluble MISRII-human Fc fusion
- AP or HP Conjugated Goat Anti-human Fc
- MIS RII-human Fc fusion binds specifically to bioactive cMIS and not to non-activated holoMIS.



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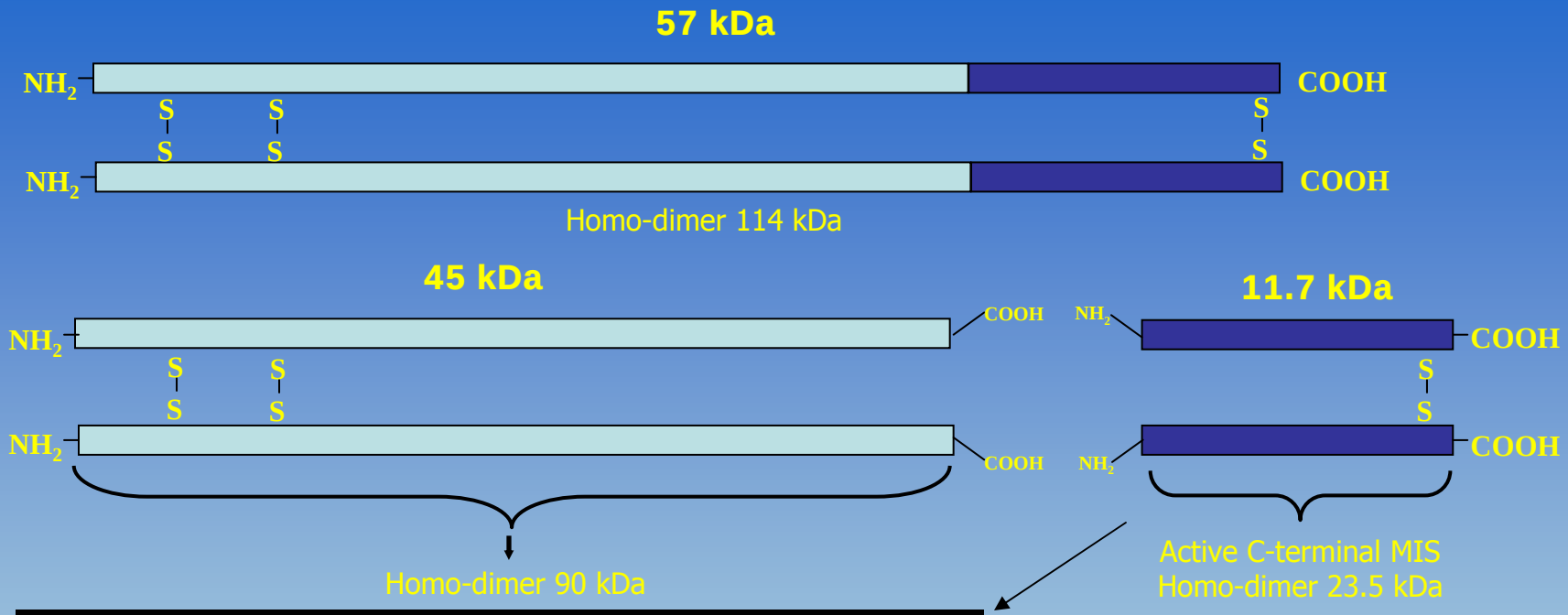
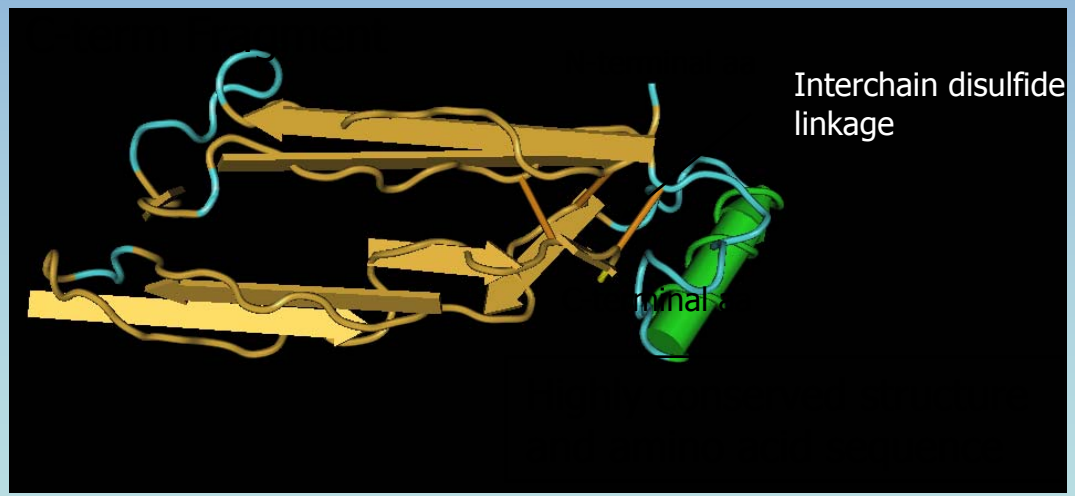
CTT Derived holo MIS





