Chemical Modulation of Gap Junctional Intercellular Communication in Toxicology

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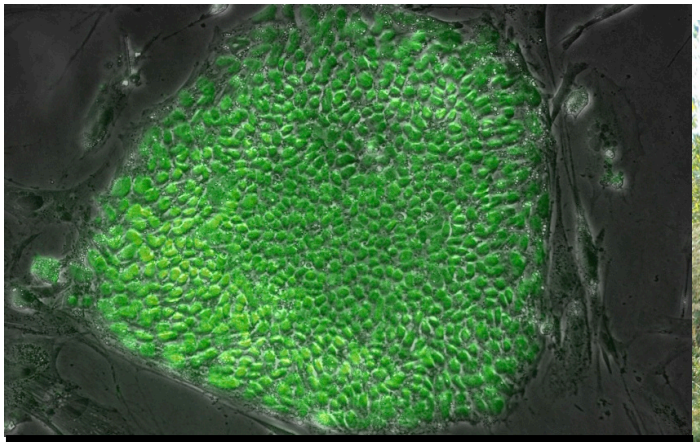
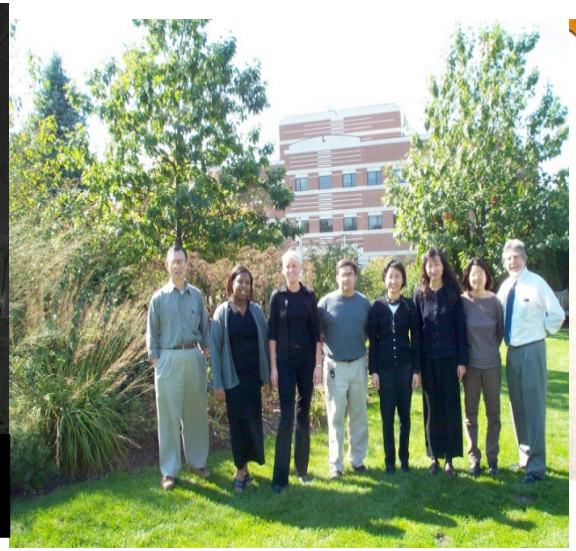
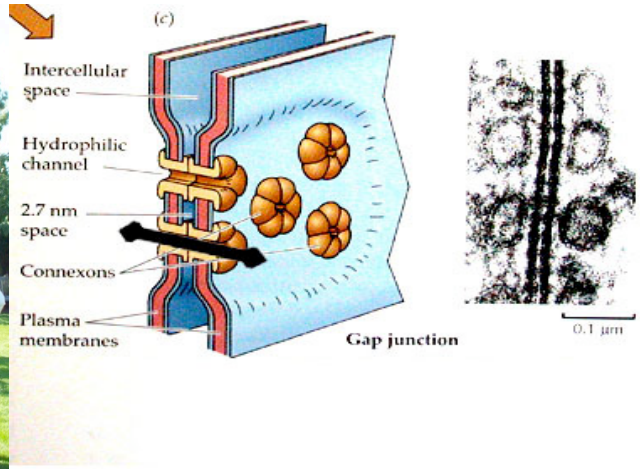


Human embryonic stem cells on feeder layer ; immunostained against Oct-4
http://www.che.wisc.edu/SPP/hescs.htm

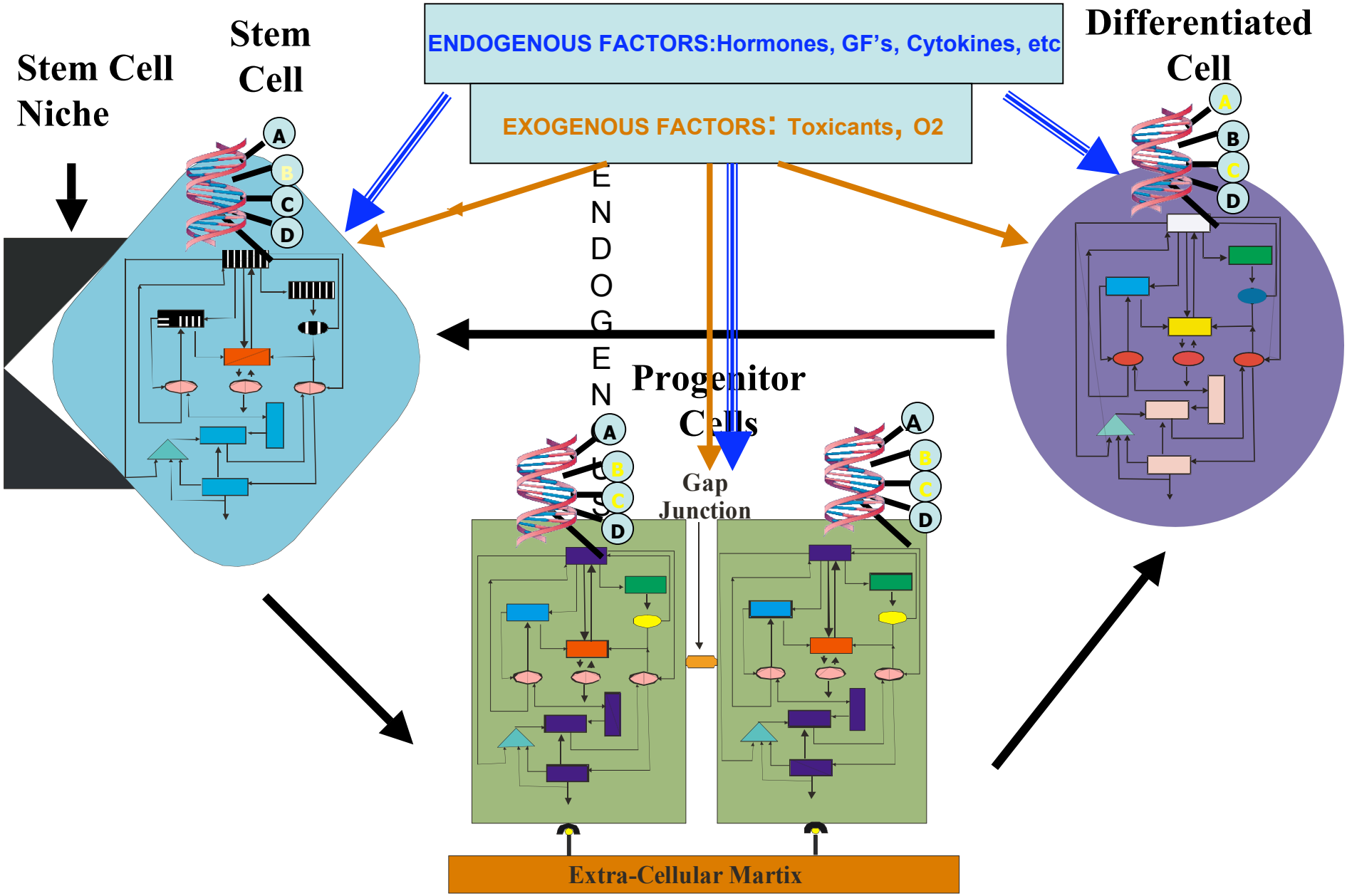




THREE ENDPOINTS OF TOXICITY

- **MUTAGENESIS-** "Genotoxicity:" POINT MUTATION & CHROMOSOME CHANGES
  - Due to errors in DNA repair or errors in replication
- **CELL KILLING-** "Cytotoxicity:" NECROSIS, APOPTOSIS, ANOIKIS
  - Due to non-specific mechanisms (necrosis) or epigenetic mechanisms (apoptosis; anoikis)
- **INAPPROPRIATE ALTERATION OF GENE EXPRESSION-** "Epigenetic toxicity:" TRANSCRIPTIONAL, TRANSLATIONAL, AND POST-TRANSLATIONAL MODULATION OF GENOME
  - Due to changes in intracellular signaling and cell-to-cell communication

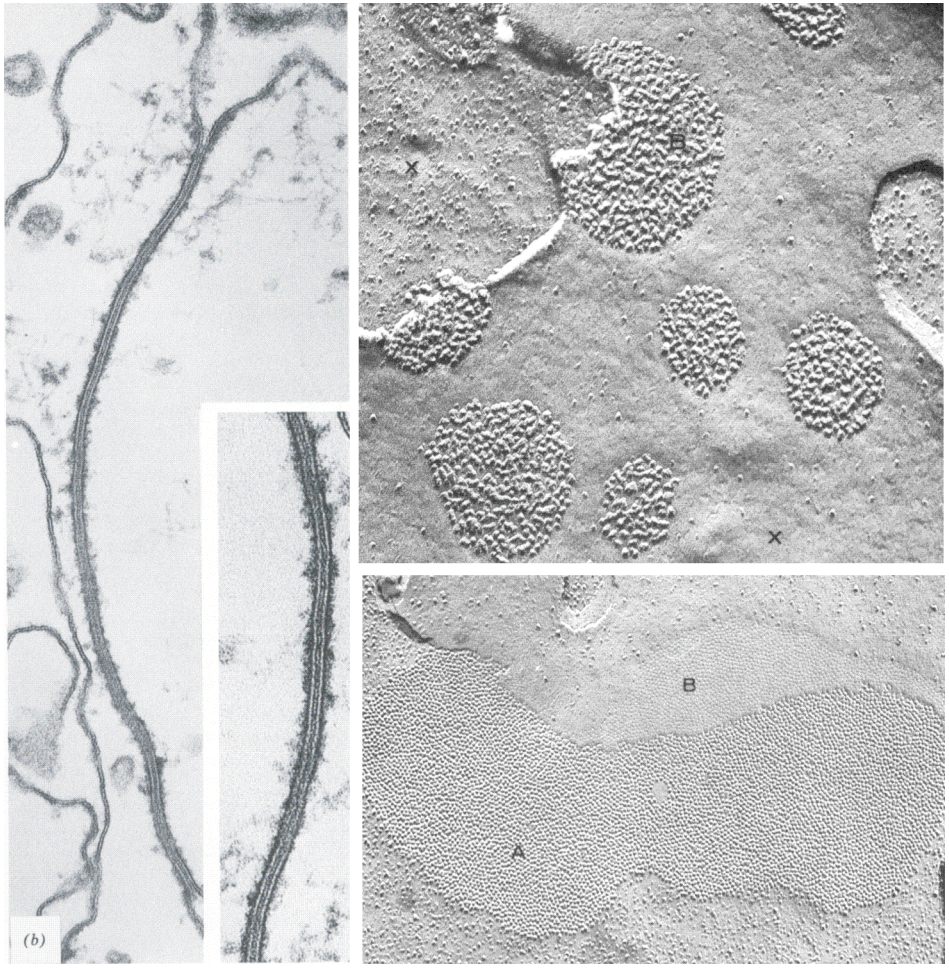
Systems Integration of Intracellular Signaling and Intercellular Signaling Of Stem Cells, Progenitor and Terminally-Differentiated Cells In Tissues

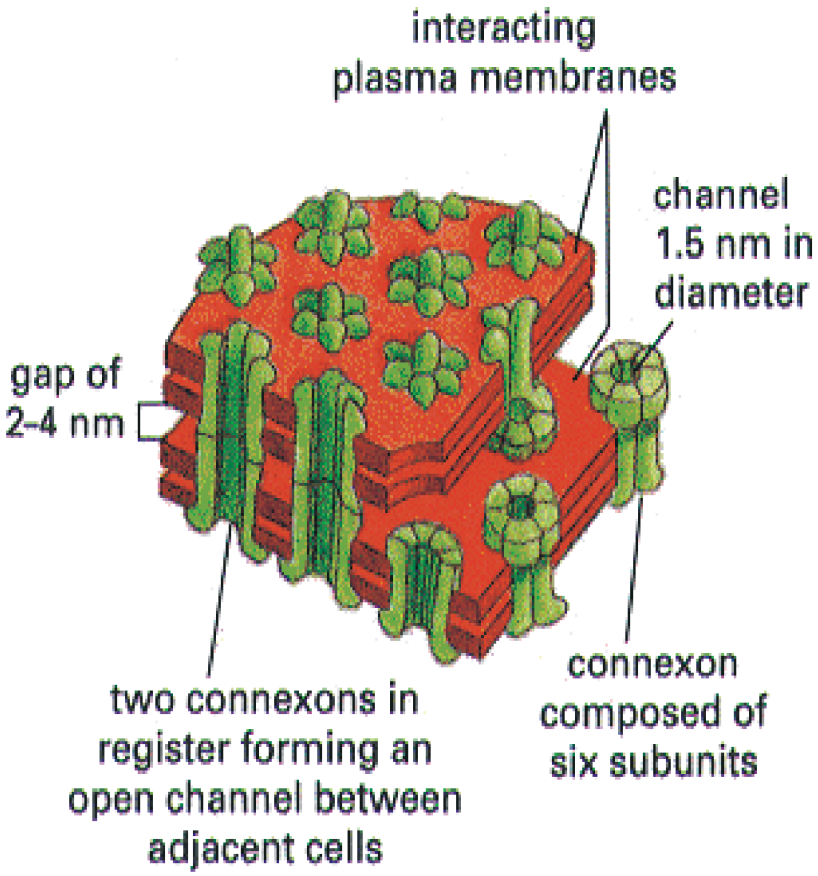


WHAT ARE GAP JUNCTIONS?

