[Case Study]

Anti-Apoptosis Engineering for Animal Cell Culture

Tai Hyun Park

School of Chemical Engineering

Seoul National University

Cell Death

Apoptosis

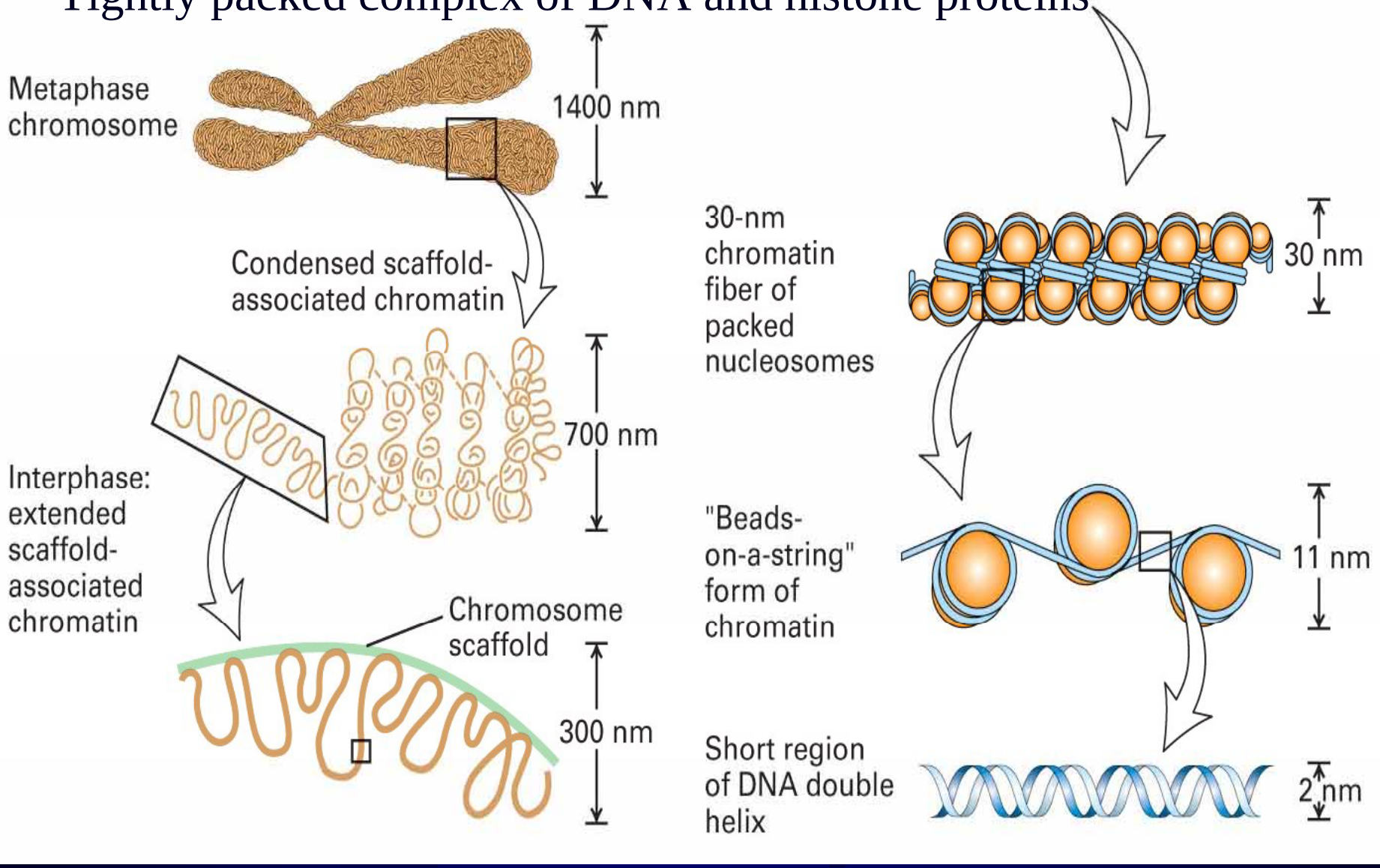
- Active, intrinsically programmed cell death
- Cell shrinkage, membrane blebbing, and DNA fragmentation

Necrosis

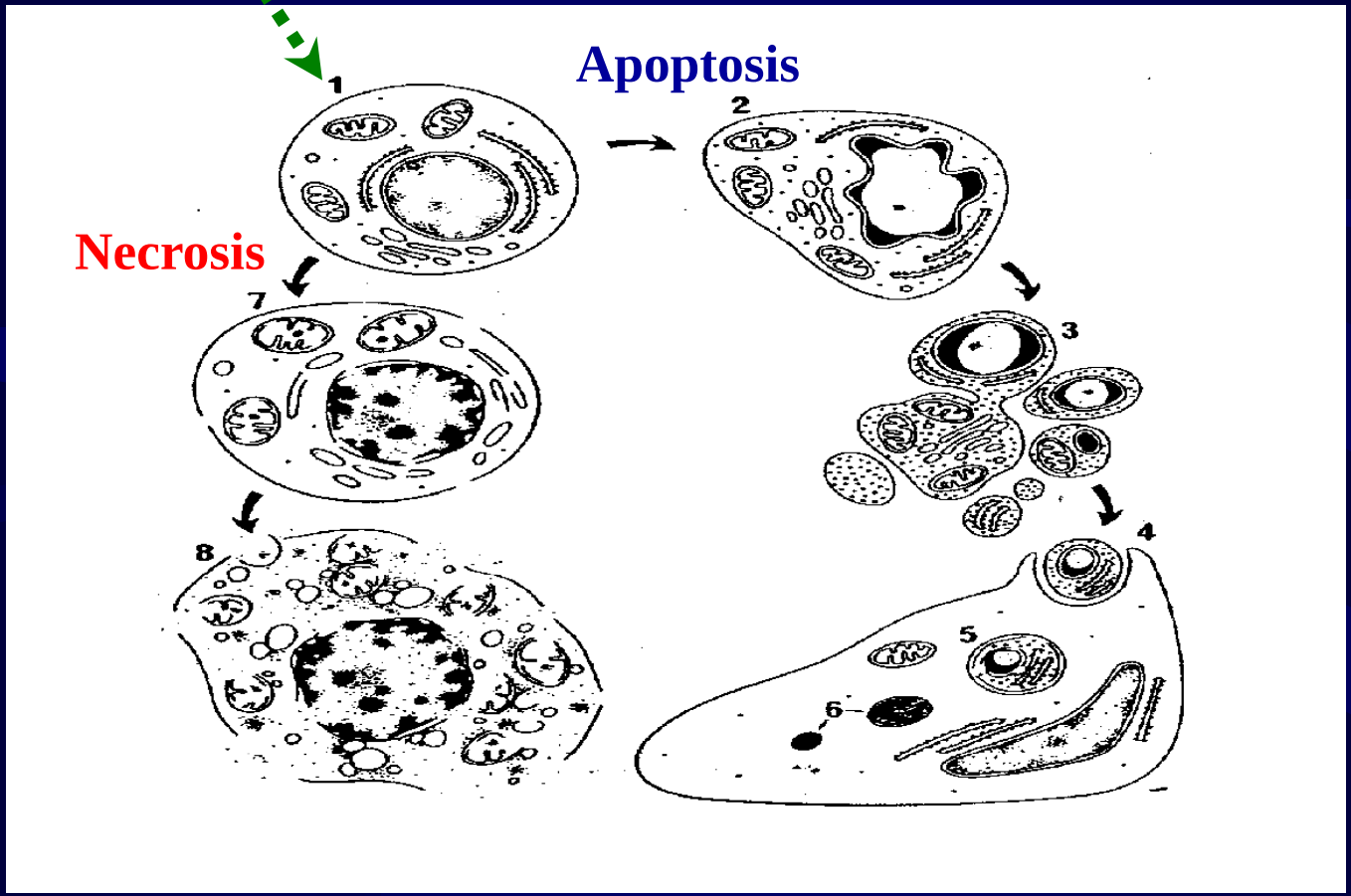
- Accidental and passive cell death due to the stress
- Cell swelling, mitochondrial swelling, and mild clamping of chromatin

Chromosome

- Tightly packed complex of DNA and histone proteins



Apoptosis and Necrosis



Seoul National University

Apoptosis in Human Disease

Disease associated with increased apoptosis

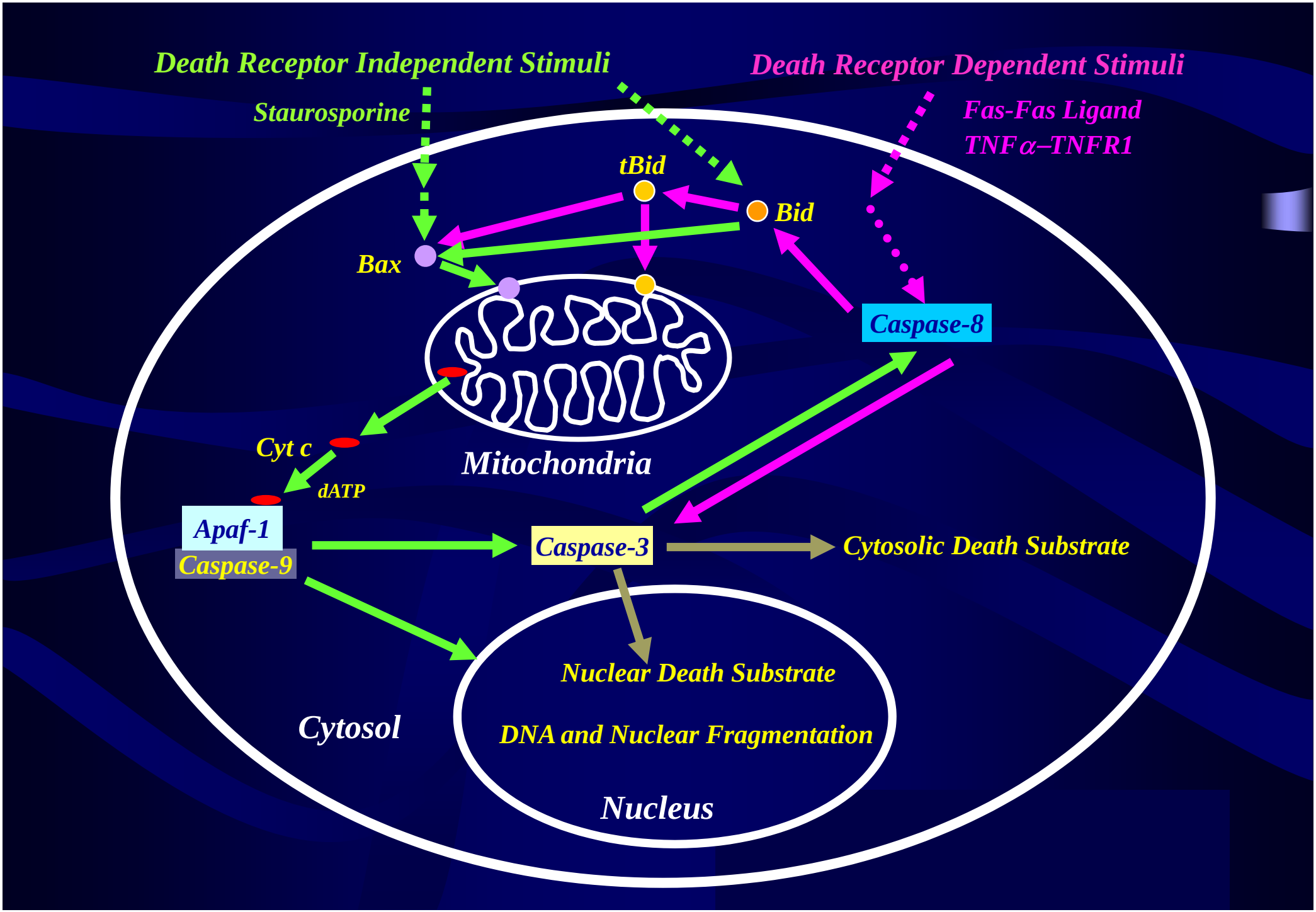
- Alzheimer's disease and Parkinson's disease (Neurodegenerative disorders)
- Bacterial and viral infection such as AIDS
- Autoimmune disorders such as Insulin-dependent diabetes mellitus