pI's:

A) HoloMIS: pH 7.2

B) cMIS: pH 8.3

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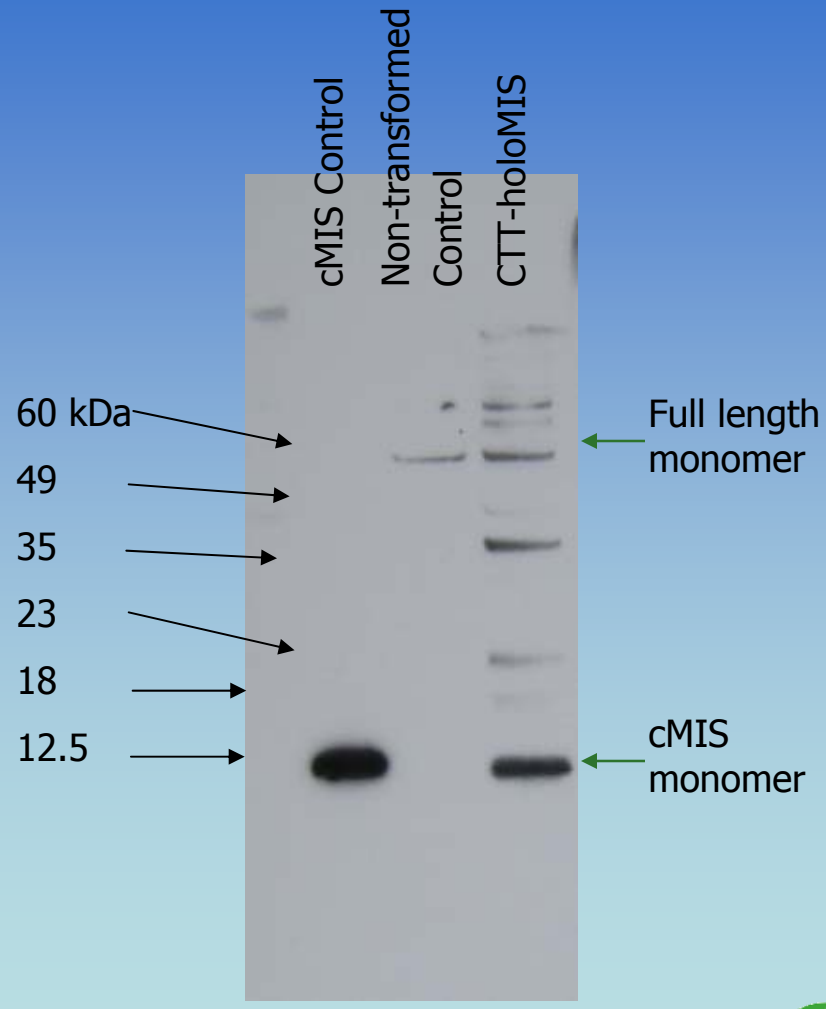
Human pro-Mullerian Inhibiting Substance from Tobacco Chloroplast