- FIRST APPEARED IN EVOLUTION OF METAZOANS.
- 20 CONNEXIN GENES ARE HIGHLY EVOLUTIONALLY CONSERVED.
- GJ's ARE FOUND IN ALL ORGANS AND TISSUES.
- ALLOW EQUILIBRATION OF IONS & SMALL SUBSTRATE MOLECULES BETWEEN COUPLED CELLS.
- ARE MODULATED BY ENDOGENOUS AND EXOGENOUS CHEMICALS.
- CANCER CELLS, WHICH DO NOT HAVE GROWTH CONTROL, DO NOT TERMINALLY DIFFERENTIATE, AND DO NOT APOPTOSE, DO NOT HAVE FUNCTIONAL GJIC.

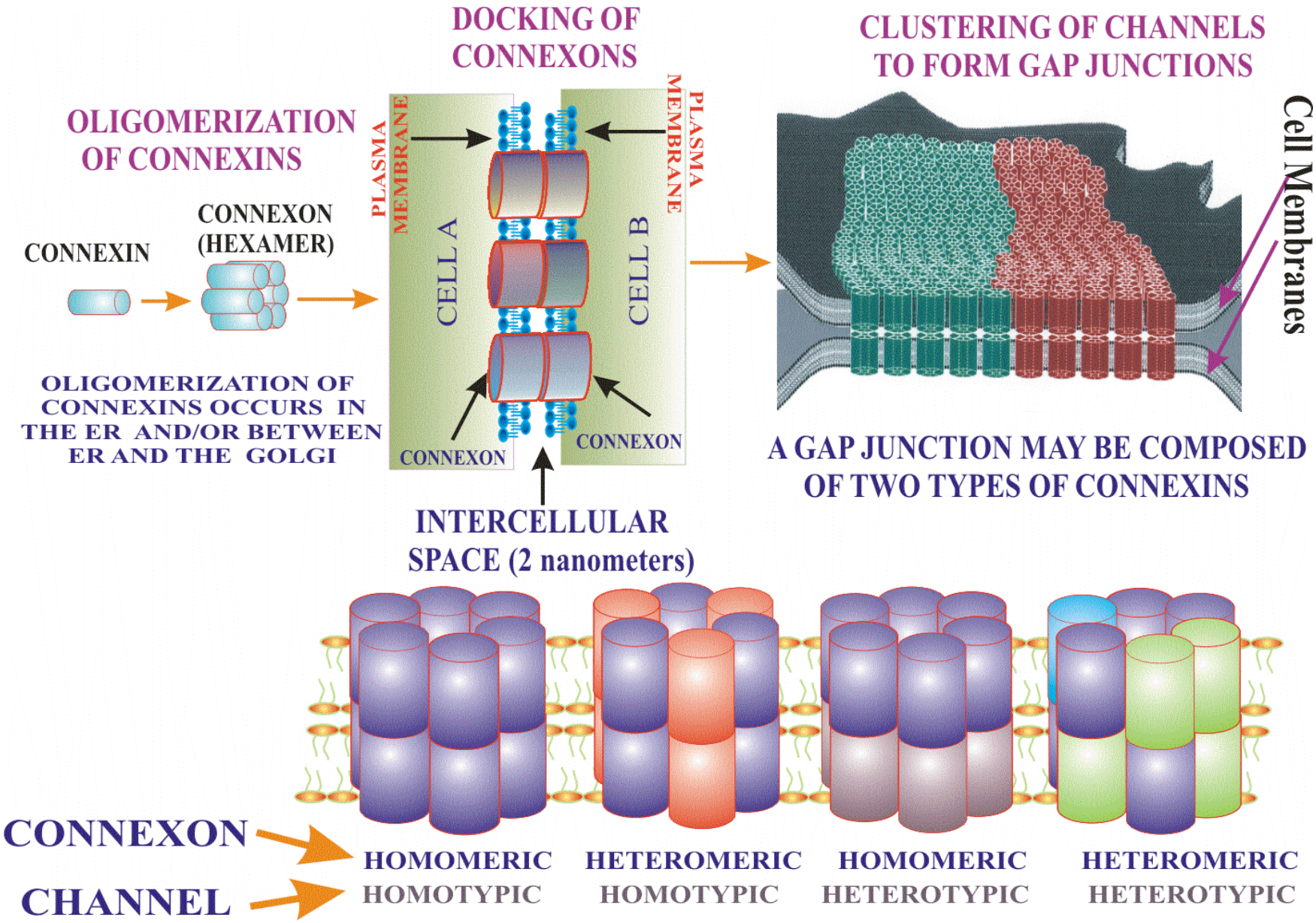
WHAT ARE GAP JUNCTIONS?





Connexin	TISSUE	Connexin	TISSUE
Cx25	?	Cx37	ENDOTHELIUM
Cx26	BREAST SKIN CHOCHLEA LIVER	Cx39 (m)	?
Cx29	MYELINATED CELLS	Cx40	HEART ENDOTHELIUM
Cx30	BRAIN CHOCHLEA SKIN	Cx40.1(H)	
Cx30.2(m)	SKIN	Cx43	REDUNDANT
Cx30.3	SKIN	Cx45	HEART NEURONS SMOOTH MUSCLE
Cx31	SKIN PLACENTA	Cx46	LENS
Cx31.1	SKIN	Cx47	BRAIN SPINAL CHORD
Cx31.9 (H)	BRAIN	Cx50	LENS
Cx32	LIVER Schwann Cells Oligodendrocytes	Cx57 (m)	OVARIES
Cx33	Testis	Cx62 (H)	OVARIES
Cx36	NEURONS	Cx59 (H)	?

GENESIS OF GAP JUNCTIONS



PROPERTIES OF CELL-CELL CHANNELS

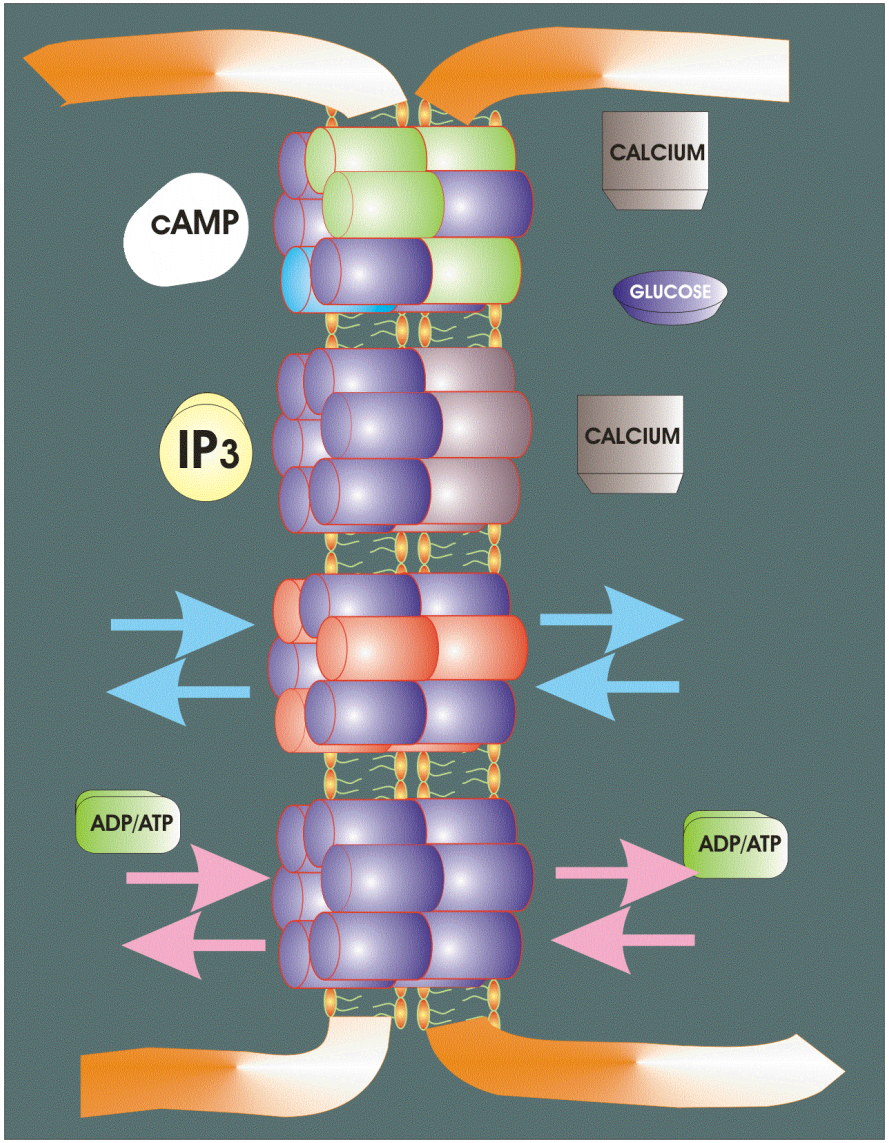
The channel is made by the collaborative efforts of two cells and traverses the membranes of two cells instead of one.

The channel is a bi-cellular structure made of two symmetrical halves which can be independently regulated by the interacting cells.

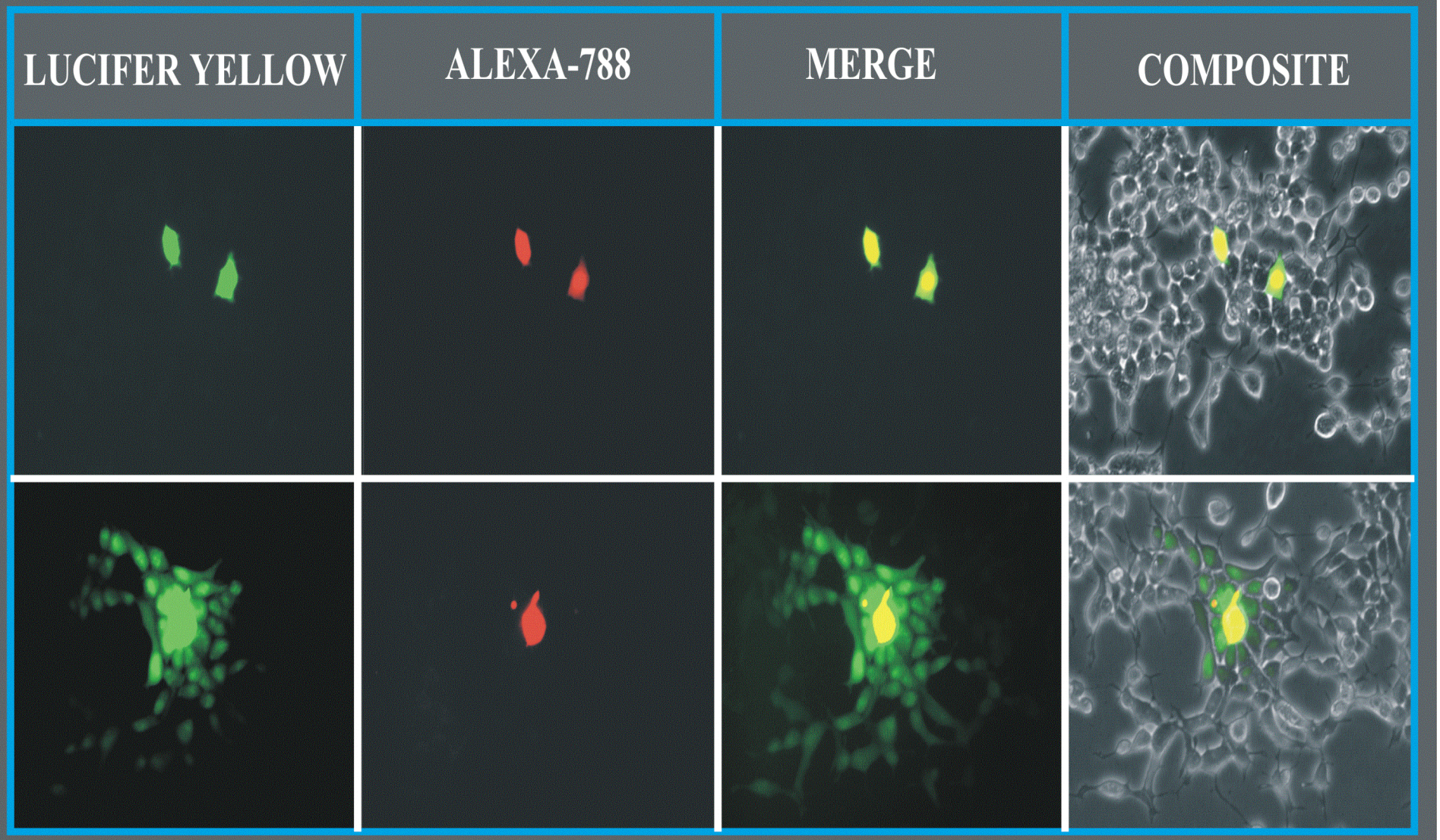
The channel has a diameter of 2 nm and molecules less than 1000 Da can traverse through.