Disease associated with decreased apoptosis

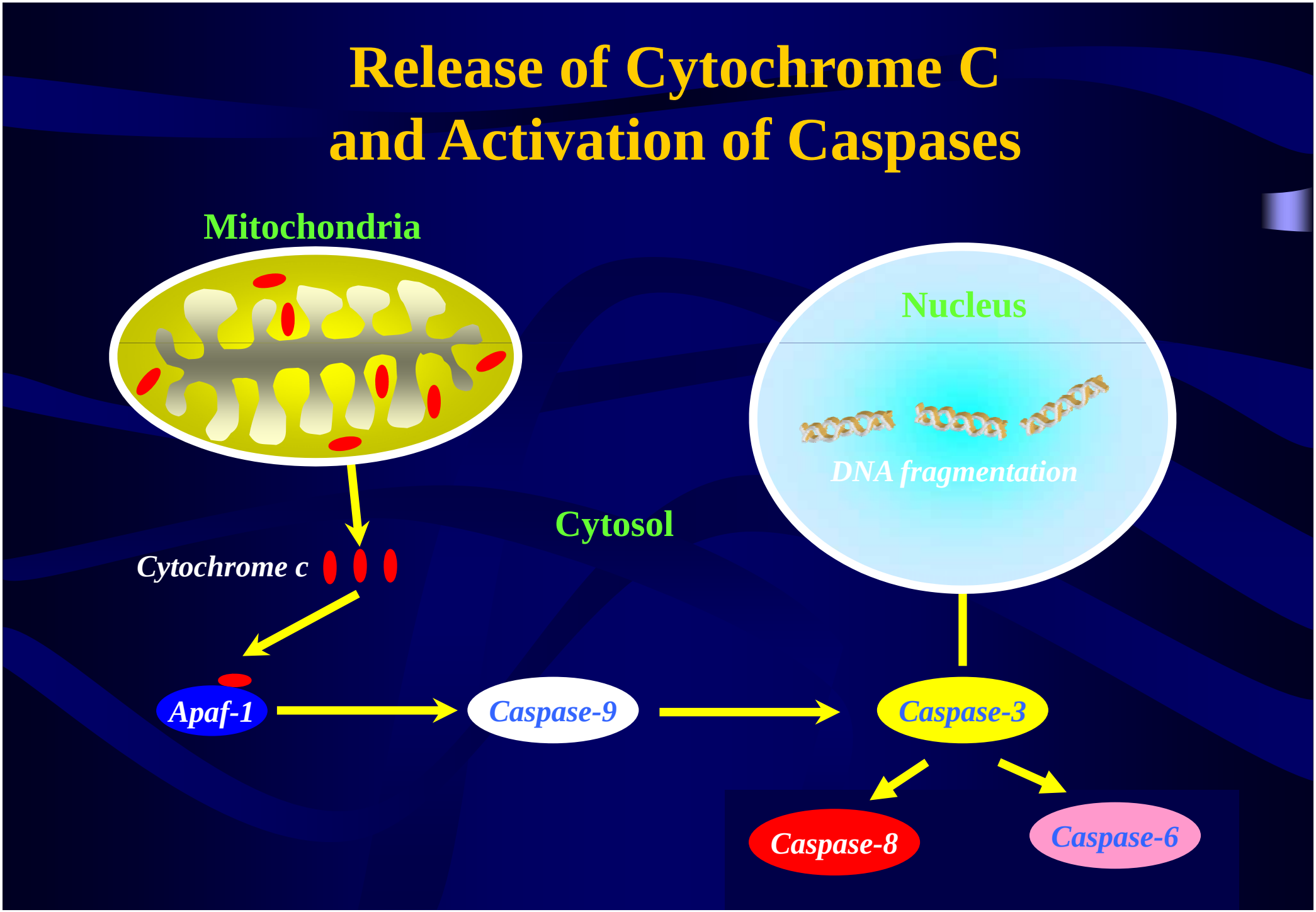
- Cancer
- Autoimmune disorders such as systematic lupus erythematosus
- Atherosclerosis

Apoptosis and Its Mechanisms

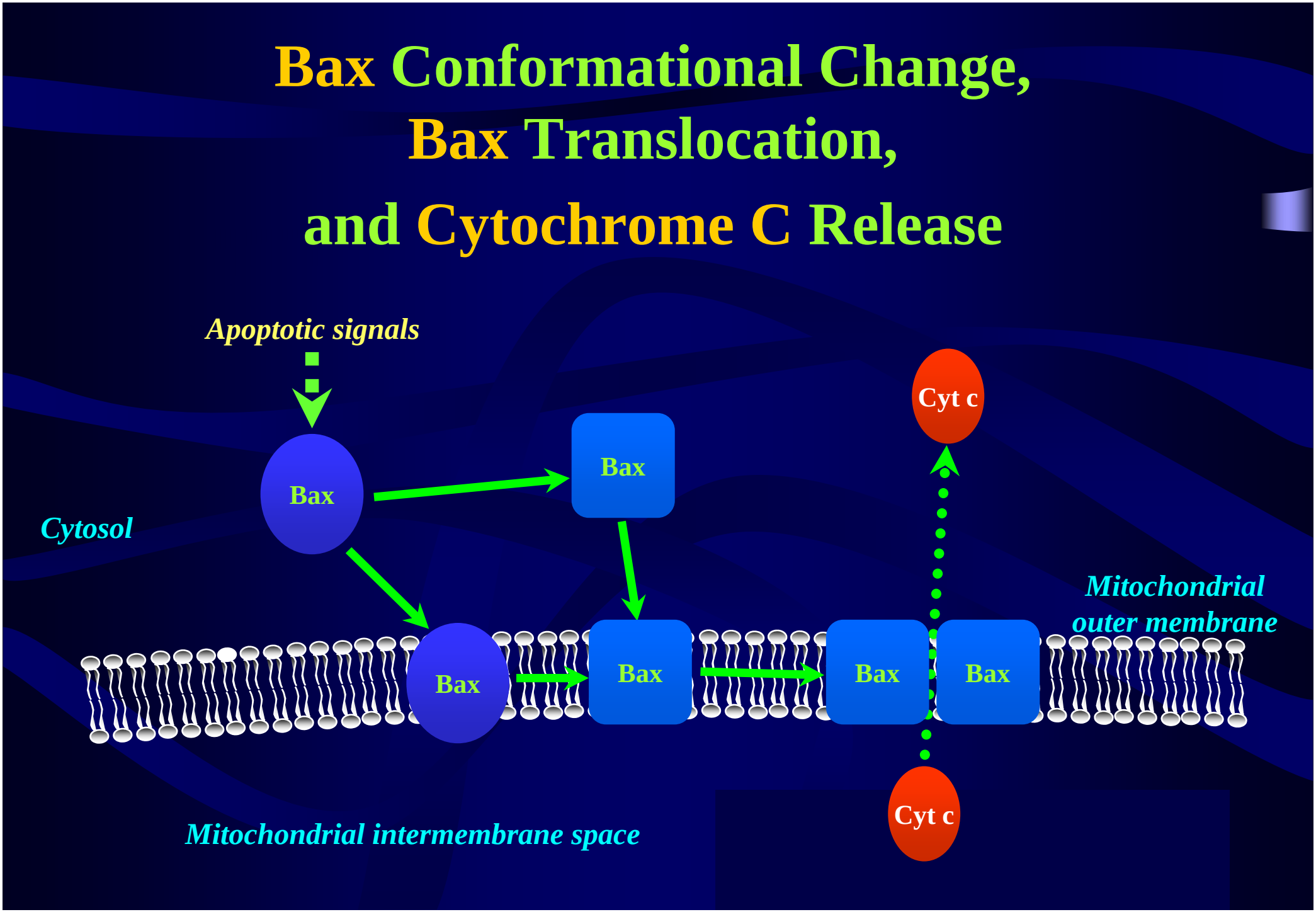
- **Some common pathways despite a wide range of inducing signals.**
- **Many death signals converge onto mitochondria and are mediated through members of the Bcl-2 protein family.**
- **Activation of the caspases and various mitochondrial changes are involved in apoptosis.**


Death Receptor Independent Stimuli
Staurosporine
Death Receptor Dependent Stimuli
Fas-Fas Ligand
TNFα–TNFR1
tBid
Bid
Bax
Caspase-8
Cyt c
dATP
Mitochondria
Apaf-1
Caspase-9
Caspase-3
Cytosolic Death Substrate
Nuclear Death Substrate
DNA and Nuclear Fragmentation
Cytosol
Nucleus

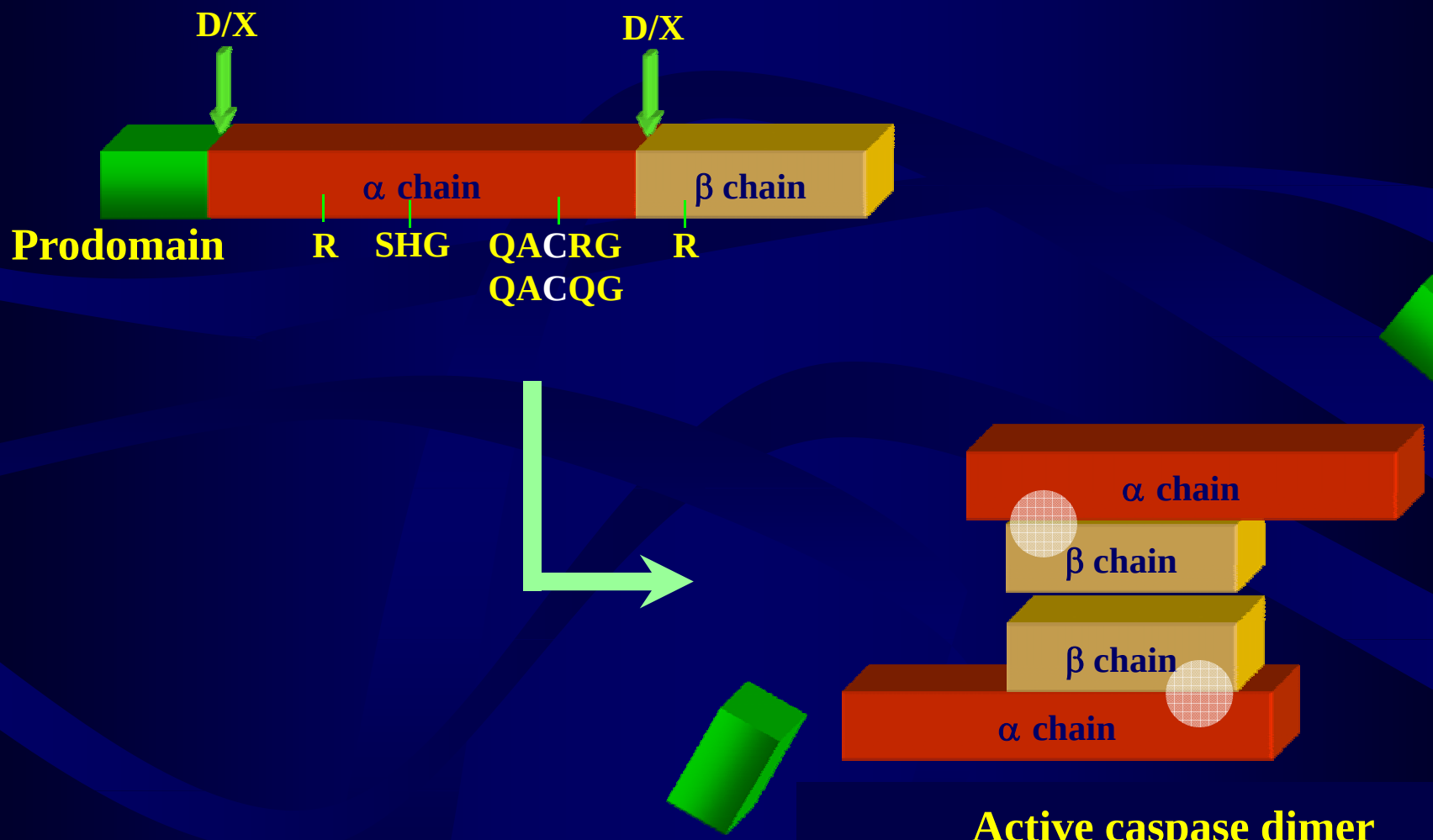
Release of Cytochrome C and Activation of Caspases



Bax Conformational Change, Bax Translocation, and Cytochrome C Release



Activation of Caspase



Why Silkworm Hemolymph (SH)?