Reduced/Denatured NuPAGE
Chicken anti-cMIS HRP

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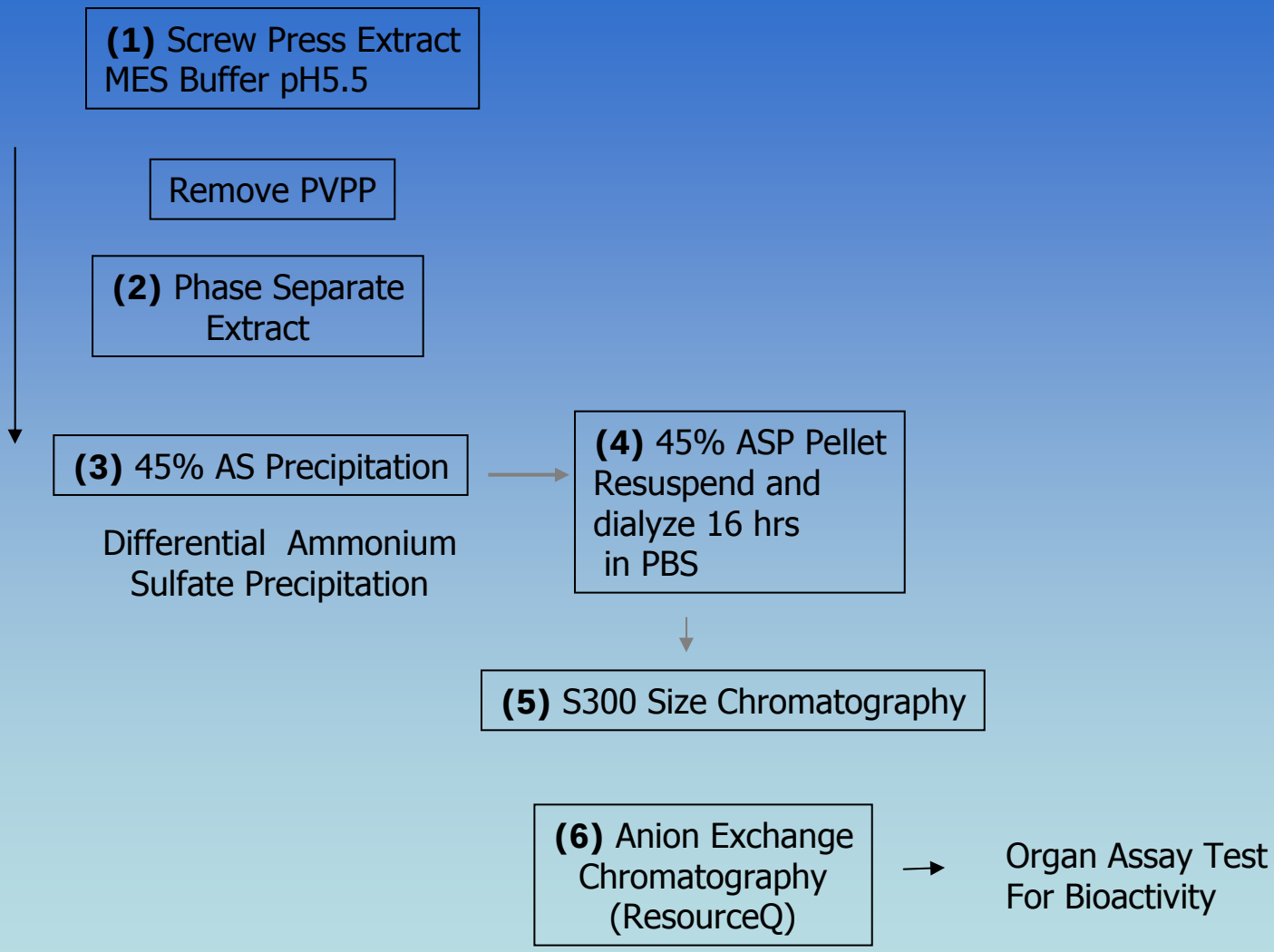
Small Scale Purification of holoMIS