The channel is not only a passive conduit but also acts as a molecular sieve.

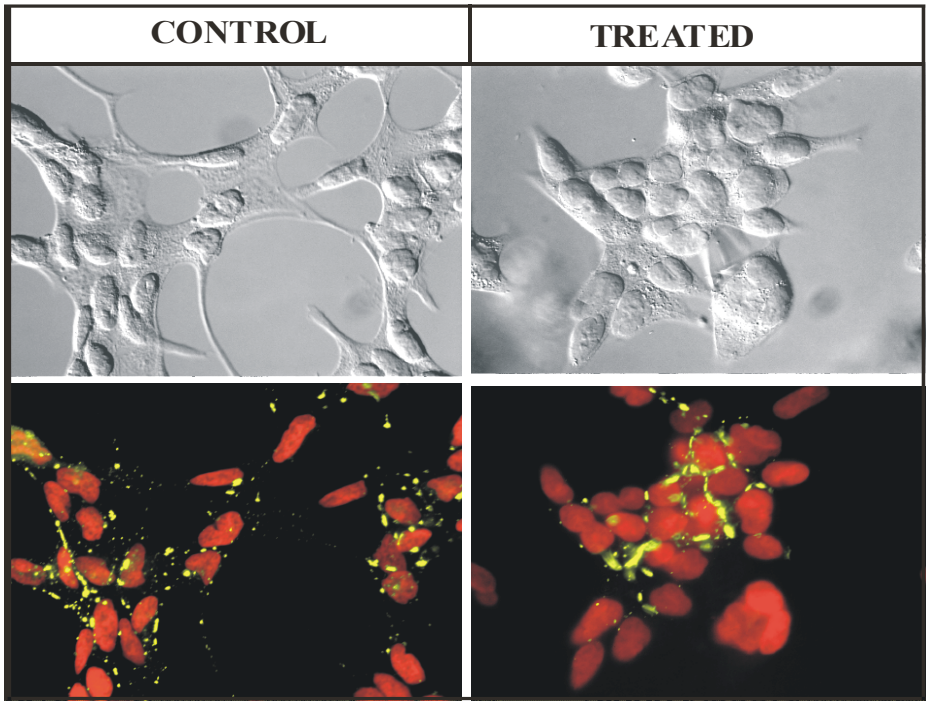
The channel is permeable to second messengers such as cAMP and IP3 and calcium.

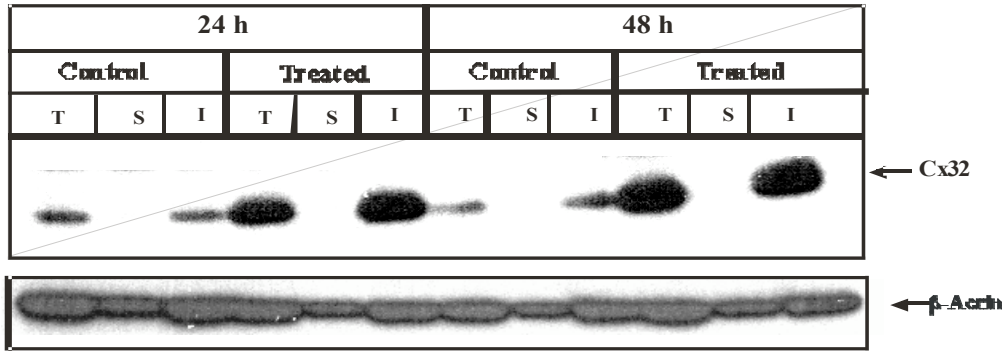


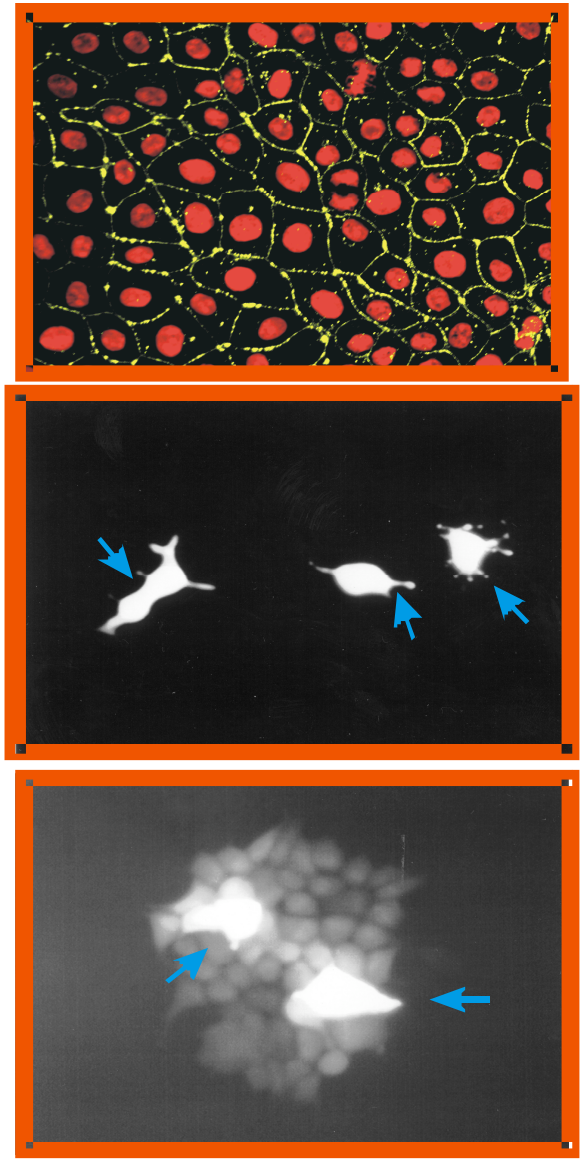
COMMUNICATION THROUGH GAP JUNCTIONS CAN BE STUDIED BY A VARIETY OF METHODS



METHODS


CONTROL
TREATED


24 h
48 h
Control
Treated
Control
Treated
T
S
I
T
S
I
T
S
I
T
S
I
Cx32
β-Actin



L. P. Yotti, C.C. Chang, J.E. Trosko, "Elimination of metabolic cooperation in Chinese Hamster Cells By A Tumor Promoter".

Science 206: 1089-1091, 1979.

operation is exemplified by the different syndromes of mucopolysaccharidosis (*6, 7*). Here cell-to-cell contact is not required, since cooperation appears to be mediated by means of a diffusible product. Metabolic cooperation has been shown to be influenced by such factors as different chemical analogs (*8*), cell lines (*9*), and membrane modifications (*10*). Cell-to-cell communication, thought to be involved in metabolic cooperation, has also been implicated in a variety of biological processes, including immune response (*11*) and growth control (*12*).

We report here a series of experiments that demonstrate the elimination of metabolic cooperation between 6-thioguanine-resistant (6-TG^r) and 6-thioguanine-sensitive (6-TG^s) Chinese hamster V79 cells by the potent tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA).

Since the original demonstration by Berenblum (*13*) of two-stage (initiation and promotion) carcinogenesis in mouse skin, a number of more recent studies have corroborated the two-stage conceptualization of tumorigenesis. Initiation seems to be the result of an irreversible cellular event that is induced by physical or chemical changes, whereas promotion appears to be a reversible process (up to a point) that depends on repeated treatment of the initiated cell by agents which are weakly carcinogenic or noncarcinogenic by themselves. Many different chemical compounds, given to animals after initiation with chemical or physical carcinogens, have been implicated as tumor promoters in several organ systems. The list includes chemicals as structurally and functionally dissimilar as butylated hydroxytoluene (*14*), phenobarbital (*15*), thyroid-stimulating hormone (*16*), bile acids (*17*), Tween 80 (*18*), alkanes (*19*), and cholesterol (*20*). Evidence favors the hypothesis that tumor initiation is a mutagenic event and promotion an epigenetic change (*21*).

In an attempt to delineate the biochemical mechanism of tumor promotion, TPA, the most potent of all known tumor promoters, has been examined quite extensively. When TPA is administered to cells in culture, a large number of responses are elicited. Among the most striking are an increase in the synthesis of DNA and RNA, stimulation of ornithine decarboxylase activity, an increase in the uptake of 2-deoxyglucose, an increase in prostaglandin synthesis, altered cellular morphology, and an increase in malignant transformations, and in the recovery of specific mutant somatic cells (*22*). Clearly, TPA is capable of inducing major cellular changes whose significance we do not yet fully understand.

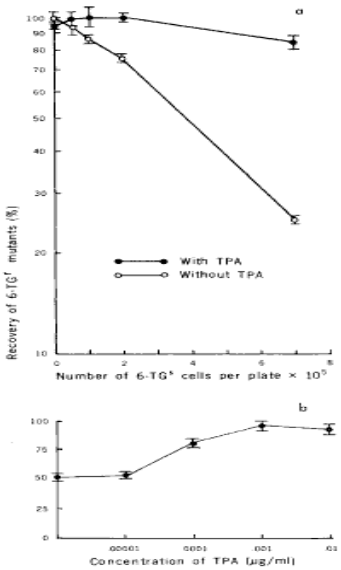


Fig. 1. (a) Effect of cell density on the recovery of 6-TG^r cells cultured with and without TPA. Wild-type 6-TG^s cells and a mixture of approximately 100 x-ray-induced 6-TG^r colonies were grown in modified Eagle's medium (Earle's balanced salt solution with a 50 percent increase of essential amino acids and vitamins) supplemented with a 100 percent increase of nonessential amino acids, 1 m*M* sodium pyruvate, and a 5 percent increase of fetal calf serum. In a humidified air atmosphere (5 percent CO_2) at 37°C, the two cell lines had a generation time of approximately 12 hours. Both cell lines were cultured simultaneously, allowed to attach themselves to the 9-cm-diameter plates (Falcon), and then were treated with TPA (1 μg/ml) and 6-thioguanine (10 μg/ml). The TPA was removed about 4 days after the cells were first cultured and replaced by a medium containing only 6-thioguanine. The colonies were fixed, stained with Giemsa, and scored for recovery about 3 days later. Percentage of recovery was determined as the average of the recovery in the ten plates in each treatment group. (b) Effect of concentration of TPA on the recovery of 6-TG^r cells. The culture conditions were identical to those in (a). In each plate, 100 6-TG^r cells were cultured with 8×10^5 6-TG^s cells. For each treatment group there were four control plates, in which 100 6-TG^r cells were cultured alone. None of the TPA concentrations had any significant effect on the efficiency with which cells in each group attached themselves to the plates and grew. Percentage of recovery was determined as the average of the recovery in the ten plates in each treatment group.

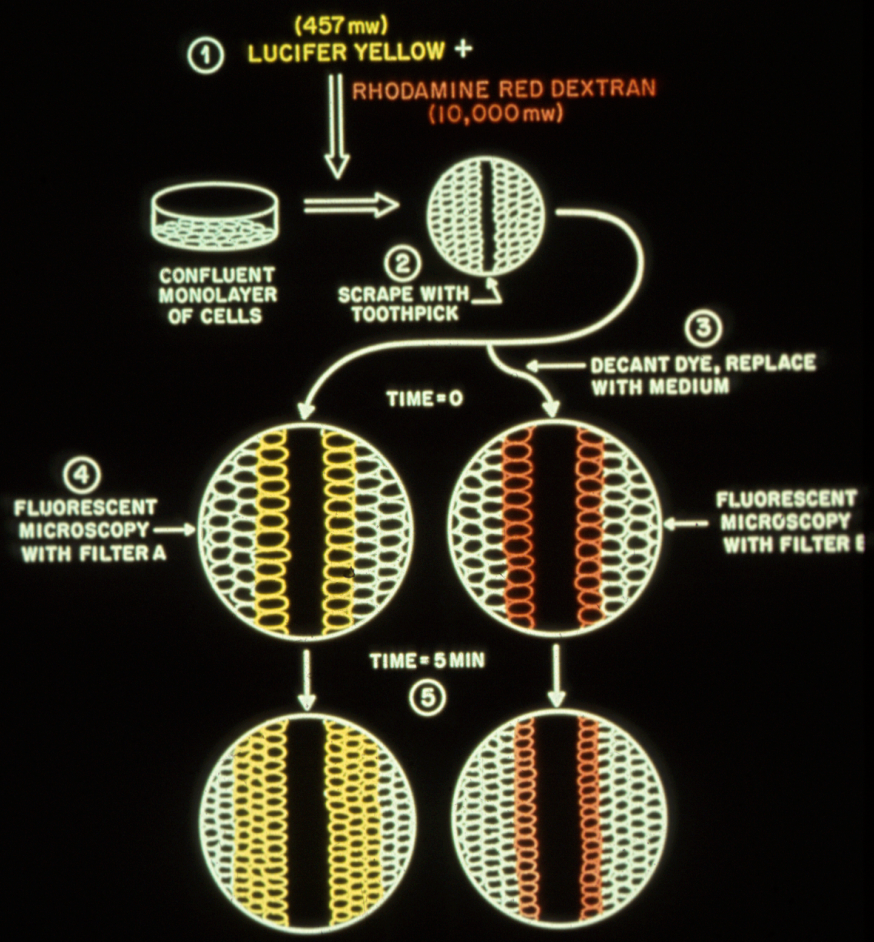
In an attempt to examine the biological effects of TPA on cellular membranes and the intercellular transport of small molecules, we cultured a small number of 6-TG^r V79 cells in the presence of various numbers of 6-TG^s cells; in each case, the number used was sufficient to reduce the recovery of the mutant cells (Fig. 1a). With no treatment, the recovery of the 6-TG^r cells diminishes precipitously when the number of wild-type cells increases. In the presence of 7×10^5 wild-type cells it is possible to recover as colonies approximately 25 percent of the 6-TG^r cells originally cultured. However, if the same experiment is conducted with the addition of TPA, the recovery of 6-TG^r cells is not significantly reduced; it is still possible to recover approximately 85 of the 100 6-TG^r cells originally cultured. In this series of experiments, TPA was present during the first 4 days of growth, at the end of which virtually all of the 6-TG^s wild-type cells had been killed. The TPA was then removed, and cultivation of the colonies in selective medium was continued for 4 to 5 days. Control experiments have clearly indicated that TPA does not enhance the efficiency with which 6-TG^r cells attach to the plate and grow when they are cultured alone (data not given). Therefore, we feel that TPA somehow blocks metabolic cooperation, thereby allowing mutant 6-TG^r cells to proliferate in the medium.