FBS (Fetal Bovine Serum)

- High Cost
- Nonreproducibility
- Contamination Risk
- Complicates Down-Stream Processing

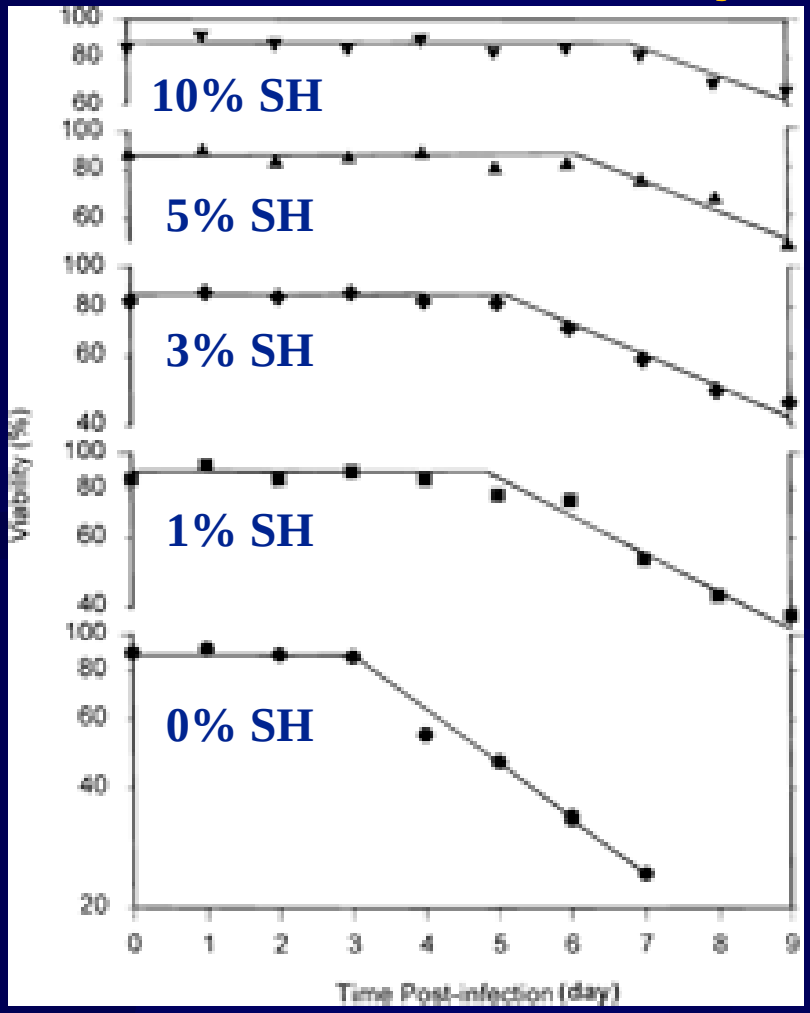
SH

- Insect Serum
- Low Cost
- Reproducibility

Beneficial Effect of SH on Insect Cell/Baculovirus System

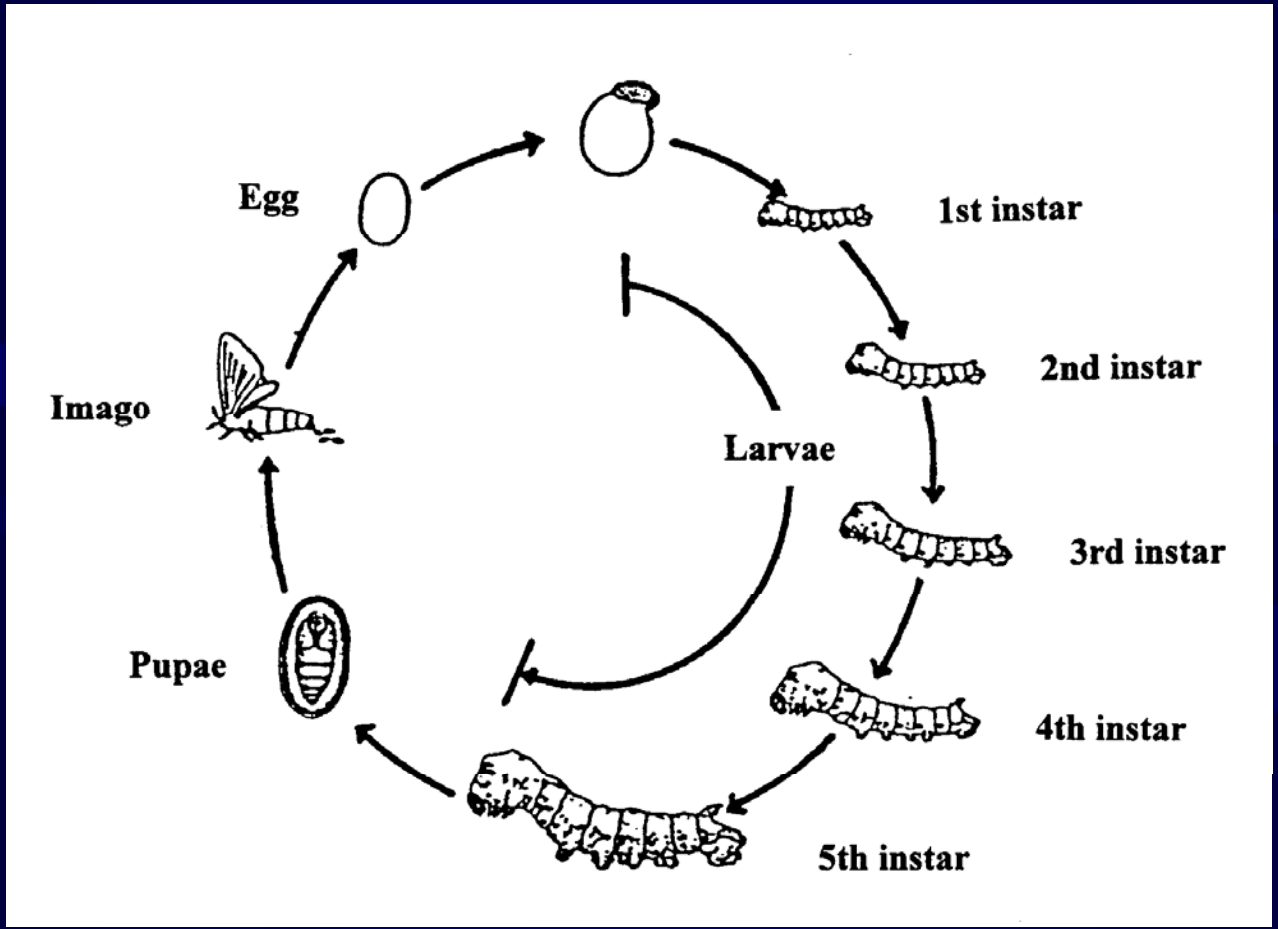
- Replacement of FBS with SH
- Production of Recombinant Protein
- Delay of Virus-Infected Host Cell Death
- Inhibition of Apoptosis

Effect of SH on Host Cell Viability
(insect cell/baculovirus system)