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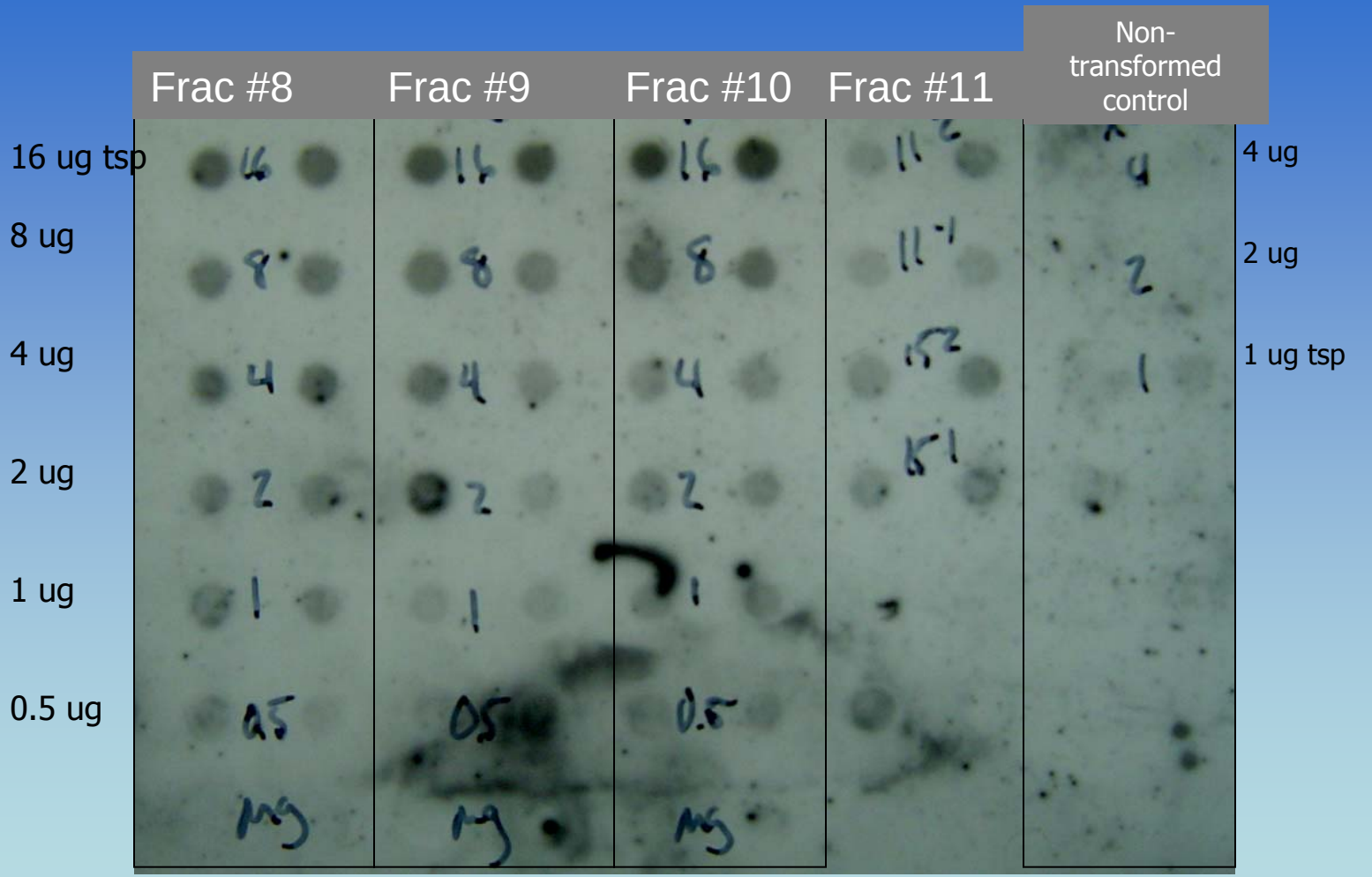
MISRII Receptor Binding Assay of Source Q Protein Fractions