In an attempt to determine whether the modification of the recovery of 6-TG^r cells by TPA was dose-responsive, we performed the following experiment. Using 8×10^5 6-TG^s cells and 100 6-TG^r cells per plate, we measured the recovery of the resistant cells after exposure to TPA (0.01 to 10 ng/ml). A dose-responsive relationship was clearly demonstrated when TPA (1 ng/ml) was sufficient to allow the recovery of almost 100 percent of the 6-TG^r cells (Fig. 1b).

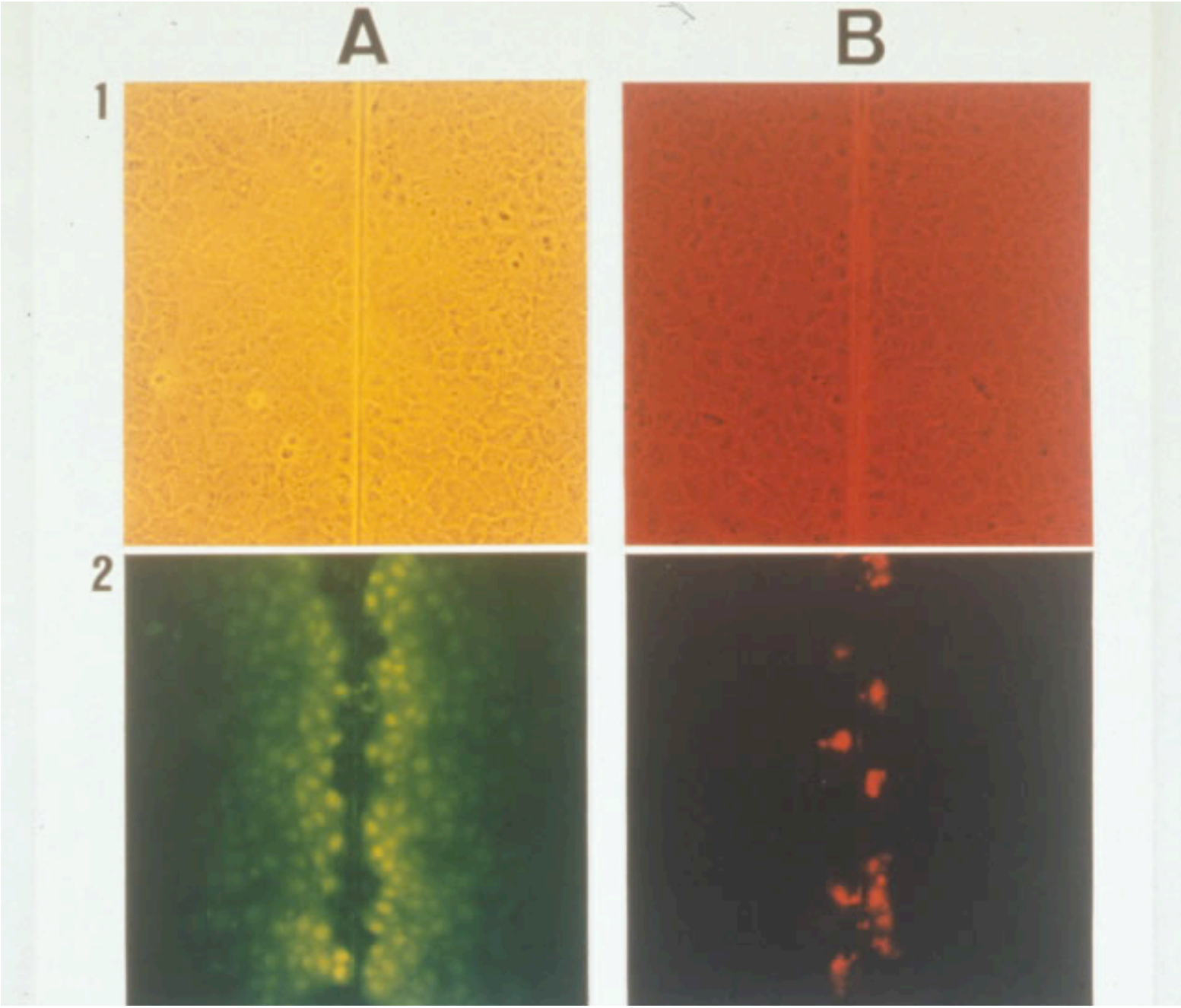
Table 1 gives the results of an experiment to determine whether this system is capable of discriminating between tumor promoters of various degrees of potency in vivo. In addition to TPA and phorbol (the parent alcohol of TPA), we examined five commercially synthesized, structural analogs of TPA. Excellent correlation was observed between the abili-

COVER OF SCIENCE ILLUSTRATING THE “FRAP” TECHNIQUE

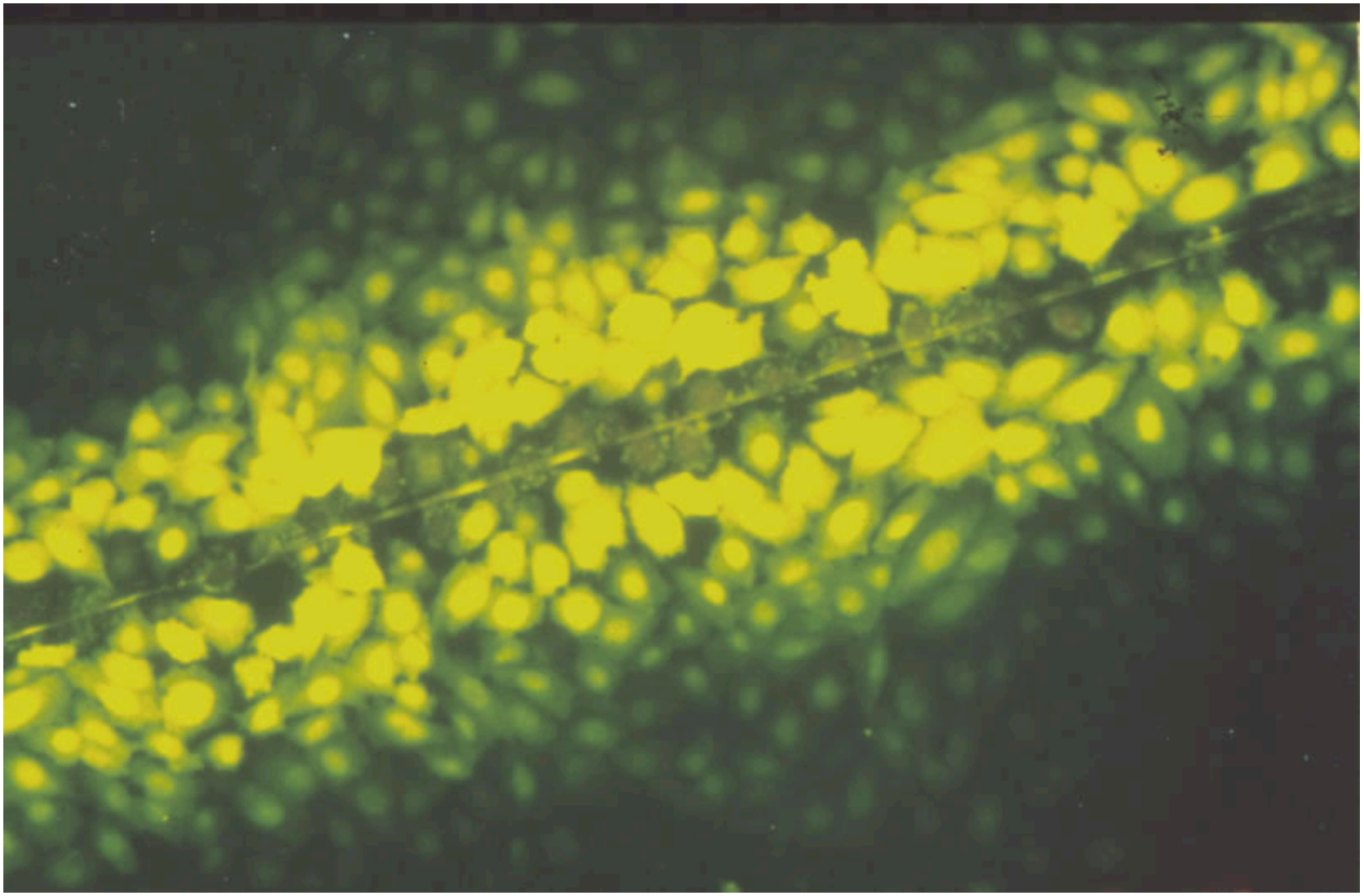
Scrape Loading / Dye Transfer Technique



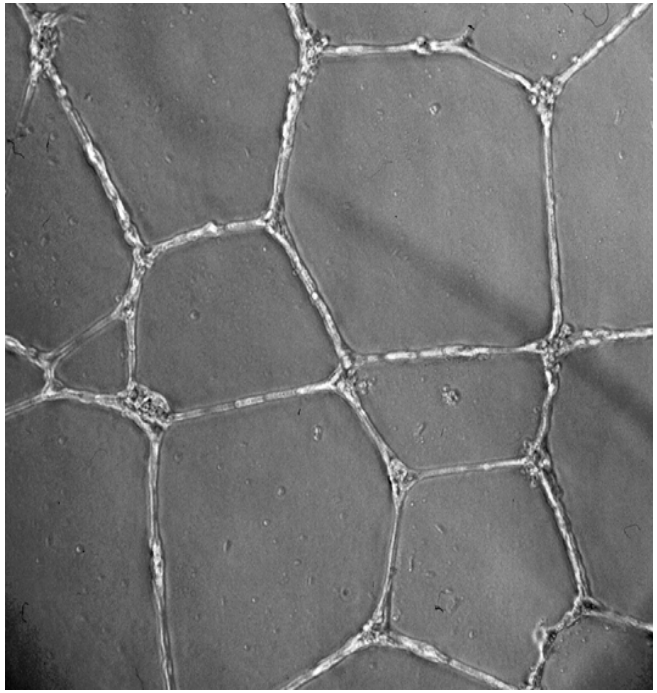
PRINCIPLE: If cells have gap junctional communication, Lucifer yellow will diffuse only thru gap junctions, rhodamine red dextran will not.