Seoul National University

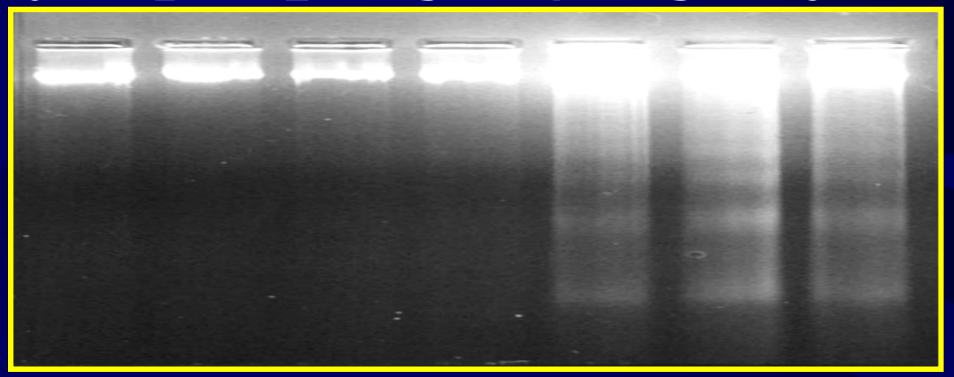
Collection of Silkworm Hemolymph



Baculovirus -Induced Insect Cell (Sf9) Apoptosis

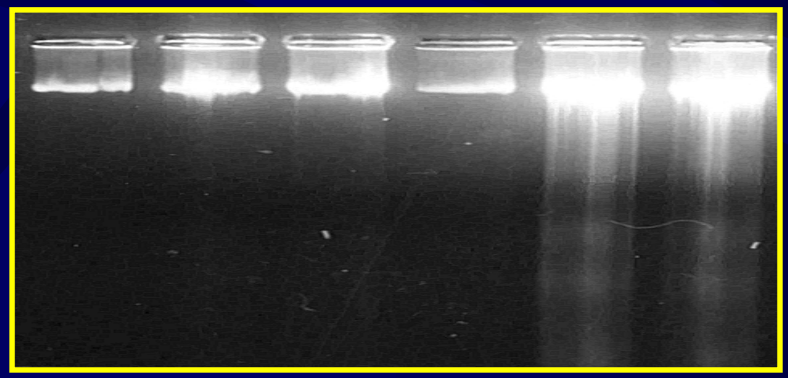
PI (day) 0 1 2 3 4 5 6

10% FBS



PI (day) 0 4 5 6 7 8

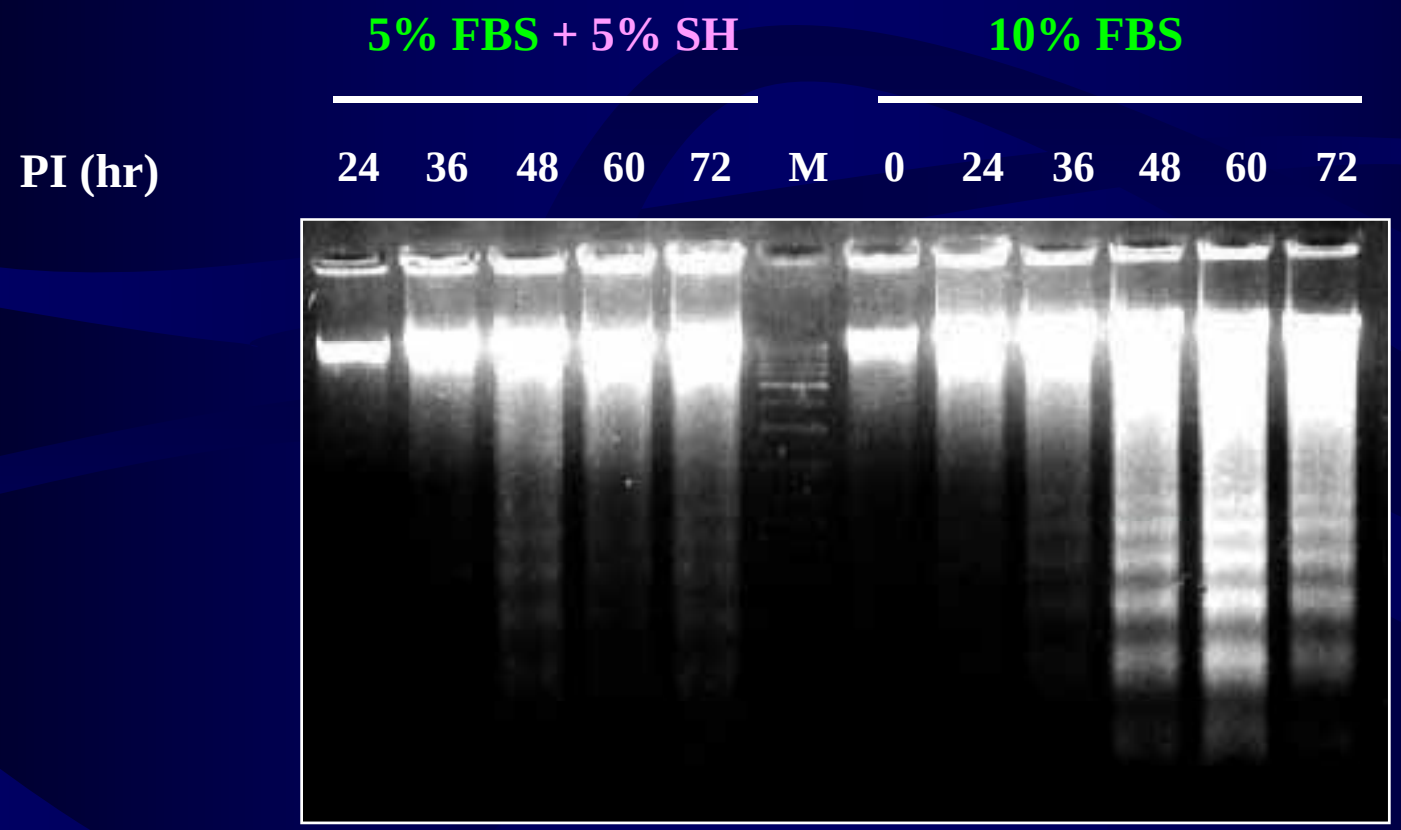
5% FBS + 5% SH



Seoul National University

Virus-Induced Human Cell Apoptosis

(Vaccinia virus, HeLa cell)



Seoul National University

Insect Cell (Sf9)

- Baculovirus-induced apoptosis
- Actinomycin D-induced apoptosis
- Camptothecin-induced apoptosis
- Staurosporine-induced apoptosis

Mammalian Cell

(i) HeLa

- Vaccinia virus-induced
- Cisplatin-induced
- Staurosporine-induced

(ii) CHO

- Sodium butyrate-induced
- Staurosporine-induced

Isolation and Identification of the Anti-Apoptotic Compound

Seoul National University

Separation Scheme

Heat Treatment (60 °C, 30 min) ⇒ Centrifugation (12,000 rpm, 1hr, 4 °C)

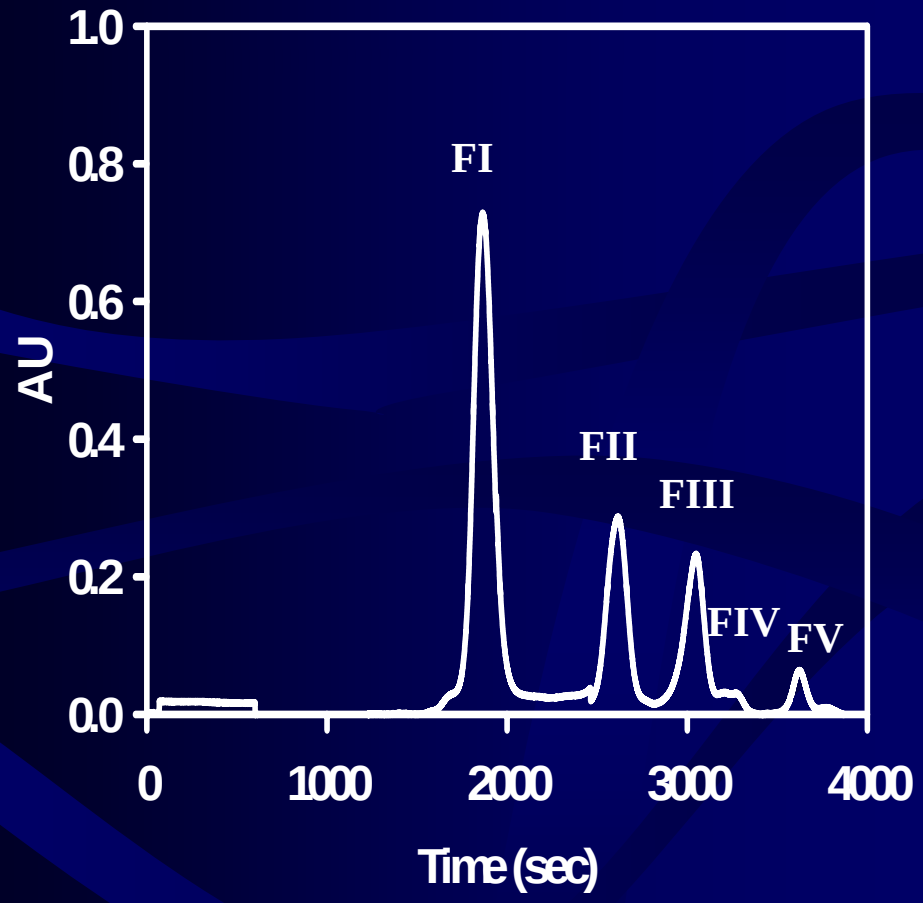
↓

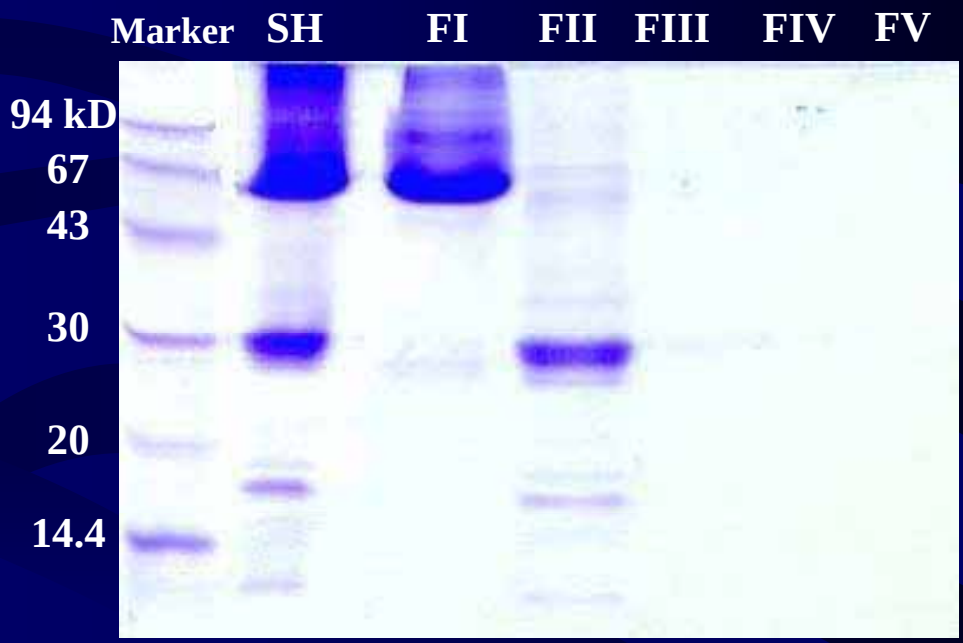
Gel Filtration Chromatography (FPLC, Superdex 200)

↓

Ion Exchange Chromatography (FPLC, Mono Q)