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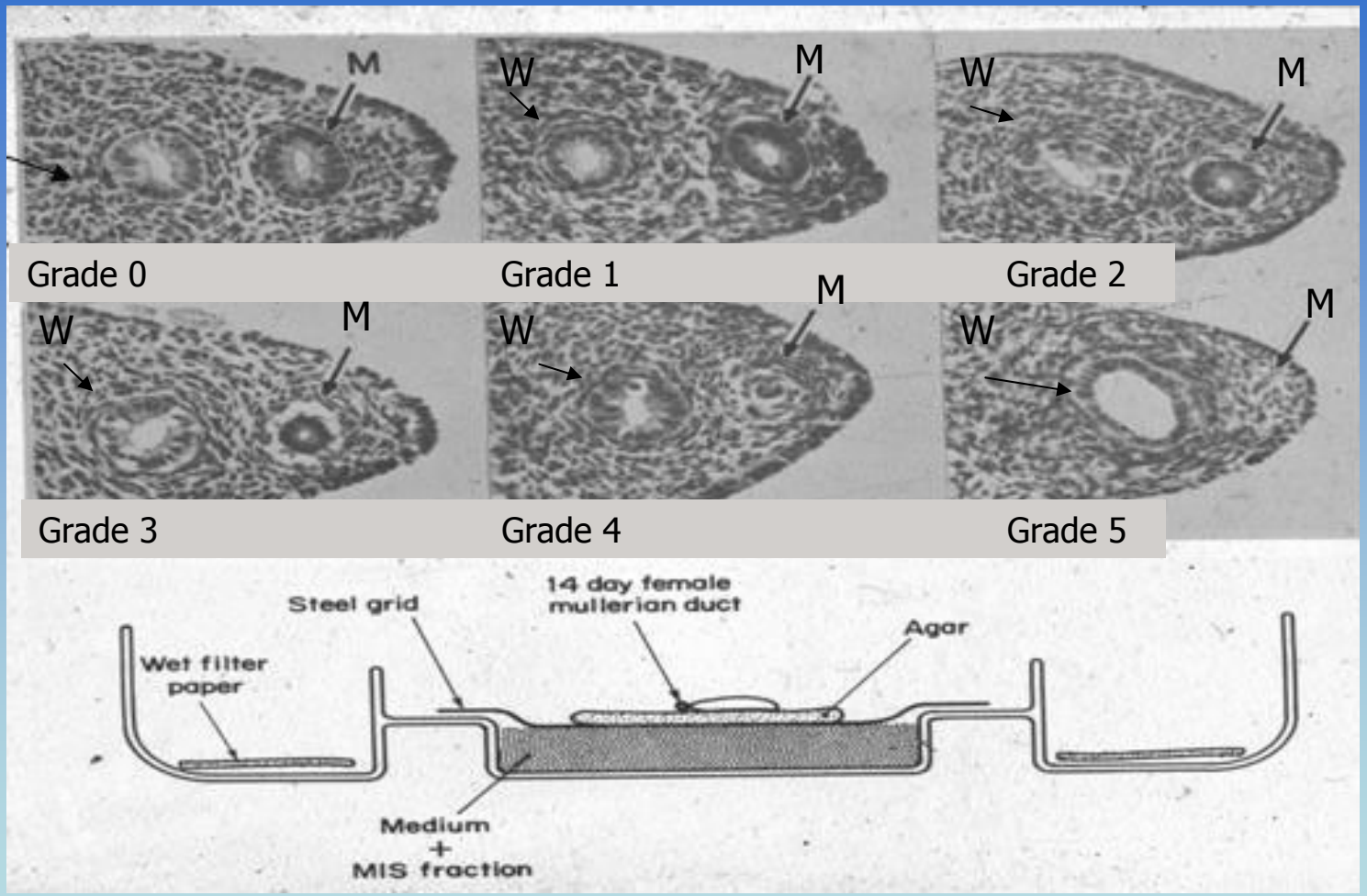
Müllerian Duct Regression Assay



Data from P Donahoe, MD and D MacLaughlin, PhD, Pediatric Surgery Research Laboratory, Massachusetts General Hospital, Charles River Plaza North, Room 6220, 185 Cambridge Street, Boston, MA 02114