A
B
1
2

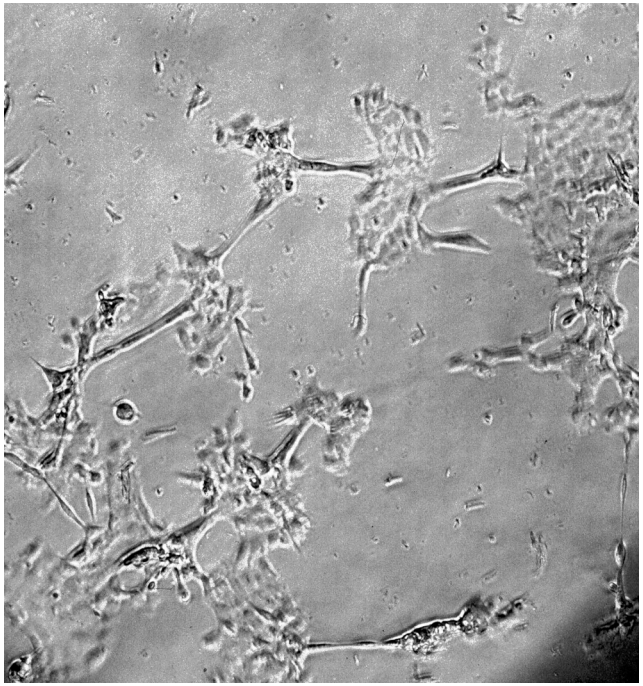
Most Normal Cells Have Functional Gap Junctional Intercellular Communication (GJIC)



WB-cells grown on Matrigel Cell-Differentiating System

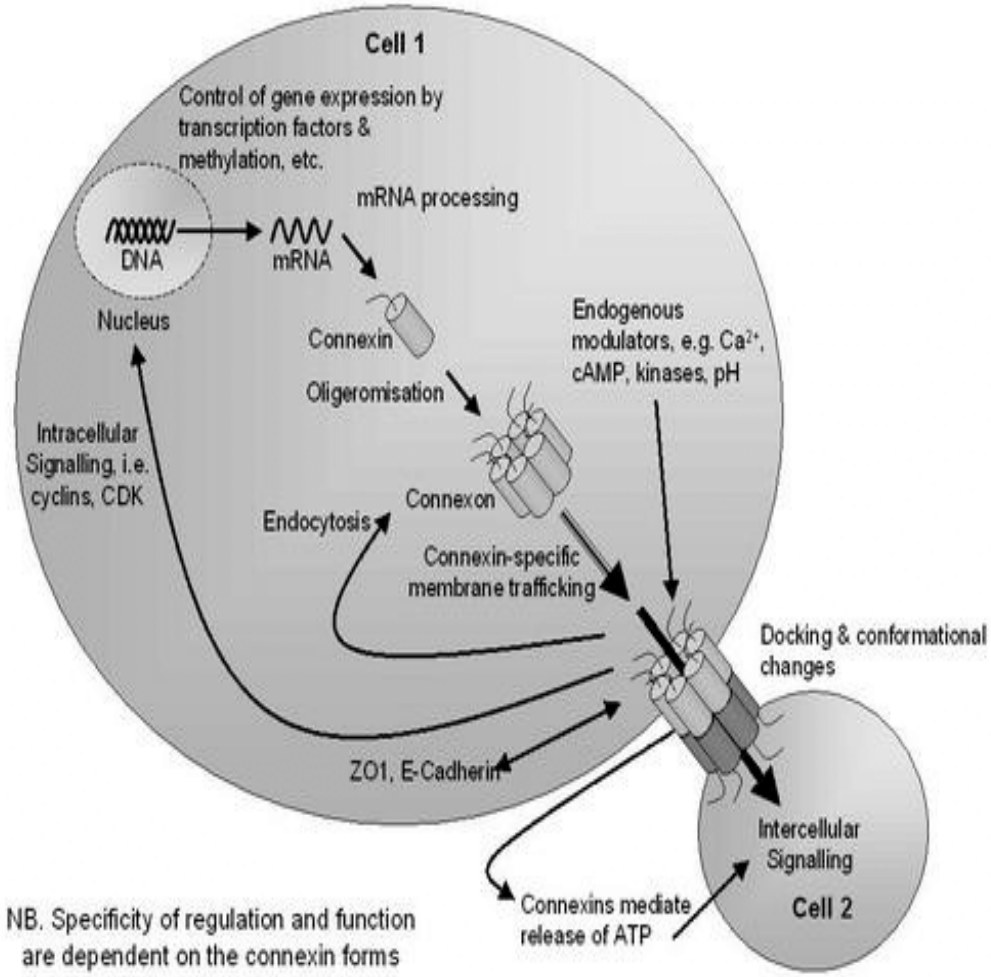


WT-WB cells

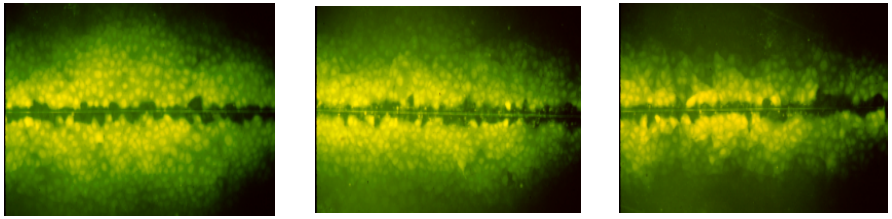


DN-Cx43WB cells

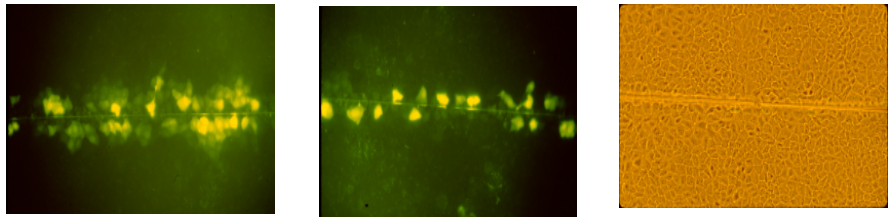
THE MOLECULAR BIOLOGY OF MODULATED GAP JUNCTIONAL INTERCELLULAR COMMUNICATION



Scrape Load/Dye Transfer Assay



0 ng/ml TPA | 1 ng/ml TPA | 2 ng/ml TPA



4 ng/ml TPA | 6 ng/ml TPA | 6 ng/ml TPA

Classes of Non-Mutagenic Chemicals Which Down-Regulate GJIC in Normal Cells: Potential Epigenetic Toxicants

- Natural chemicals: phorbol esters
- Toxins: vomatoxin, T-2 toxic and LPS
- Hormones: estrogens
- Growth factors: EGF, PDGF, TGF-α and TNF-α
  Pesticides: DDT and dieldrin
- Herbicides: 2,4 D and 2,4,5-T
- Cytokines: interleukin-1α, ceramides and prostaglandins

Chemicals That Down-Regulate GJIC in Normal Cells: Potential Epigenetic Toxicants

- Pollutants: PCBs, PBB and TCDD
- Heavy metals: methylmercury and cadmium
- Solid particles: airborne particulates and [60] fullerene (nano-particules)
- Nutrients: unsaturated fatty acids
- Drugs: phenobarbital
- Food additives: saccharin and carrageenan
- SO-CALLED MUTAGENIC-CARCINOGENS: MNNG, DMBA, nitrosamines, DNFB, estrogen, cigarette smoke or grill proteins-PAH's

EVIDENCE OF THE REVERSIBILITY OF TCDD'S TOXIC EFFECT: PROMOTION OF ADULT SKIN STEM CELLS?

CHEMICALS THAT UP-REGULATE or PREVENT THE DOWN-REGULATION OF GAP JUNCTIONS

- BOTH CHEMOPREVENTIVE and CHEMOTHERAPEUTIC AGENTS THAT HAVE BEEN DOCUMENTED AS MODULATORS OF GJIC.

- THE DISCREPANCIES IN THE LITERATURE ARE DUE, IN LARGE PART, TO THE LACK OF KNOWLEDGE OF HOW CHEMICALS INHIBIT OR ENHANCE GAP JUNCTIONS.

- BEST EXAMPLE IS THE FAILURE OF THE CARET & ATBC HUMAN INTERVENTION STUDIES. RETINOIDS, FOR EXAMPLE, HAVE BEEN SHOWN TO BE PRO-OXIDANTS and ANTI-OXIDANTS, DEPENDING ON CIRCUMSTANCES, AND THEY CAN UP- OR DOWN-REGULATE GJIC.

Chemicals Which Up-Regulate GJIC in Cancer Cells: Chemopreventive/Chemotherapeutic Agents

- **Carotenoids** – L Zhang et al., Carcinogenesis 12:2109-2114, 1991.
- **Retinoids** – T Shuln et al., Gann 74:100-105, 1983.
- **Green Tea Components** – K Sigler and R Ruch, Cancer Letters 69:15-19, 1993.
- **Vitamin D** – A Clairmont et al., Carcinogenesis 17:1389-1391, 1996.
- **Japanese Soy Extract: "Natto"** – C Takahashi et al., Carcinogenesis 16:471-476, 1995.

Chemicals Which Up-Regulate GJIC in Cancer Cells: Chemopreventive/Chemotherapeutic Agents

- **Glycyrrhetic Acid** – JE Davidson and J Pharm, Exper. Therap. 246:1104-1107, 1988.
- **Lovastatin –** R Ruch et al., Molec. Carcinogenesis 7:50-53, 1993.
- **Irsogladine** – F Ueda et al., J. Pharm Experm. Therap. 274:815-819, 1995.
- **Theophylline, Forskolin, Isoprotererol** – Traub et al., Euro J. Cell Biol. 43:48-54, 1987.
- **Glucocorticoids** – P Ren et al., Carcinogenesis 15:1807-1813, 1994.

Chemicals Which Up-Regulate GJIC in Cancer Cells: Chemopreventive/Chemotherapeutic Agents

- **Chinese Medicine Mixture L2** – ZQ Zhang et al., Beijing Institute for Cancer Research 16:88-92, 1994.
- **Lycopene** – V Krutovskikh et al., Japan J Cancer Research 88:1121-1124, 1997.
- **Hexamethylene Bisacetamide** – T Ogawa et al., Kidney International 60:996-1008, 2001.
- **Caffeic Acid Phenethyl Ester** – H-K Na et al., Cancer Letters 157:31-38, 2000.
- **Melatonin** – T Kojima et al., Cell Struc. Funct. 22:347-356, 1997.

Chemicals Which Up-Regulate GJIC in Cancer Cells: Chemopreventive/Chemotherapeutic Agents

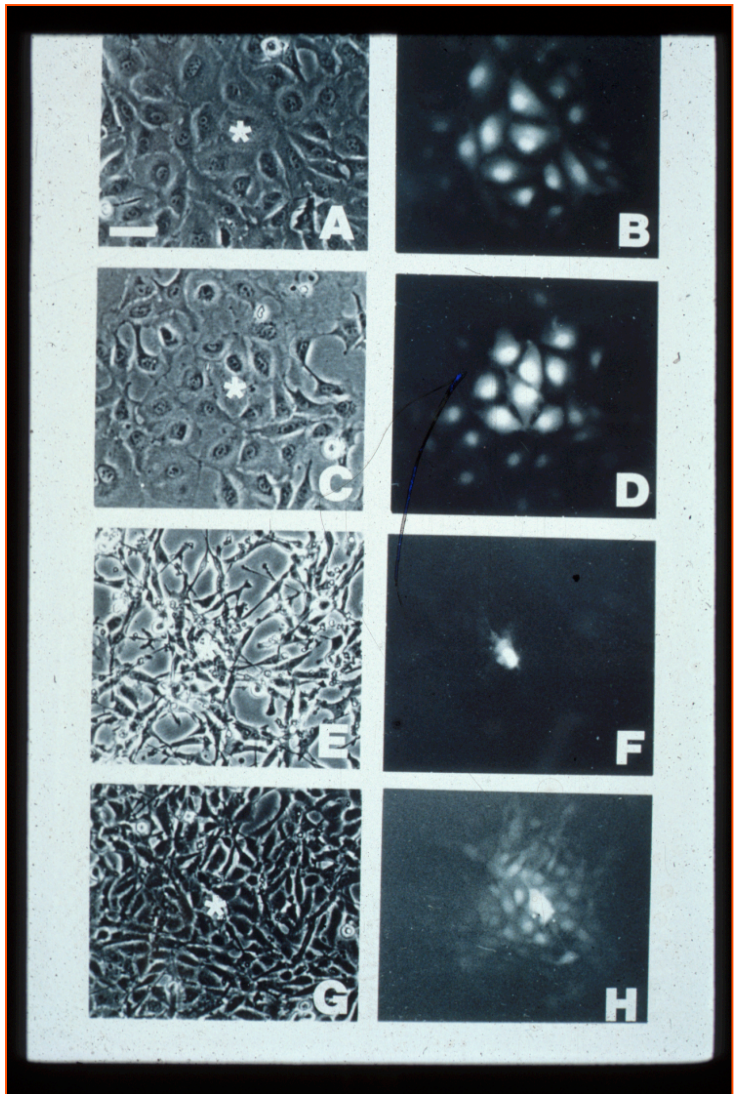
- "Augmentation of differentiation and gap junction function by kaempferol in partially-differentiated colon cancer cells". Nakamura, Y., Chang, C.C., Mori, T., Sato, K., Ohtsuki, K., Upham, B.L., and Trosko, J.E. Carcinogenesis 26: 665-671, 2005.

"Beta- Sitosterol from psyllium fibers". :Y. Nakamura et al, Nutrition and Cancer, 51:218-225, 2005.

" Inhibition of gap junctional intercellular communication and activation of mitogen-activation protein kinase by tumor-promoting organic peroxides and protection by resveratrol". Upham,B.L., Guzvic, M., Scott, J., Carbone, J.M., Blaha, L., Coe, C., Li, L.L., Rummel, A.L. and J.E. Trosko, Nutrition Cancer 57: 38-47, 2007

"Suberoylanilide hydroxamic acid-SAHA (Histone deacetylase inhibitor)". T. Ogawa, T. Hayashi, K.. Nakakachi, J.E. Trosko, C.C. Chang, and N. Yorioka, Cancer Res. 65: 9771-9778, 2005.