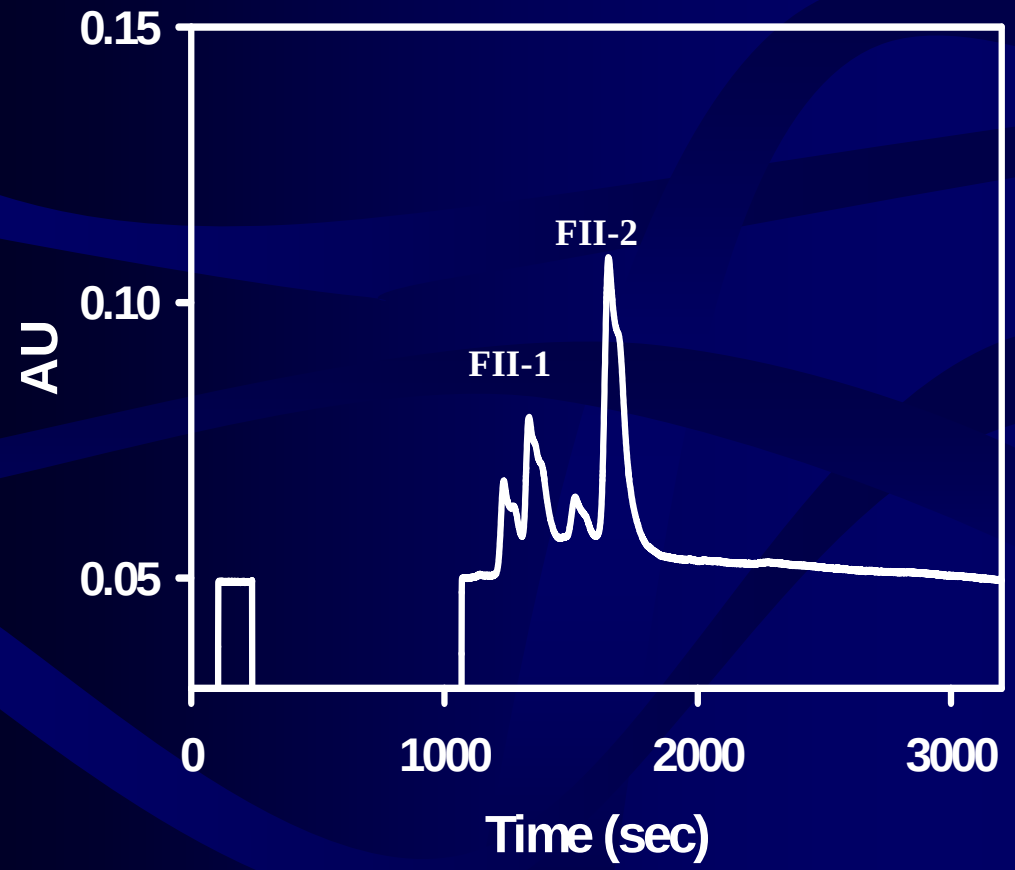
Gel Filtration Chromatography

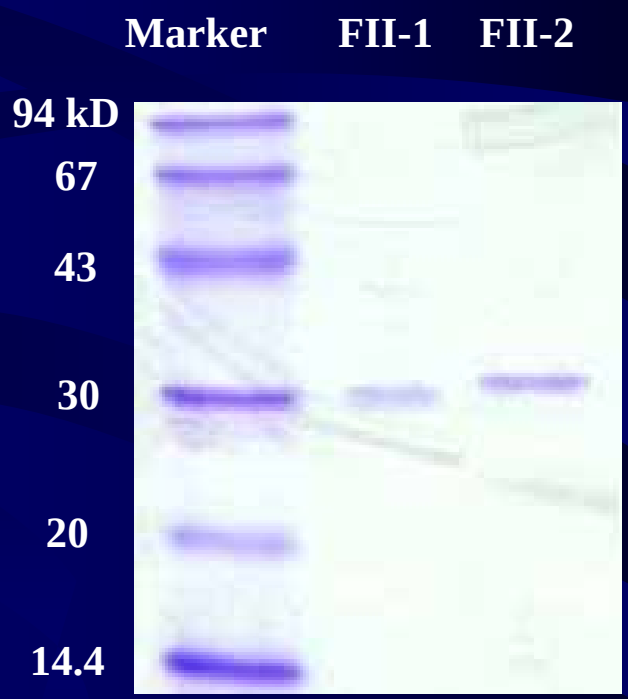




Seoul National University

Ion Exchange Chromatography





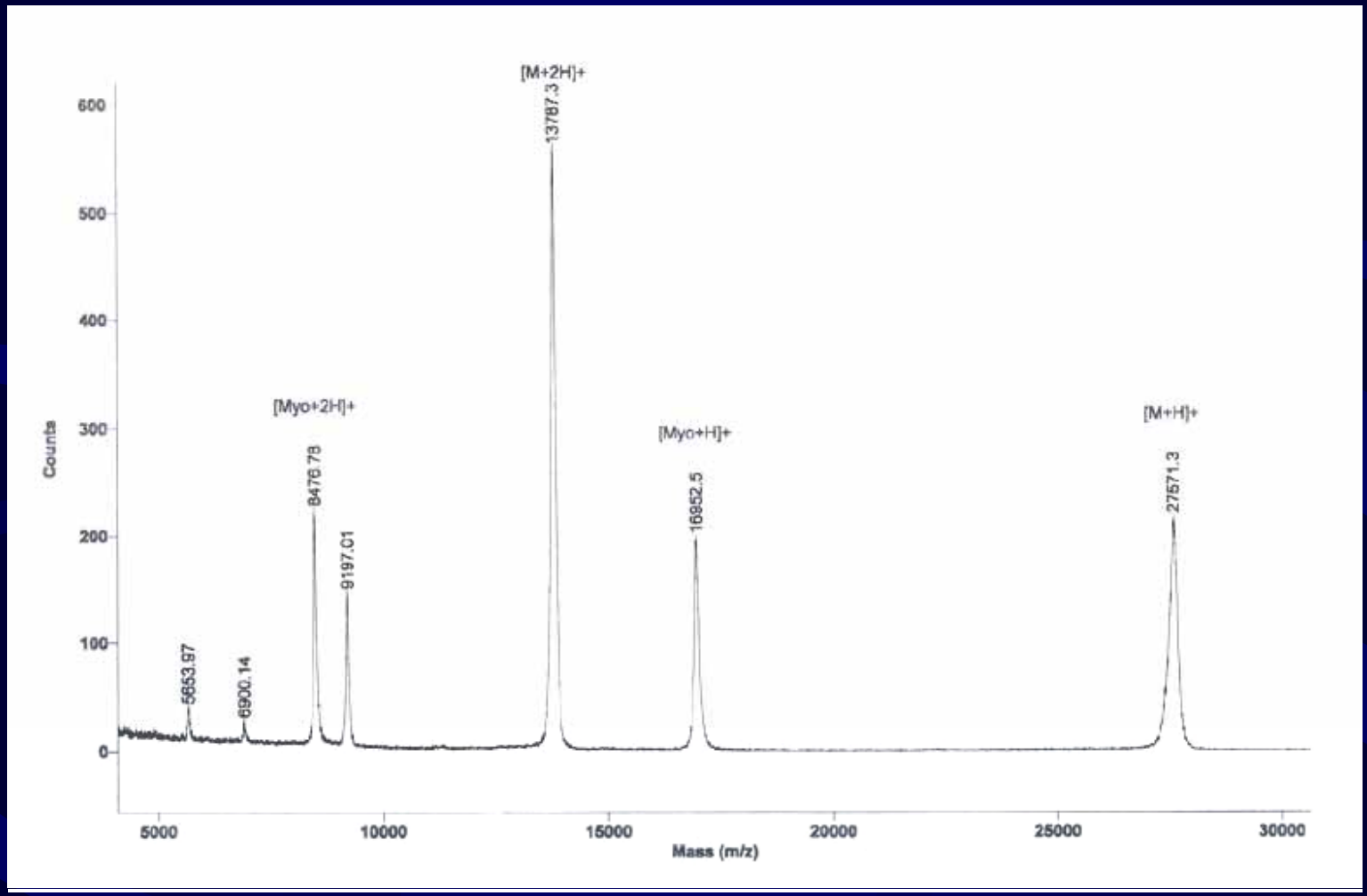
Seoul National University

Apoptosis Inhibiting Components in Silkworm Hemolymph

	Protein (mg)/ SH(mL)	Protein (mg)/ medium(mL)	Ratio % (each fraction/total)	Relative apoptosis-inhibiting activity (%)
Heat-treated SH	35.43	1.77	100	100
FI	20.62	1.03	58.19	
FI-1	5.49	0.27	15.50	48.25±4.51
FI-2	5.93	0.29	16.75	57.13 ±1.75
FI-3	5.82	0.29	16.43	61.53 ±0.70
FII	10.80	0.54	30.47	
FII-1	4.43	0.22	12.49	73.40 ±1.74
FII-2	4.17	0.21	11.78	92.16 ±3.26
FIII~FV	4.02	0.20	11.34	Negligible

Seoul National University

MALDI-TOF Mass Spectrometry Analysis of FII-2



Seoul National University

Amino Acid Sequence Homology

	1	10
30K Protein	M K P A I V I L C L	F V A S L Y A A D S
FII-2		A D S

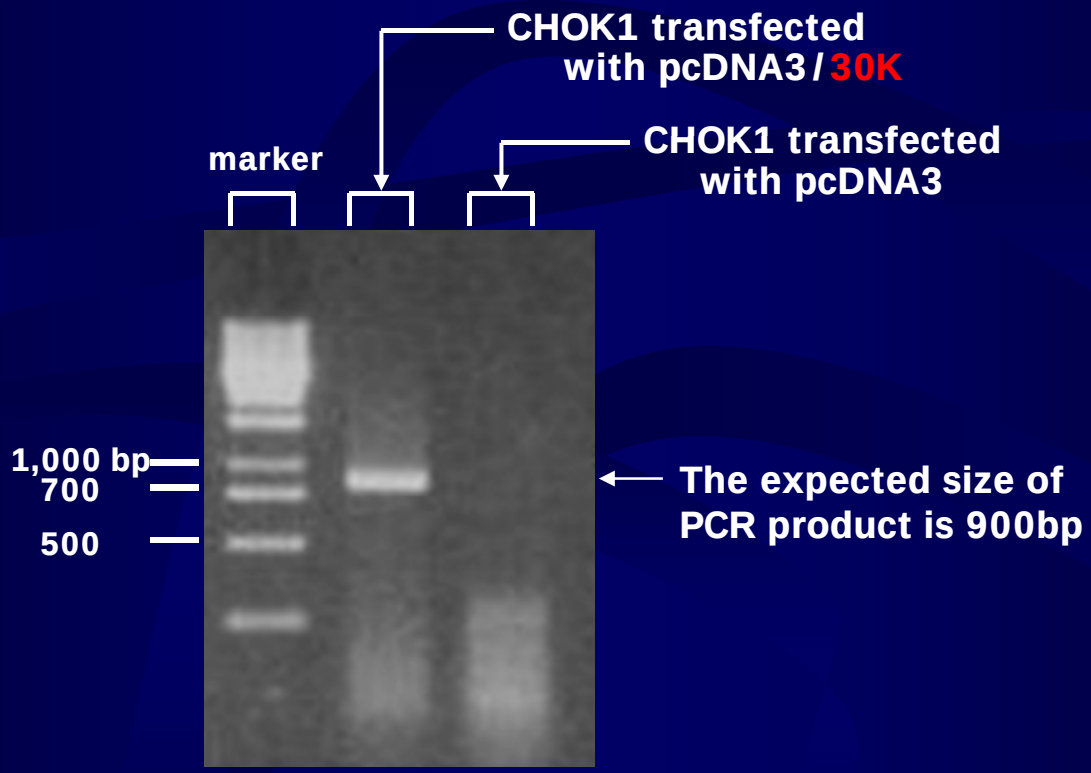
	21	31
	D V P N D I L E E Q	L Y N S V V V A D Y
	D V P N D I L E E Q	L Y N S V - V A D -

Seoul National University

Anti-Apoptosis Engineering by Expressing the 30K gene in CHO Cells

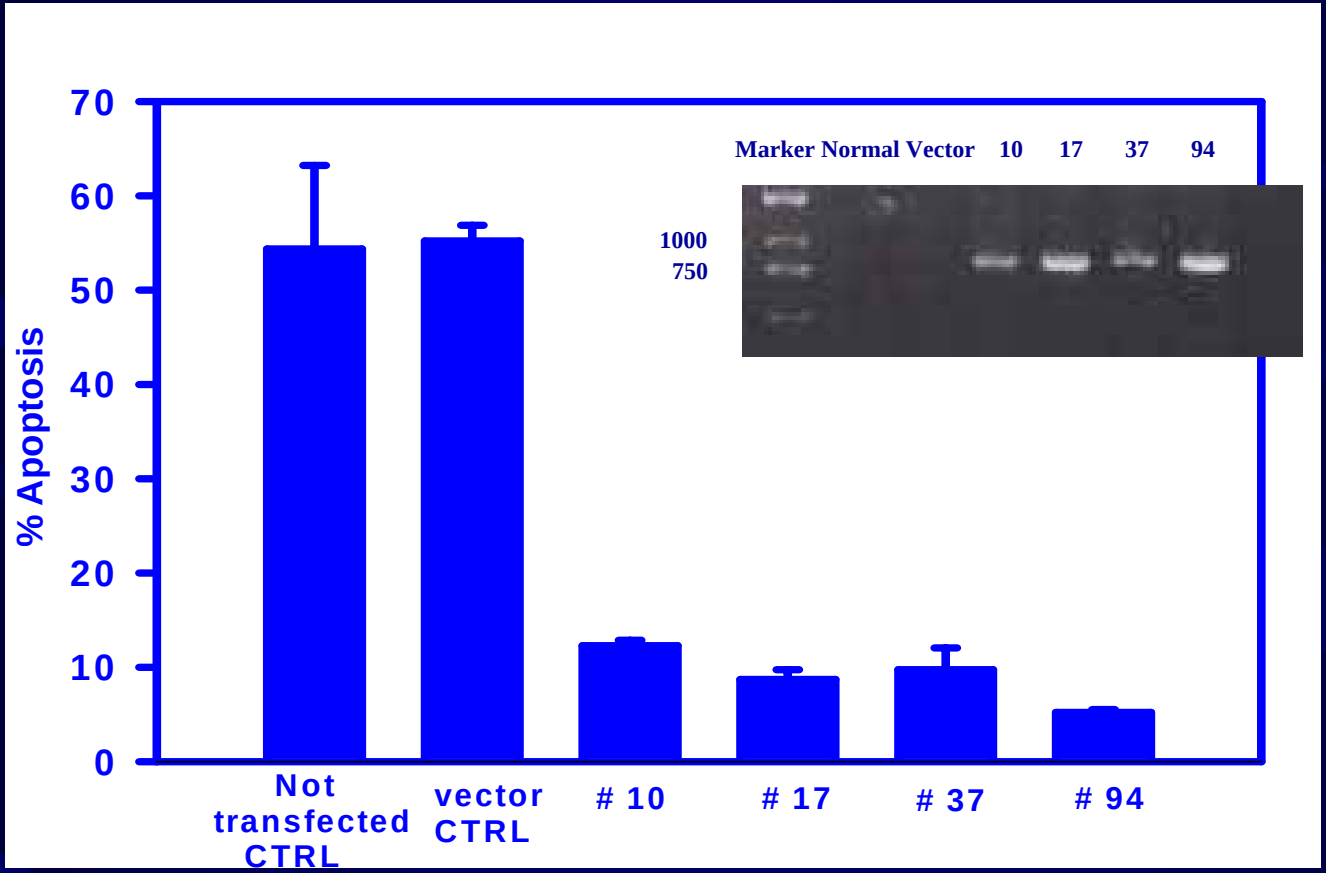
Seoul National University

30K Protein Expression in CHO Cell (analyzed by RT-PCR)