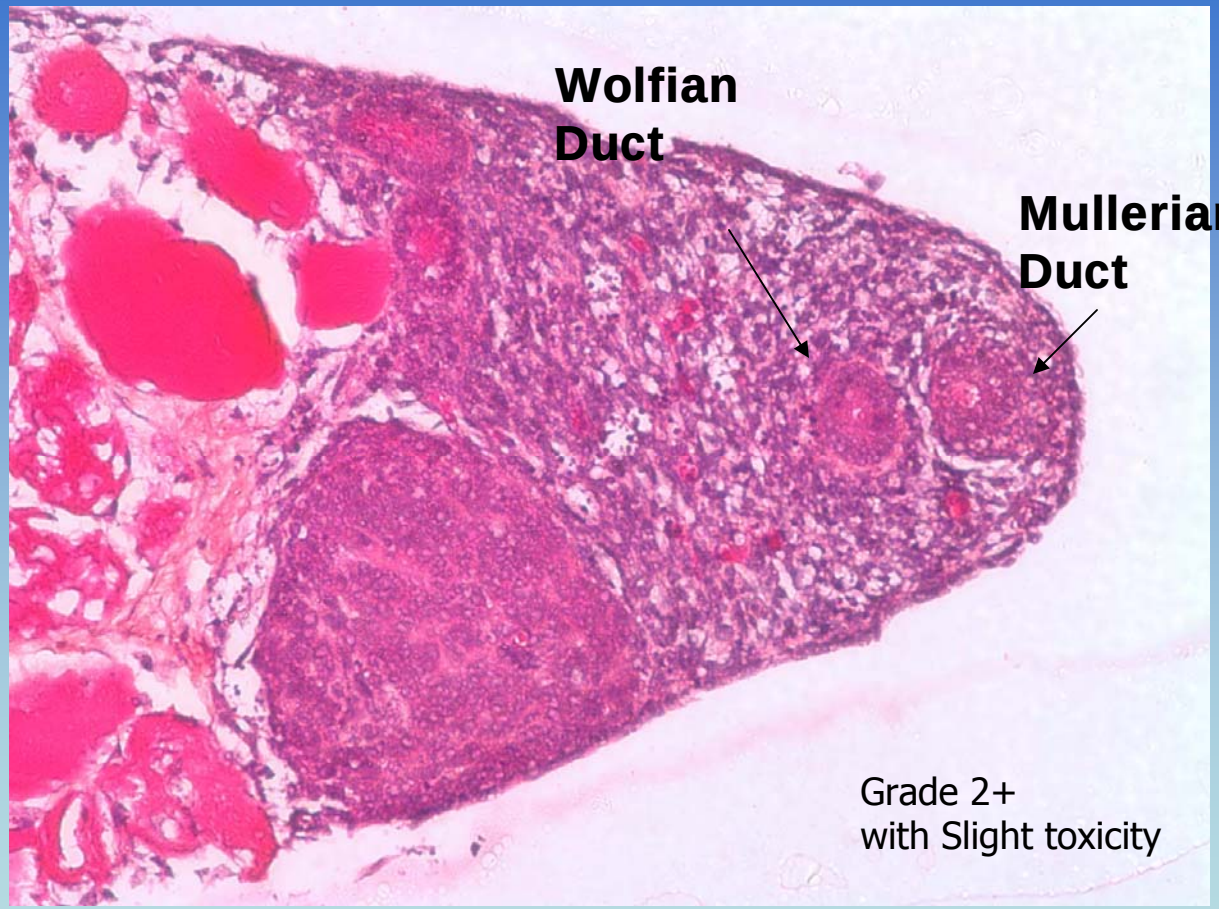
Mullerian Duct Regression Assay

Highly specific and sensitive assay for bioactive MIS

Mullerian Duct (Grade 0)	Mullerian Duct (Grade 1-2)	Mullerian Duct (Grade 2-3)	Mullerian Duct (Grade 3-4)	Mullerian Duct (Grade 4-5)
				
	size reduction	Disruption of internal structure	Duct is breaking apart	No Duct is visible