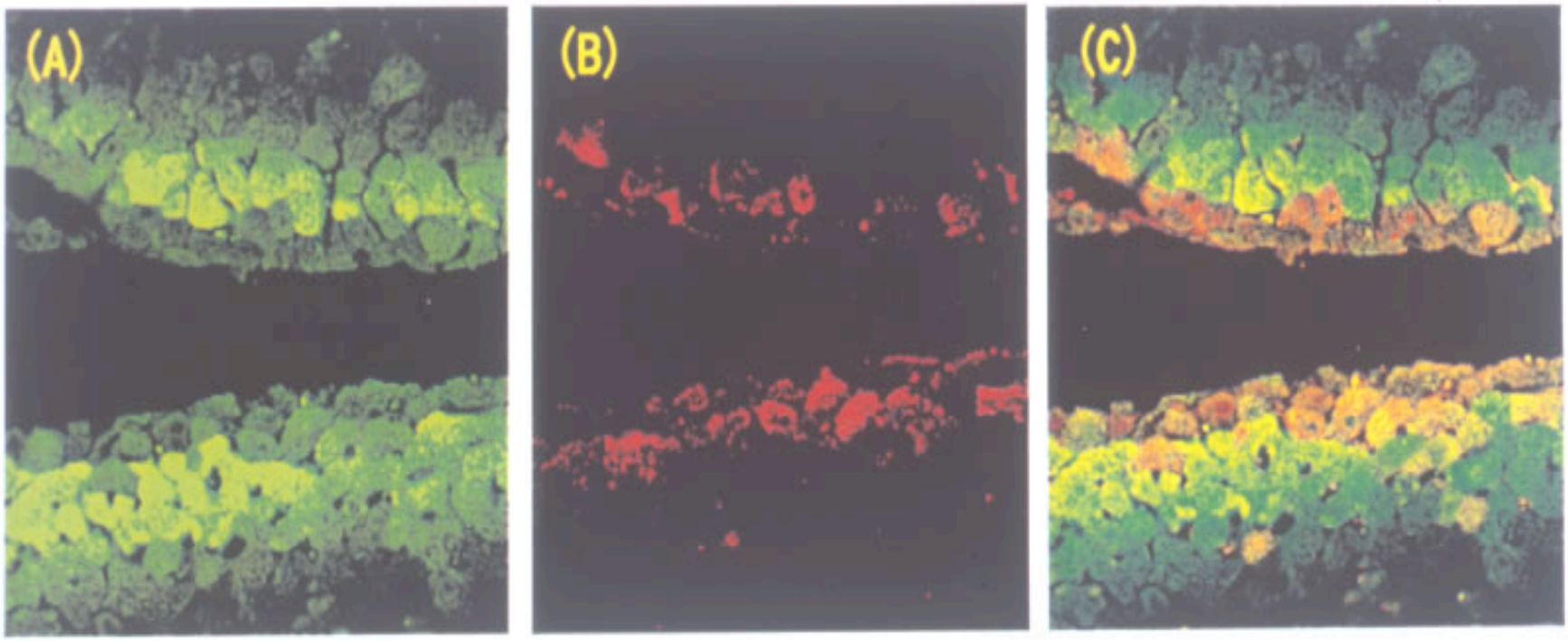
Anti-Oncogene Drugs Such as Lovastatin, Specifically Reverse ras-Down Regulation of GJIC



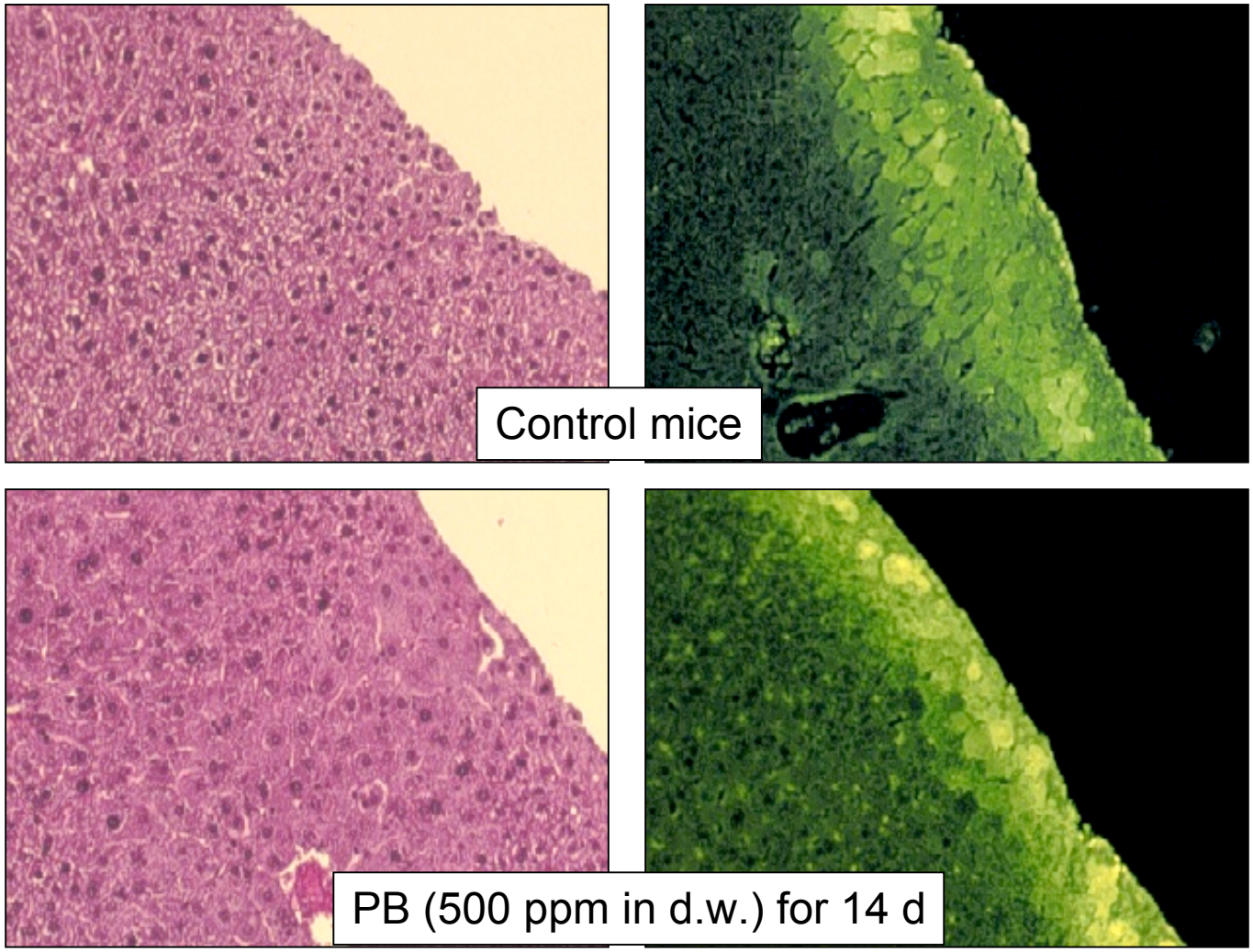
DEMONSTRATION OF GAP JUNCTIONAL COMMUNICATION IN VIVO

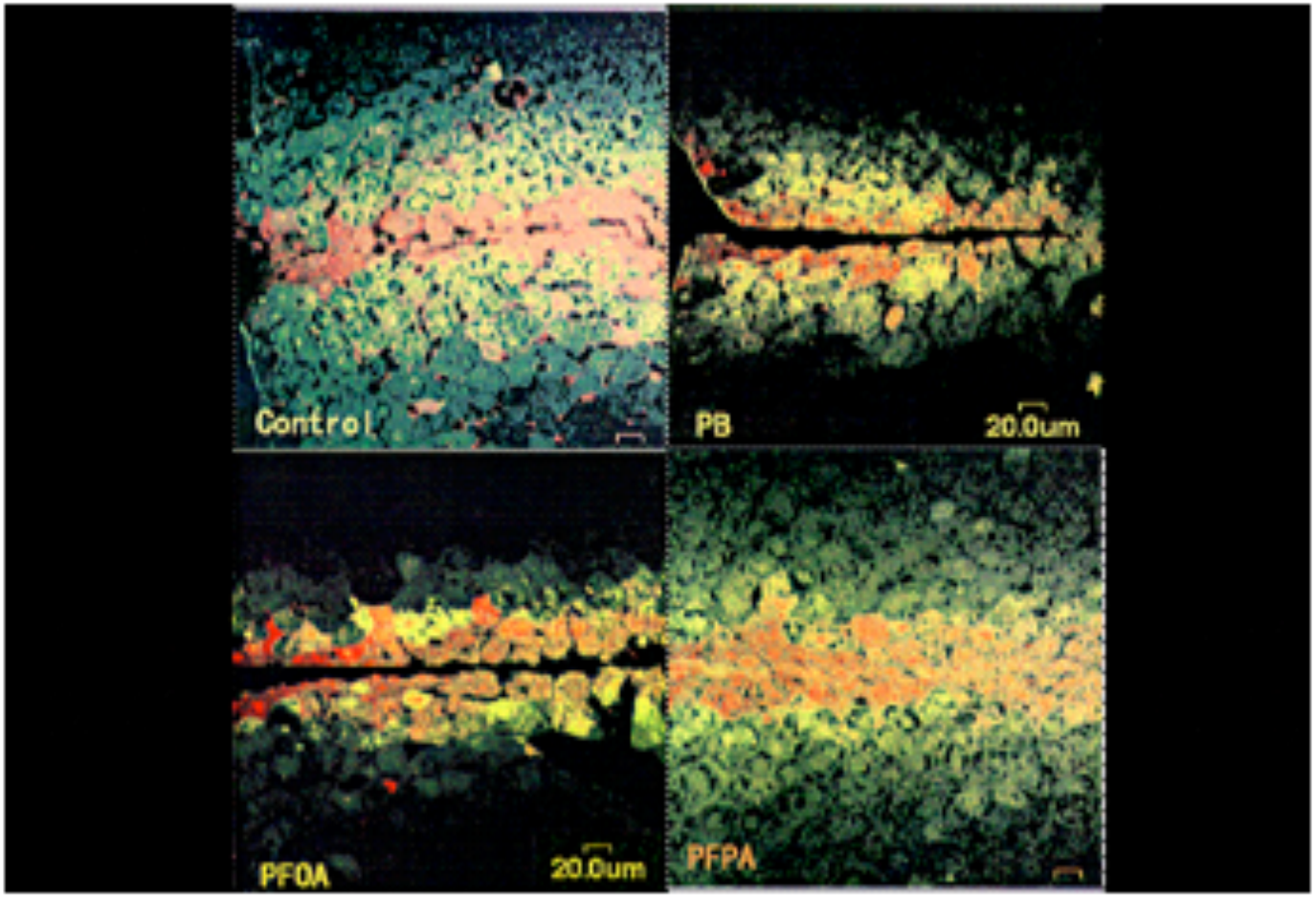
“INCISION DYE-TRANSFER” to MEASURE GAP JUNCTION FUNCTION IN LIVE ANIMALS

Demonstration that GJIC Could be Detected In Vivo By Incision Loading of Rat Liver



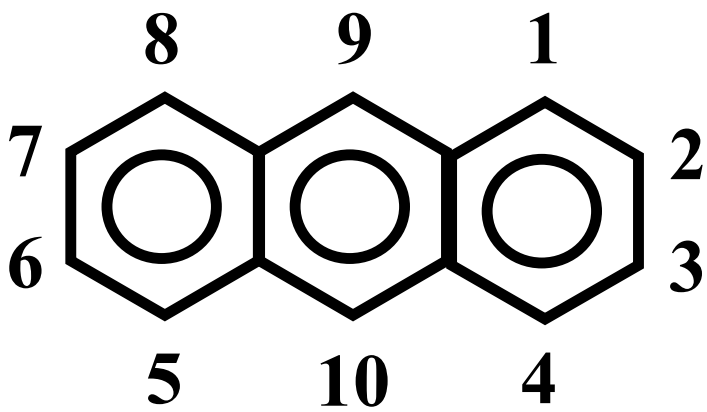
Phenobarbital Decreases B6C3F1 Mouse Hepatocyte GJIC *In vivo*



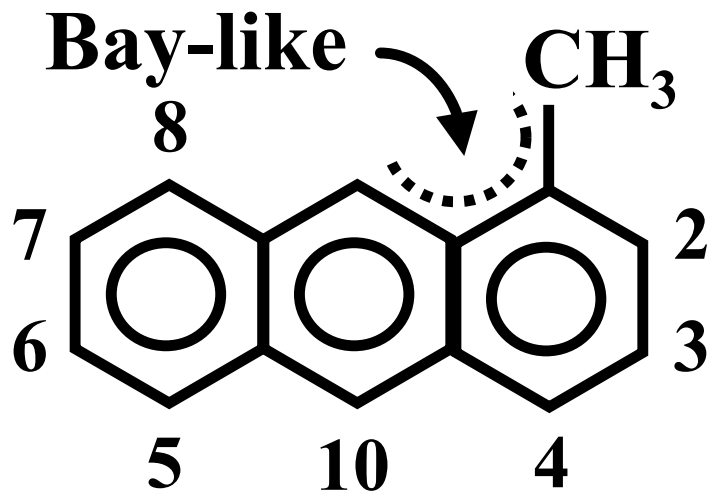

Control
PB
20.0um
PFOA
20.0um
PFPA

Structure/Function Relationship to the Modulation of Gap Junctional Intercellular Communication