Seoul National University

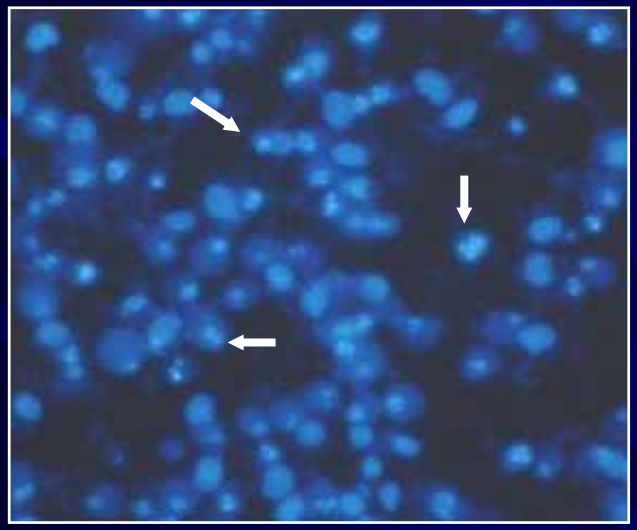
Selection of Stable Transfectant



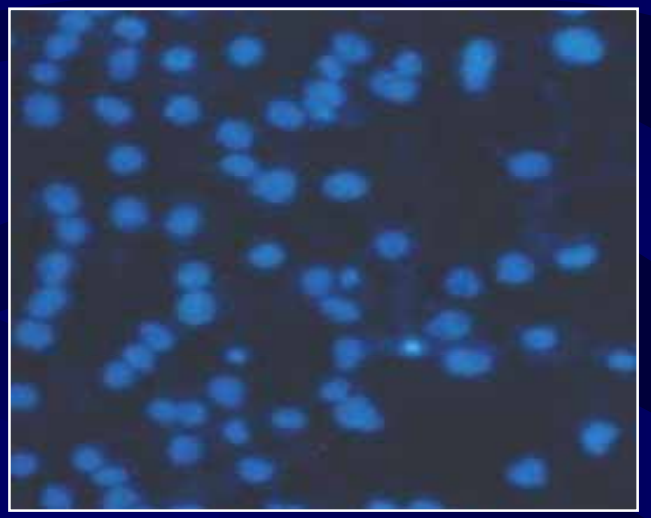
Seoul National University

Effect of 30K Protein Expression on Apoptosis in CHO Cell

CHOK1 cells transfected with pcDNA3



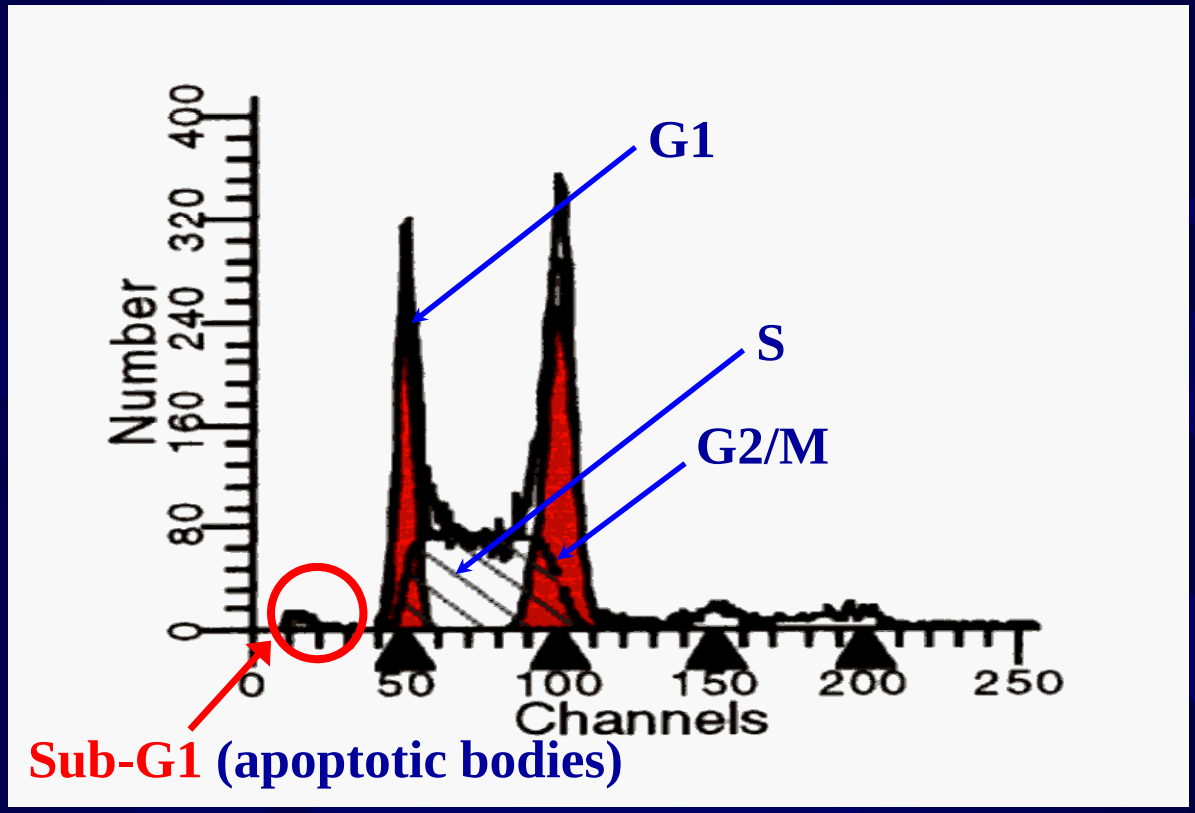
CHOK1 cells transfected with pcDNA3/30K



Apoptosis was induced with staurosporine (STS) for 12 h.
The nuclear chromatin was stained with Hoechst 33258 fluorescent dye.

Seoul National University

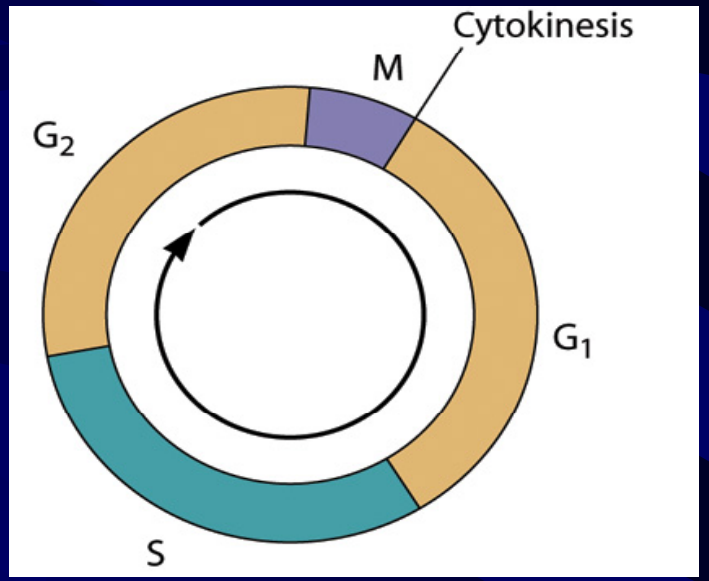
Flow Cytometric Analysis of Apoptosis



Cell Cycle

The Cell Cycle

- Cell Cycle
  - S phase:
    --- DNA synthesis
    (DNA replication)
  - M phase:
    --- mitosis
  - G_1, G_2 :
    --- G strands for gap
    between S and M phase
    --- Cell growth

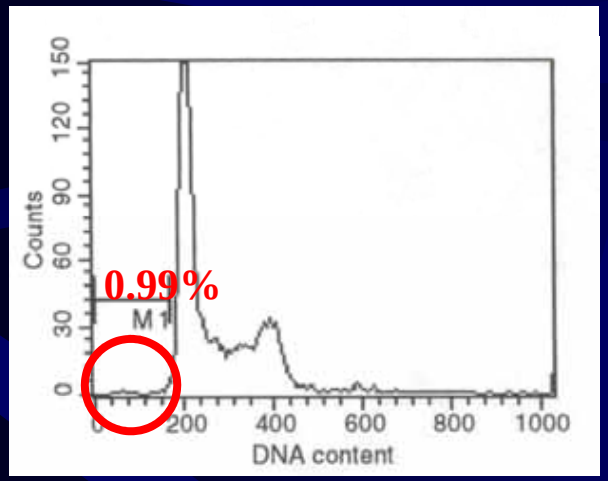


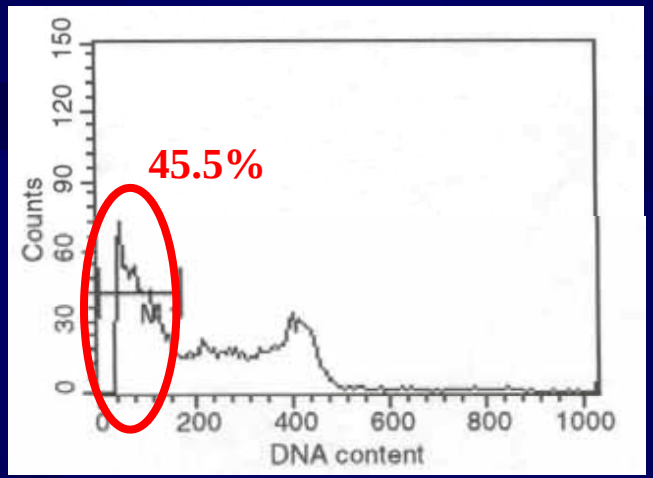
Effect of 30K Protein Expression on Apoptosis in CHO Cell

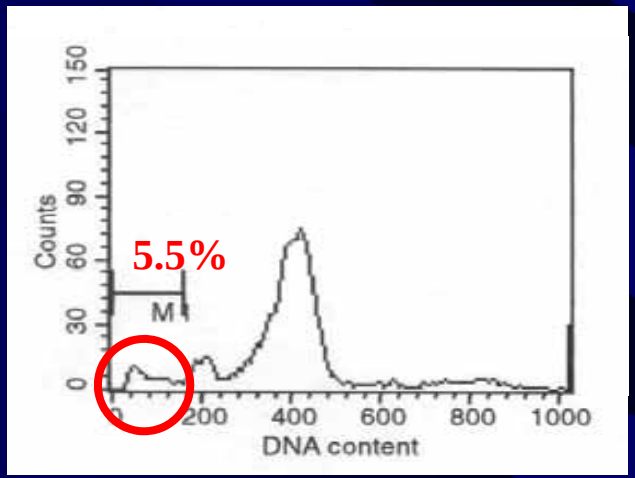
Before the induction of apoptosis

12 h after induction
(Apoptosis was induced with staurosporine.)