8 ug/mL
Bioactive (plasmin Cleaved) holoMIS for full regression

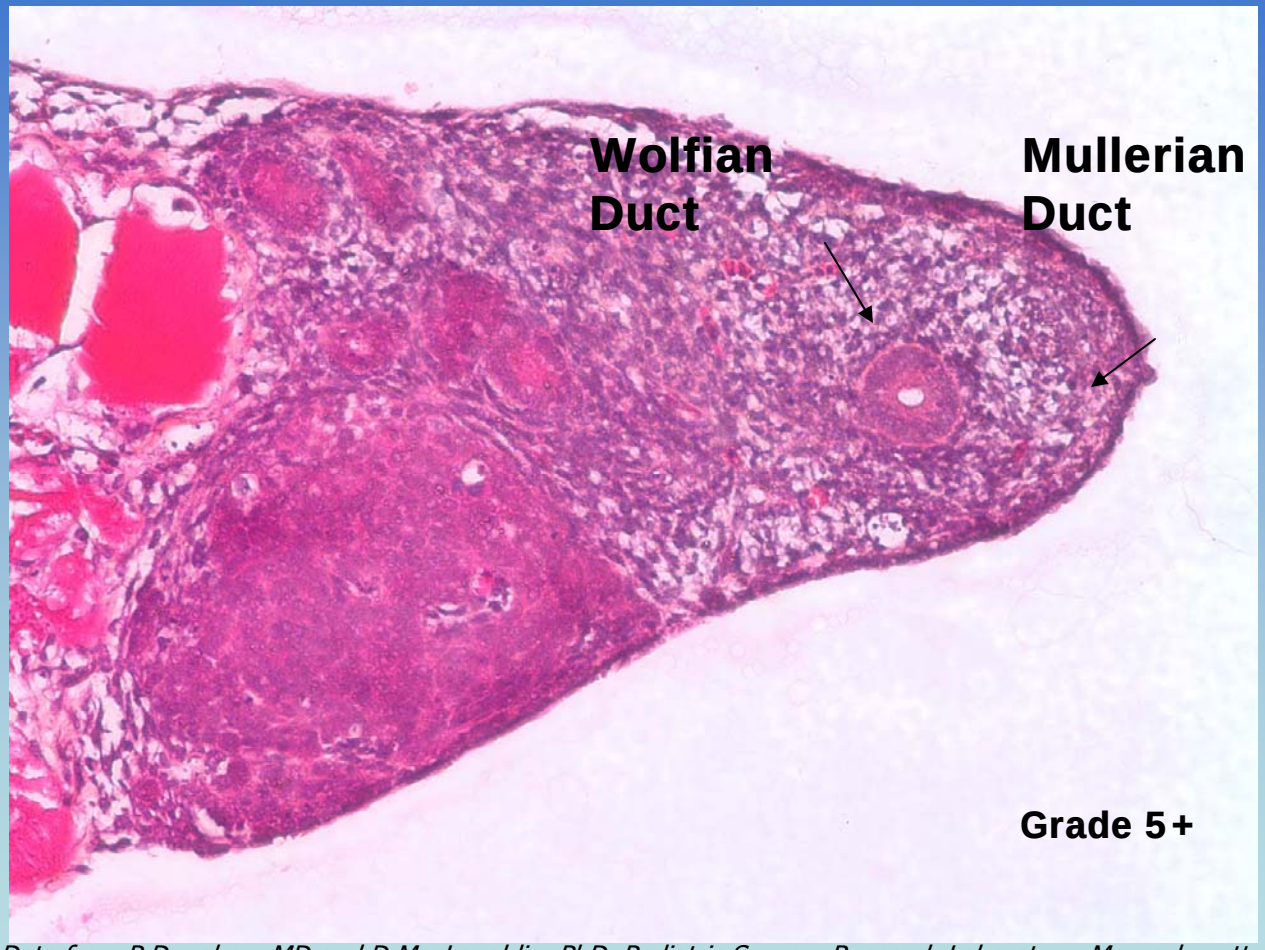
CTT MIS Resource Q Fraction #8 (100 uL)



- Fraction #8 was the least active by MISRII:Fc receptor binding assay.
- Mullerian duct has size reduction as well as membrane disruption = 2+

Data from P Donahoe, MD and D MacLaughlin, PhD, Pediatric Surgery Research Laboratory, Massachusetts General Hospital, Charles River Plaza North, Room 6220, 185 Cambridge Street, Boston, MA 02114

CTT MIS ResourceQ Fraction #9 (100 uL)