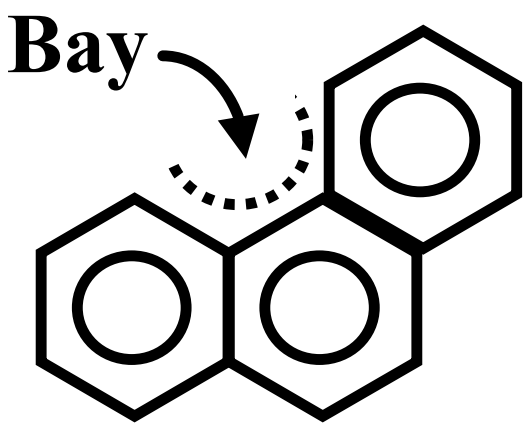
Polycyclic Aromatic Hydrocarbon Examples



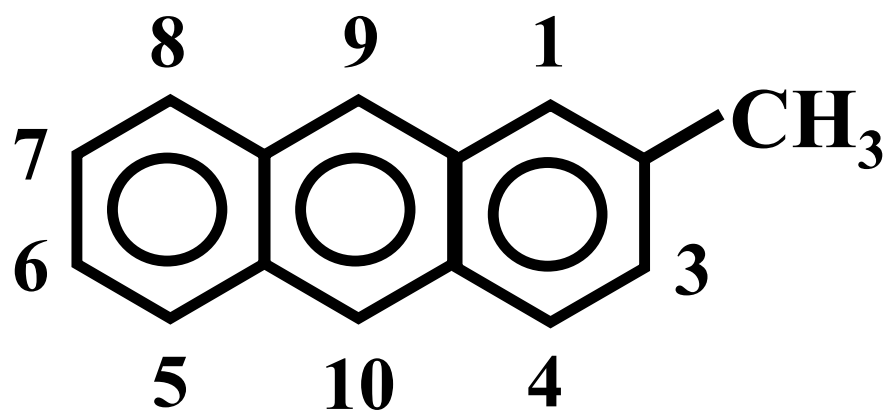
anthracene



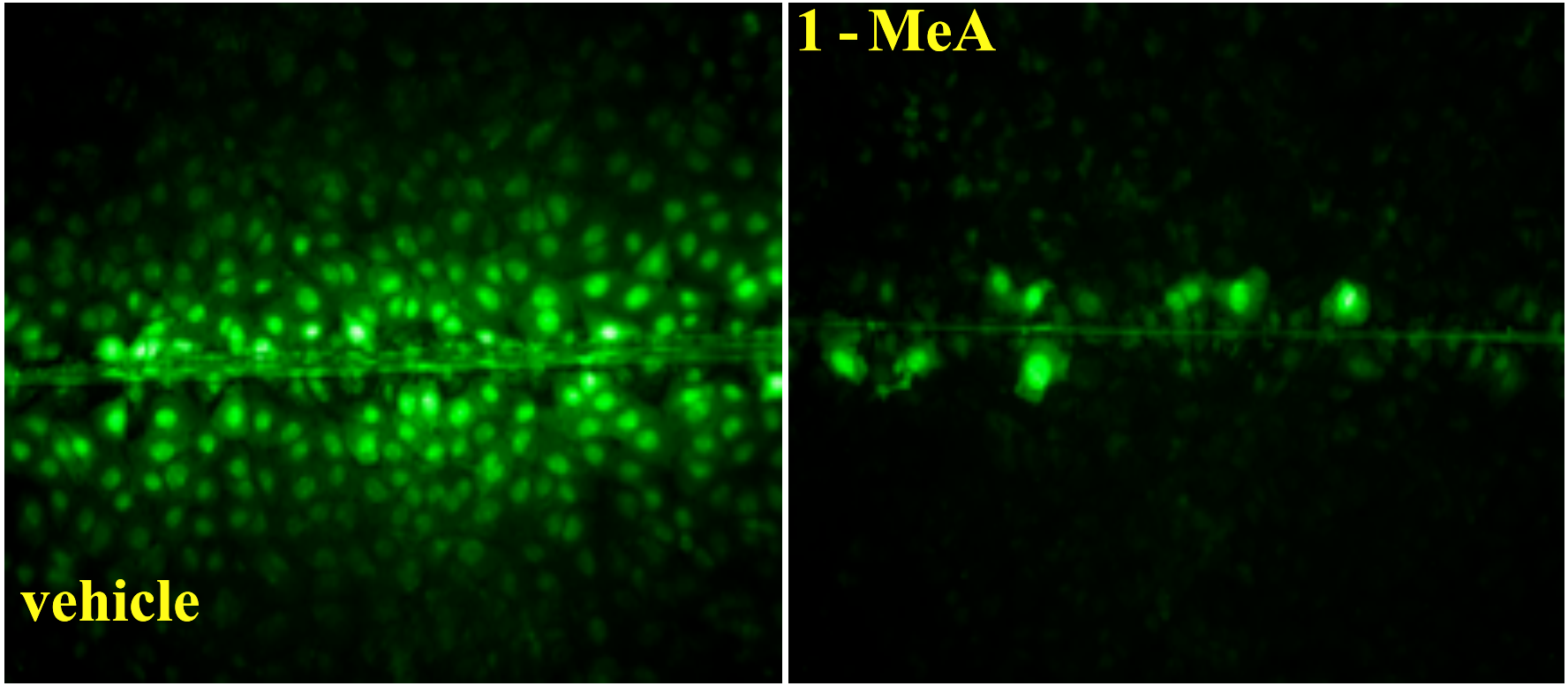
1-methylanthracene

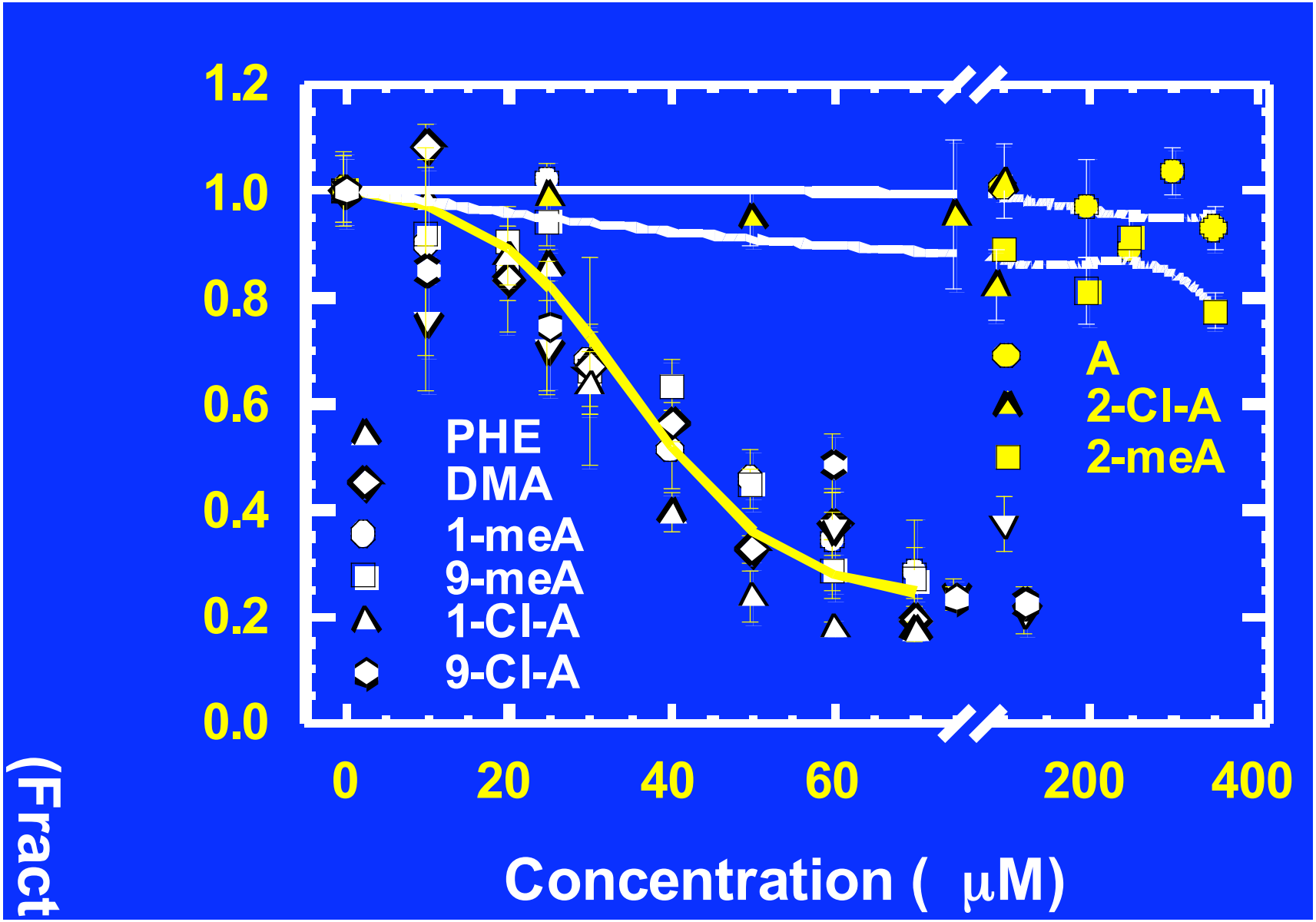


phenanthrene



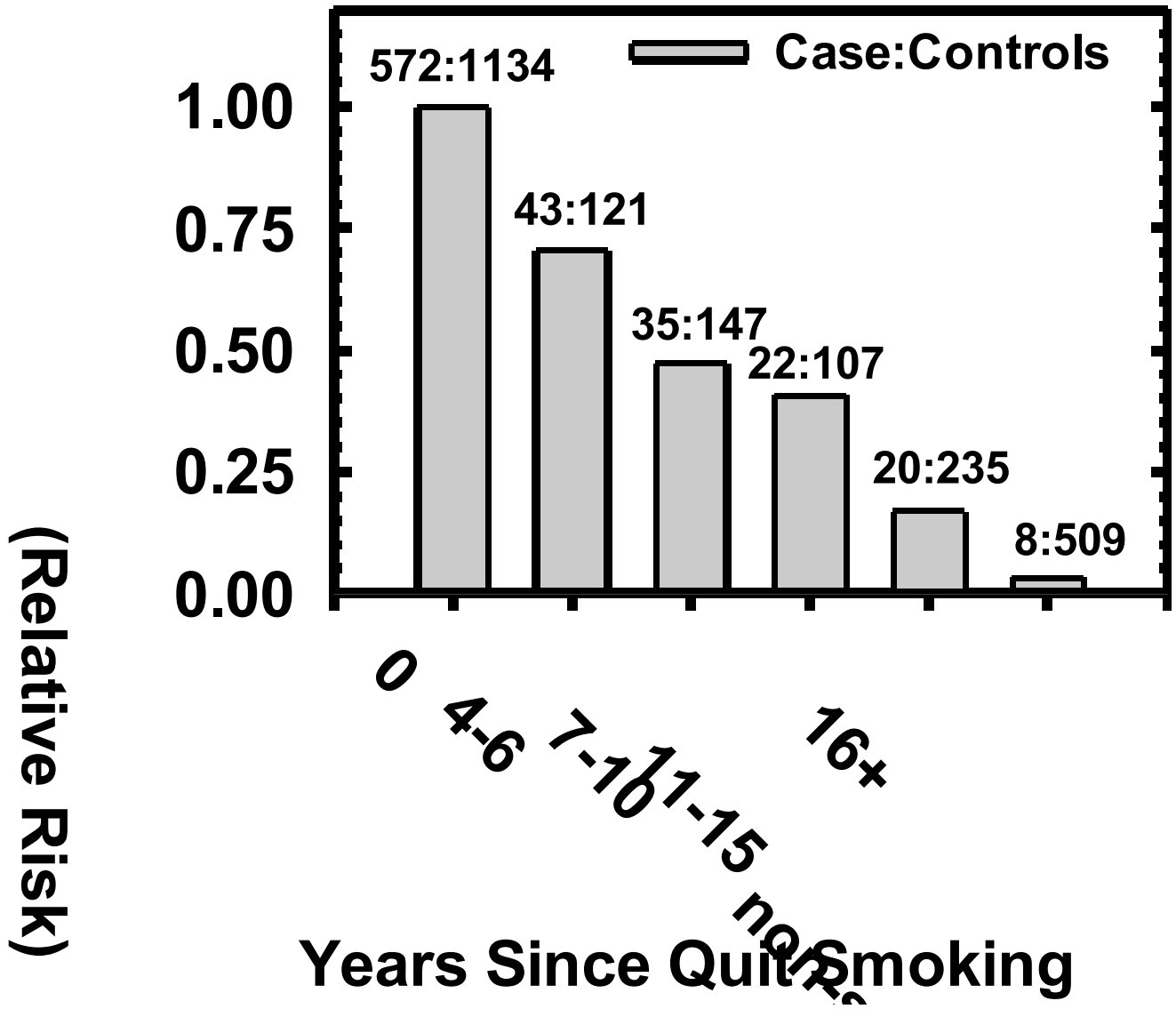
2-methylanthracene


vehicle
1 - MeA



Bay or Bay-like PAHs = Inhibition

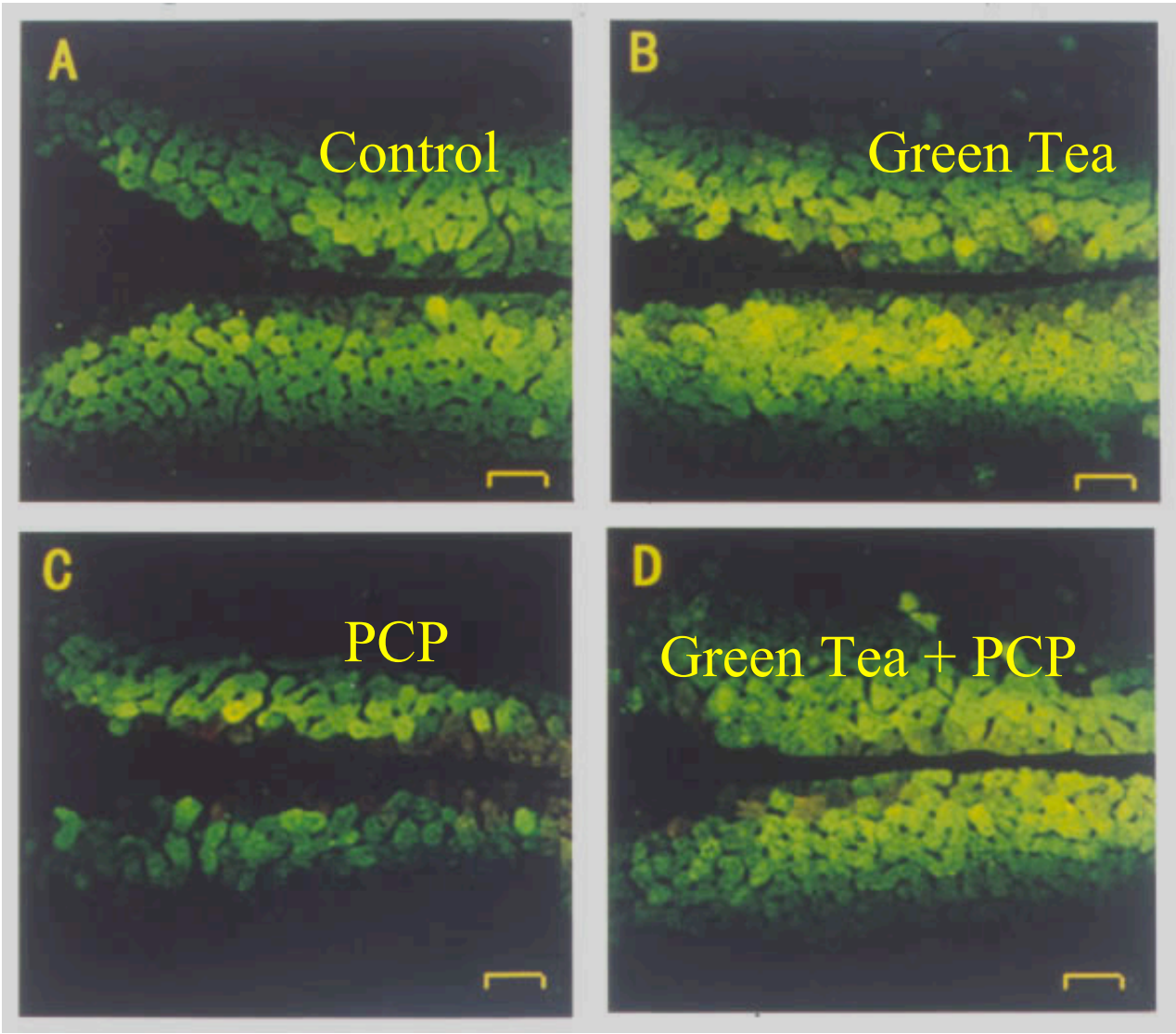
Linear PAHs = No Effect


Case:Controls
572:1134
43:121
35:147
22:107
20:235
8:509
1.00
0.75
0.50
0.25
0.00
(Relative Risk)
0
4-6
7-10
11-15
16+
Years Since Quit Smoking

IN VIVO DEMONSTRATION THAT GREEN TEA CAN PREVENT THE INHIBITION OF GJIC BY A KNOWN LIVER TUMOR PROMOTER

- ENVIRONMENTAL, NON-GENOTOXIC CHEMICAL, PCP, INDUCES OXIDATIVE STRESS IN RAT LIVER.
- PCP INHIBITS GJIC, REVERSIBLY, IN VITRO OR IN RAT LIVER STEM CELLS.
- PCP WAS GIVEN TO RATS IN WATER OR GREEN TEA (THEY WERE JAPANESE RATS!).

Rat Liver In Vivo Cell-Cell Communication With and Without Exposure to PCP +/- Green Tea



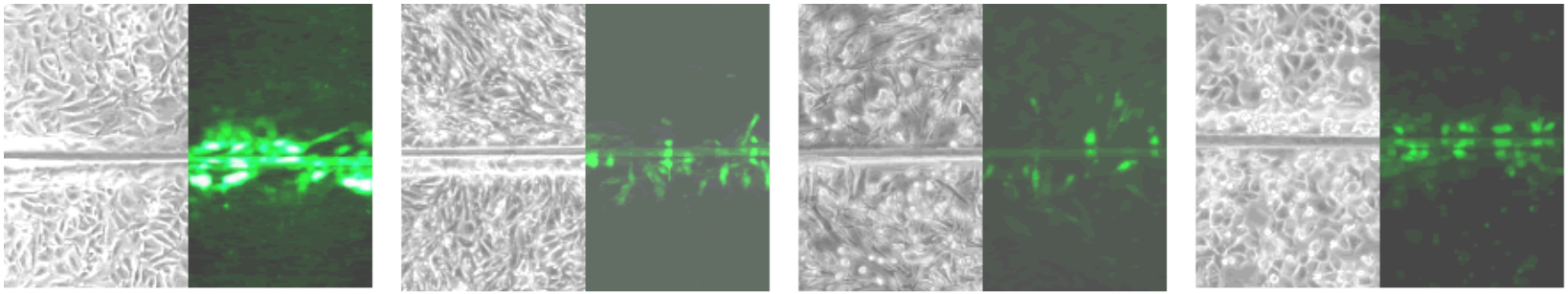
THERE WILL BE NO “SILVER BULLET” CHEMOPREVENTIVE OR CHEMOTHERAPEUTIC AGENTS

- One example is of a demonstrated chemopreventive-chemotherapeutic chemical, psyllium extract- beta-sitosterol, can only restore GJIC in tumor cells expressing Ha-ras. It does not affect src-, neu, myc-ras transformed cells.

- Each tumor and each oncogene trigger specific signaling mechanism. Chemopreventive and chemotherapeutic agents act on specific signaling mechanisms. They do not act “universally”.

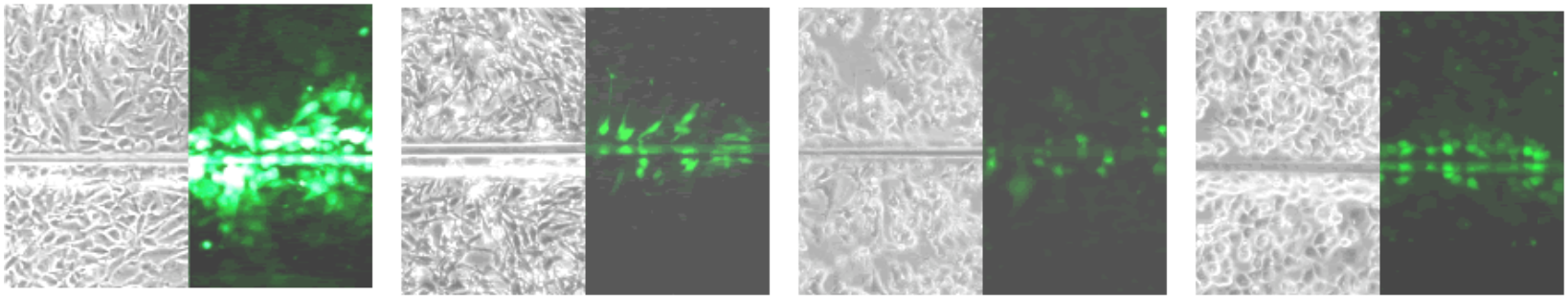
Restoration of GJIC With EtOH Extract From Psyllium Seed Husk in Various Strains of WB Cells Transfected With Different Oncogenes