CHOK1 cell | **30K-transfected CHOK1 cell**





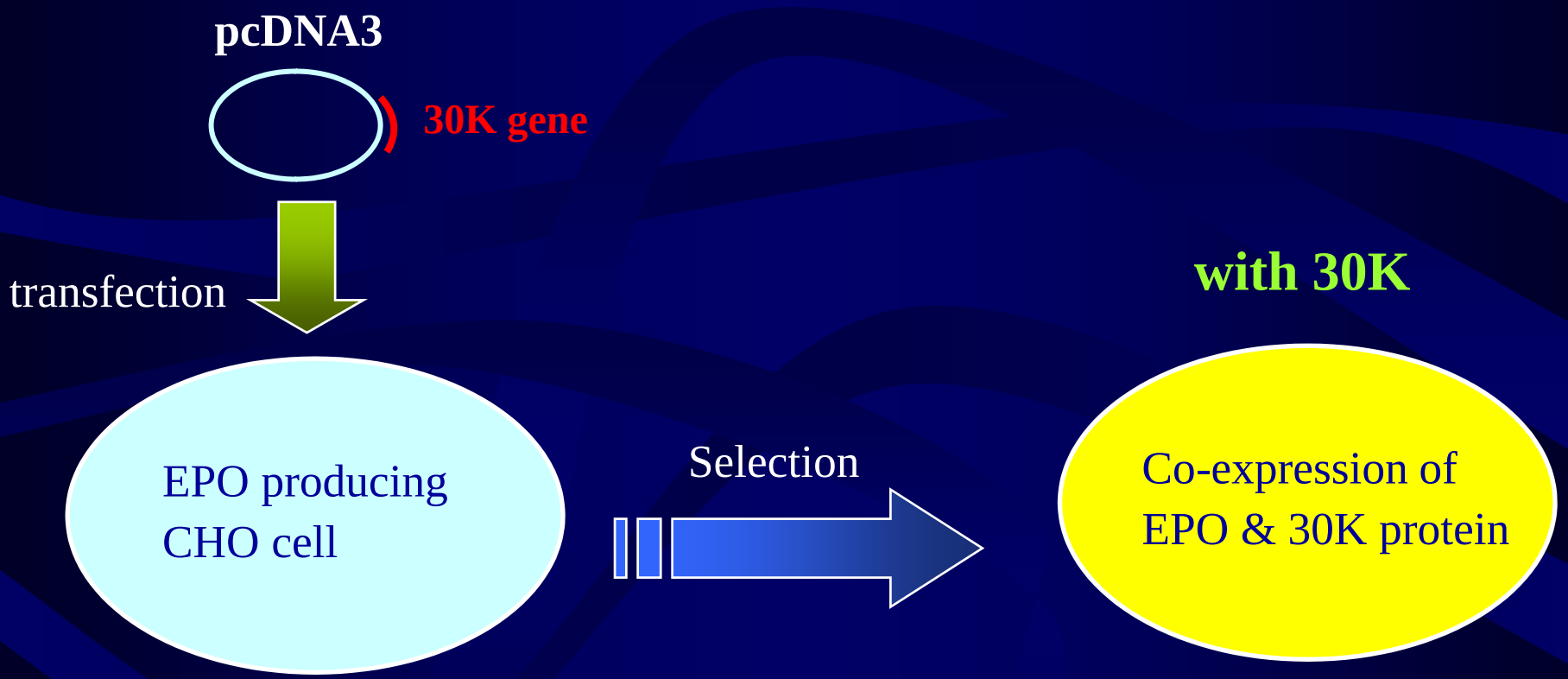


Seoul National University

Enhancement of Product Quantity and Quality by the Expression of 30K Protein

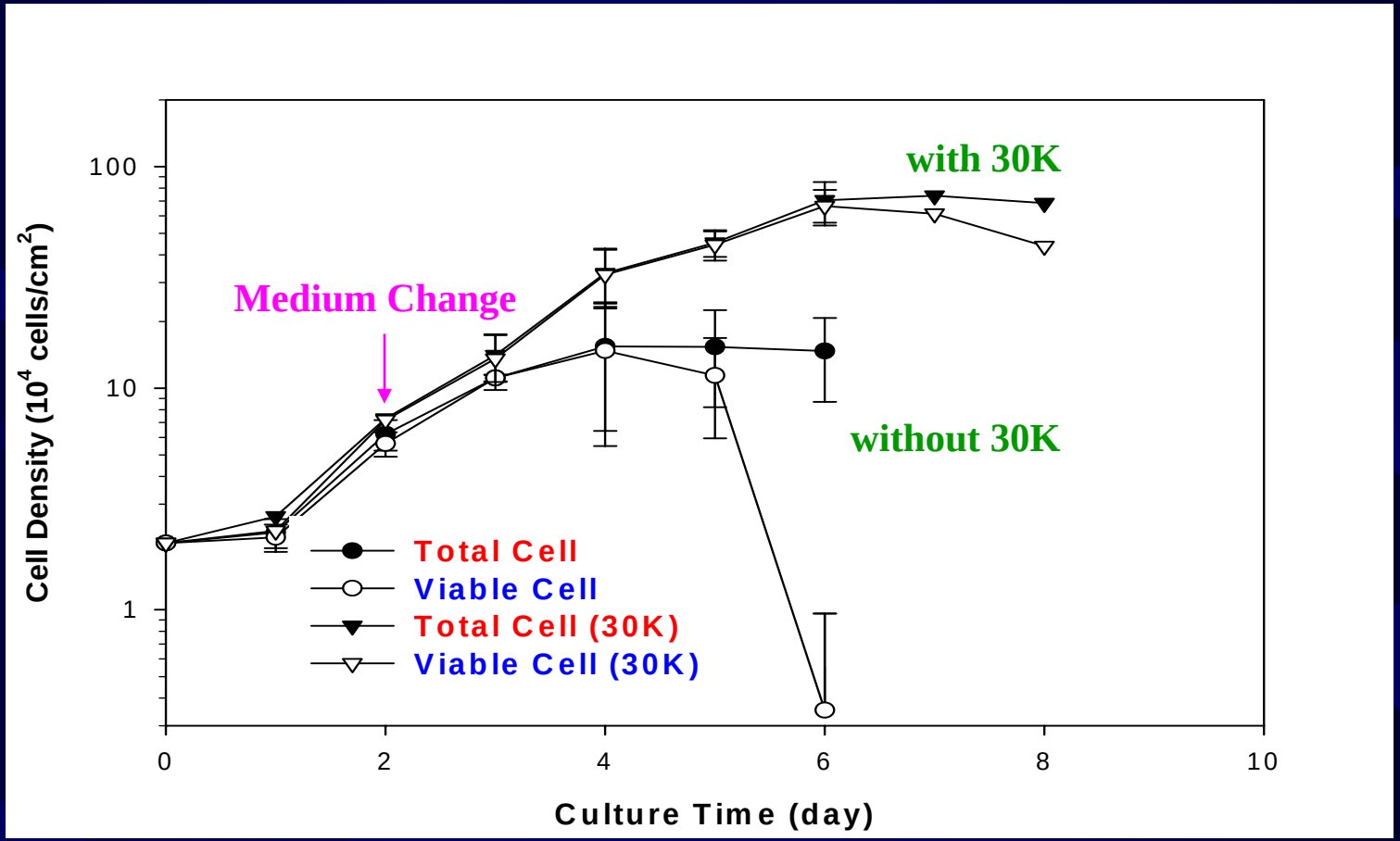
Seoul National University

Transfection of EPO Producing CHO Cell with 30K gene



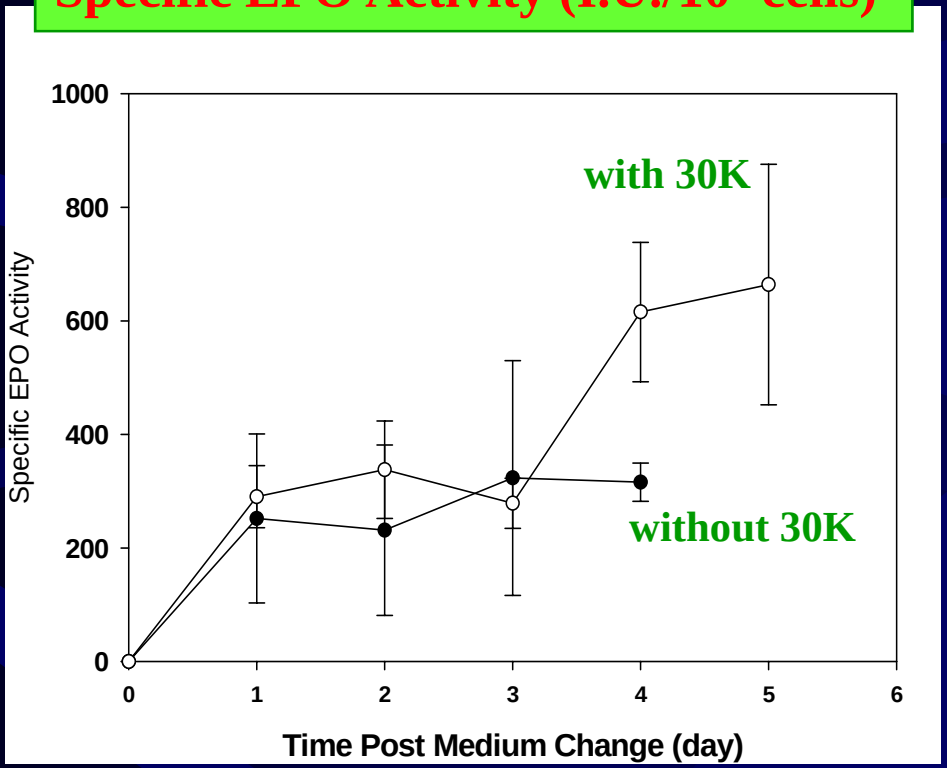
Seoul National University

Effect of 30K Expression on Cell Growth and Viability

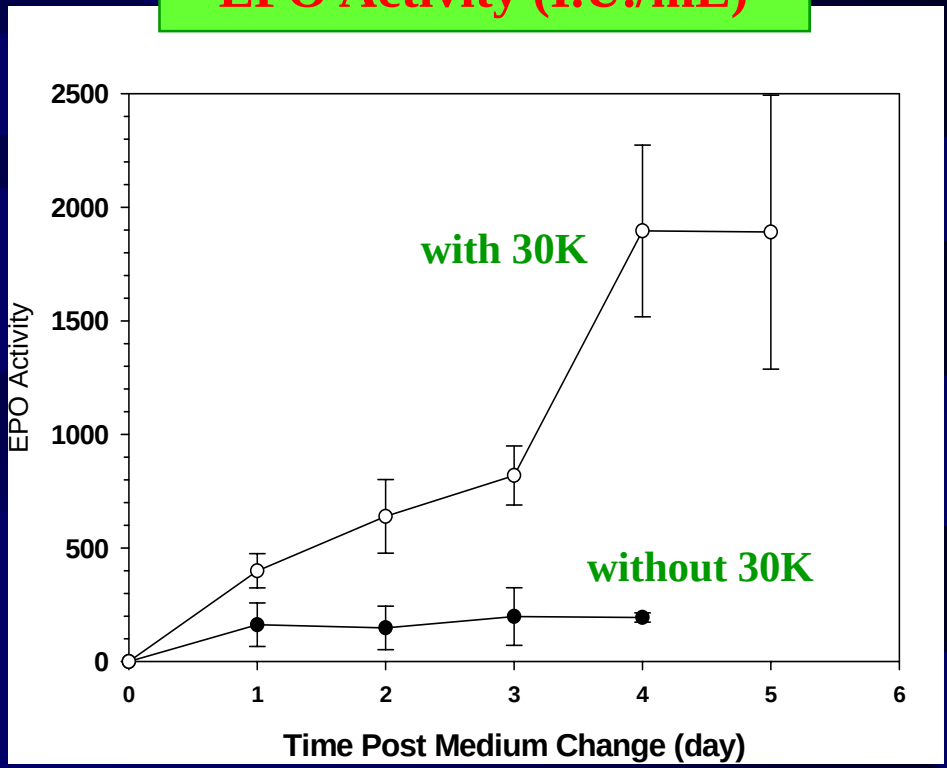


Effect of 30K Protein Expression on EPO Production

Specific EPO Activity (I.U./10^6 cells)