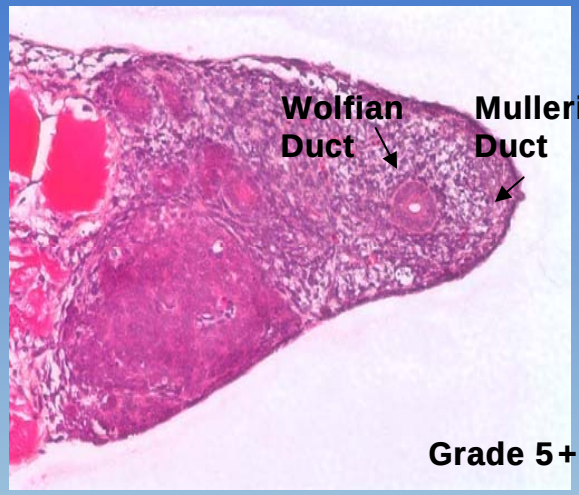


- Fraction #9 had the second best MISRII:Fc receptor binding activity.
- No visible presence of Mullerian Duct = 5+

Data from P Donahoe, MD and D MacLaughlin, PhD, Pediatric Surgery Research Laboratory, Massachusetts General Hospital, Charles River Plaza North, Room 6220, 185 Cambridge Street, Boston, MA 02114

Process for Bioactivity Testing of CTT-MIS

Mullerian Duct Regression Assay



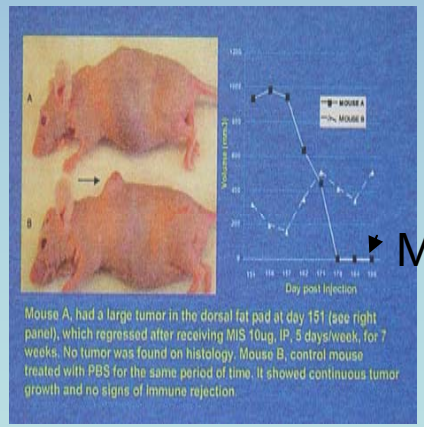
2

Apoptosis of Human Ovarian Cancer Cell Lines

3

Nude Mice (Immuno compromised) Xenographs of ovarian cancer cells

4



Mouse A

Allographs Mice – ovarian cancer mouse model

5

Prepre-animal Studies:
- Generation of dose response curve
- Safety (side effects)

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Significant Results

- Non-glycosylated TGF-β (MIS) can be assembled properly using CTT
- Non-glycosylated TGF-β (MIS) can be processed in plants into a bioactive form of MIS *in vivo*
- CTT has the potential to produce large-quantities of MIS for the ovarian, breast and other potential markets

Exclusive Rights from Tobacco Ventur, LLC:

Karen Keiko Oishi, Leonard Comaratta (2002) " Gene Expression and Production of TGF-beta Proteins Including Bioactive Mullerian Inhibiting Substance from Plants" US 60/295,545 (National and International Filing)

Chlorogen IP:

Karen Keiko Oishi, David Williams (2005) "Expressing TGF-beta Proteins in Plant Plastids".

Acknowledgements

Chlorogen, Inc (Creve Coeur, MO)

Karen K Oishi, PhD
David Corbin, PhD
Deb Weissenborn, PhD
Joshua Huffer
Lauren Gunther
Tammy Kershner
Kathy Schaberg
Sharon Berberich
Nick Duncan
David Williams
Barry Tulk, PhD
Melinda Mulesky, PhD
Steve Hosack
Bob Heeren
Steve Hill
Dave Berry

Danforth Plant Science Center (Creve Coeur, MO)
Roger Beachy, PhD
Karel Schubert, PhD
Ed Fisher
Dean Rochester

Mass General Hospital/Harvard (Boston, MO)
P Donahoe, MD
D MacLaughlin, PhD
Herb Lin, PhD

MGH Collaboration Funded by
NIH SBIR Chlorogen Grant #7 R43 CA093058

U Missouri, Delta Center (Portageville, MO)
Gene Stevens, PhD
Matt Rhine

Kentucky Tobacco Development Research Center (Lexington, KY)
Maelor Davis, PhD
Orlando Chambers

Kentucky BioProcessing,LLC (Owensboro, KY)
Barry Bratcher
Ernie Hiatt, PhD
Steve Hume
Jim Phelps

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A Cure for Cancer from Tobacco!

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