WB ras **WB neu** **WB src** **WB myc-ras**



Control

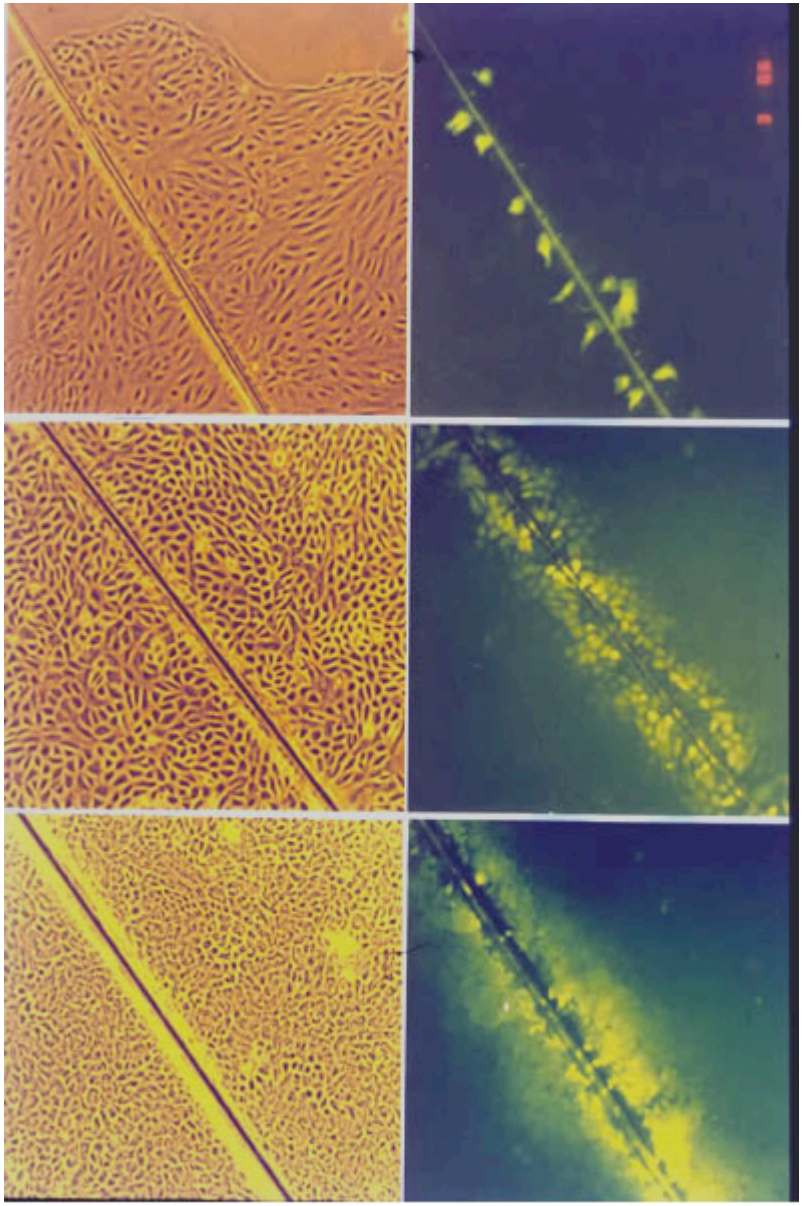
EtOH ext (50 μg/ml for 48h)



Type I Stem Cell

Young Type II Cell

Mature Type II Cell



SUMMARY

- While assays for genotoxicity and cytotoxicity exist, assays to detect "epigenetic toxicants" are needed
- Many, if not all chemicals (natural, synthetic) induce intra-cellular signaling at non-cytotoxic levels to induce gene expression changes and cellular biological responses in a structure/function relationship (cell division, cell differentiation, cell necrosis or apoptosis, cell senescence and differentiated cell responses).
- Intra-cellular signaling is associated with an epigenetic chemical's ability to modulate (increase or decrease) gap junction intercellular communication (GJIC).
- GJIC is a fundamental biological process needed for physiological homeostasis in all organs during all stages of human development, which is a species-, gender-, developmental state-, organ- and threshold-dependent process.
- To illustrate the importance of an GJIC assay, chemicals such as phorbol ester (plant toxicant); ochratoxin (microbial toxin); phenobarbital, thalidomide (drugs); DDT (pesticide); 2,4-T (herbicide); TCDD, PBBs, PCBs, PFOA (pollutants); phthalates (plasticizer); green tea, lycopene, retinoids, resveratrol (chemopreventive agents); lovastatin (anti-cardiovascular and anti-cancer agent); estrogen, dexamethasone, melatonin (hormones); fullerenes (solid particles); IL-6 (cytokine); and methyl anthracenes (cigarette smoke component and grilled protein) all modulate GJIC.