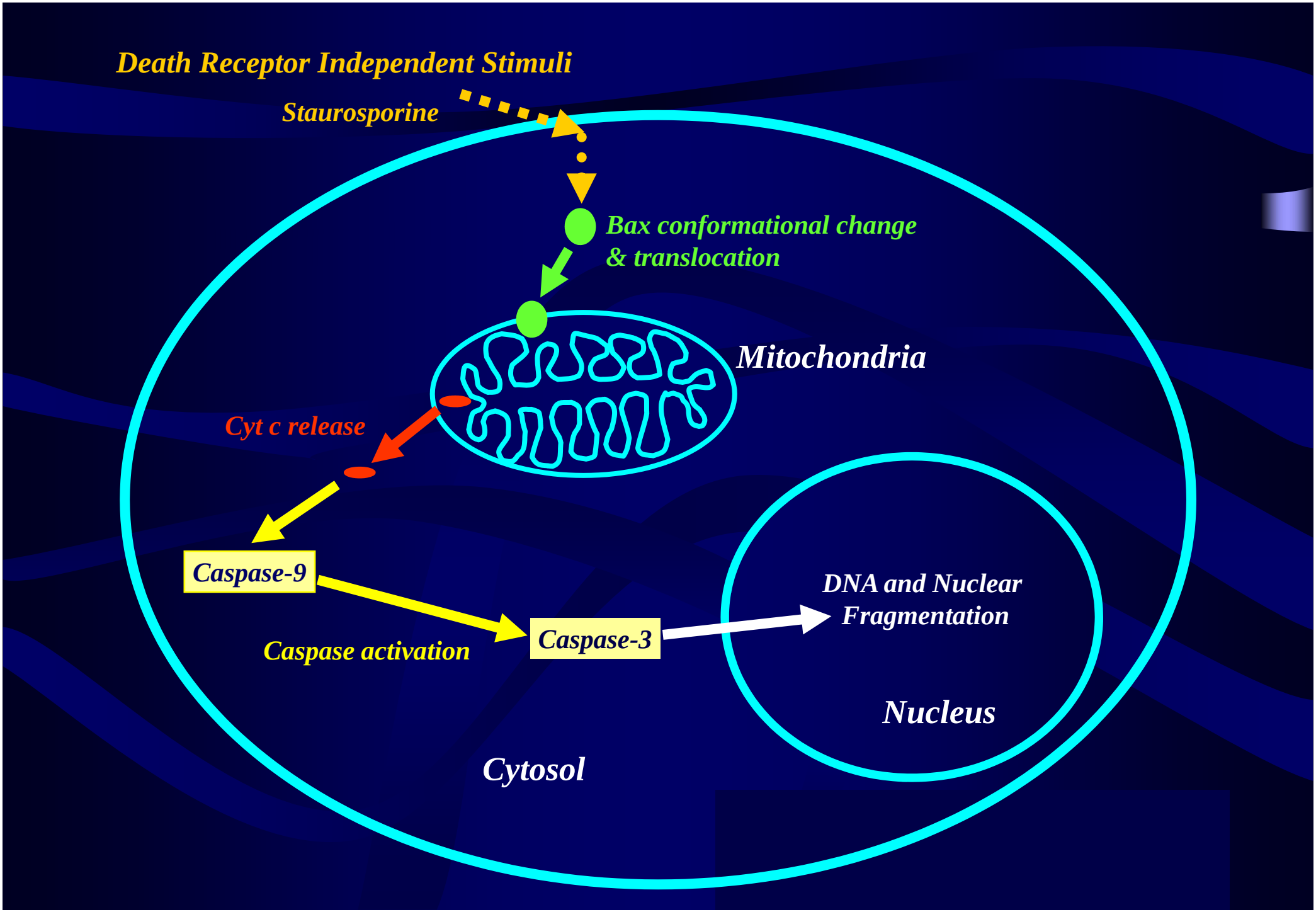
EPO Activity (I.U./mL)

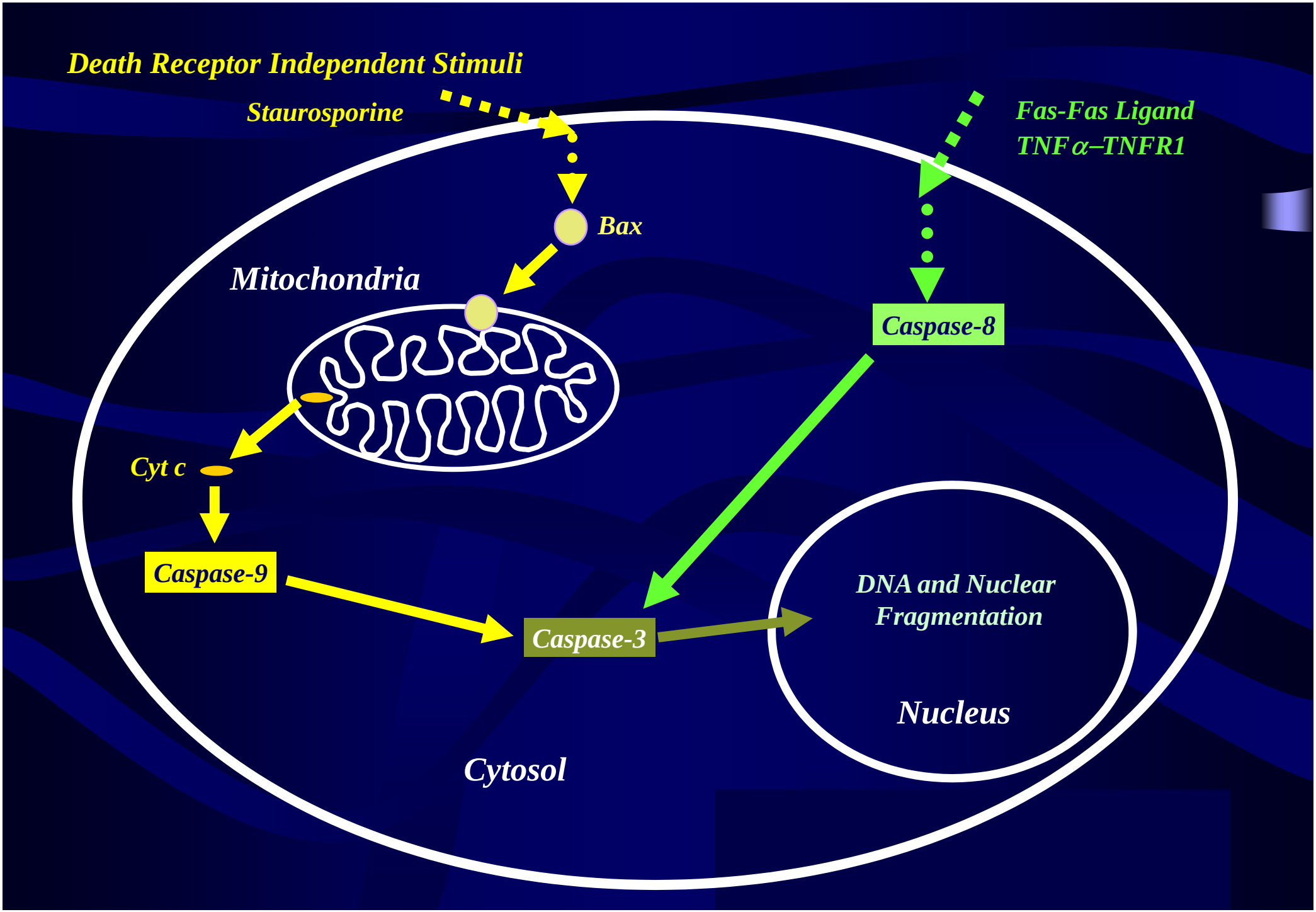


Seoul National University

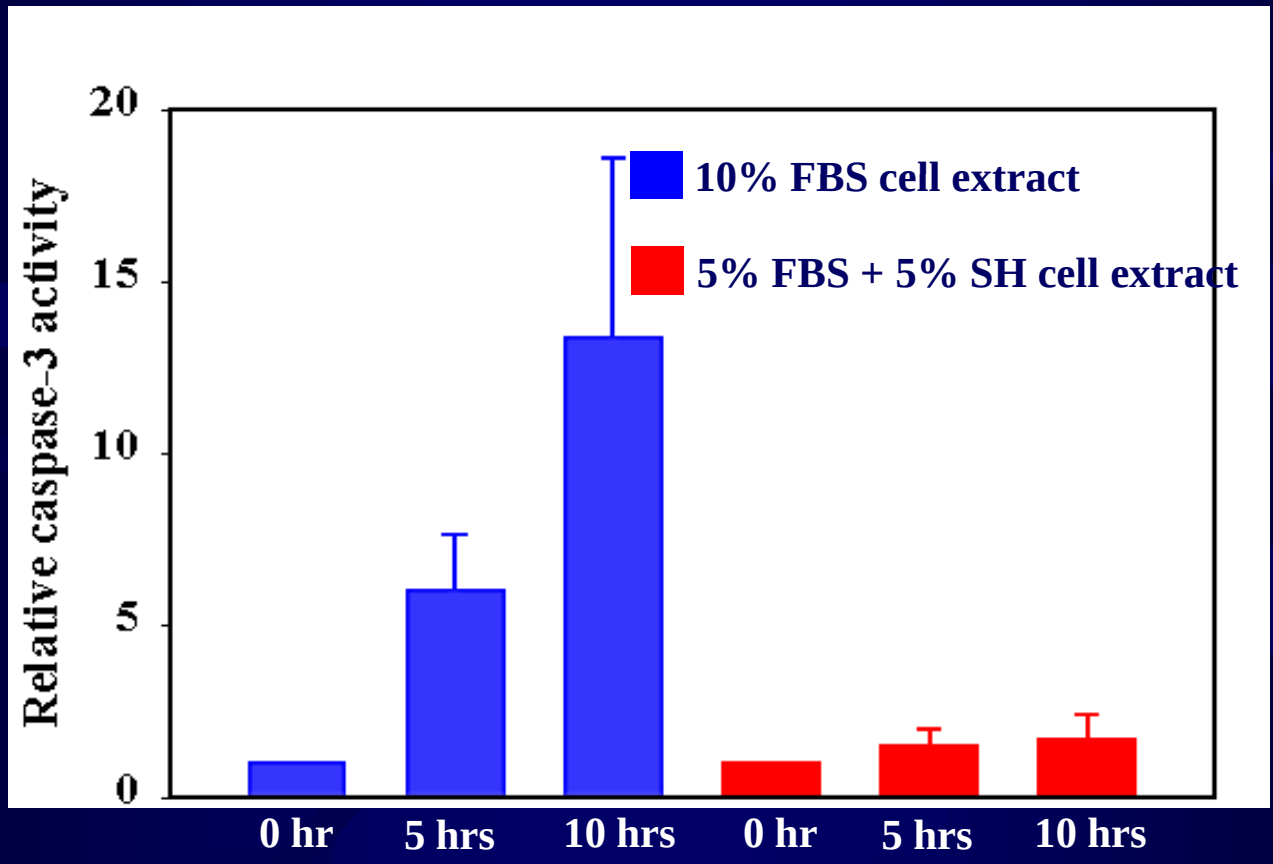
Apoptosis Inhibition Mechanism

Seoul National University


Death Receptor Independent Stimuli
Staurosporine
Bax conformational change
& translocation
Mitochondria
Cyt c release
Caspase-9
Caspase activation
Caspase-3
DNA and Nuclear
Fragmentation
Nucleus
Cytosol


Death Receptor Independent Stimuli
Staurosporine
Fas-Fas Ligand
TNF α–TNFR1
Bax
Mitochondria
Caspase-8
Cyt c
Caspase-9
Caspase-3
DNA and Nuclear
Fragmentation
Nucleus
Cytosol

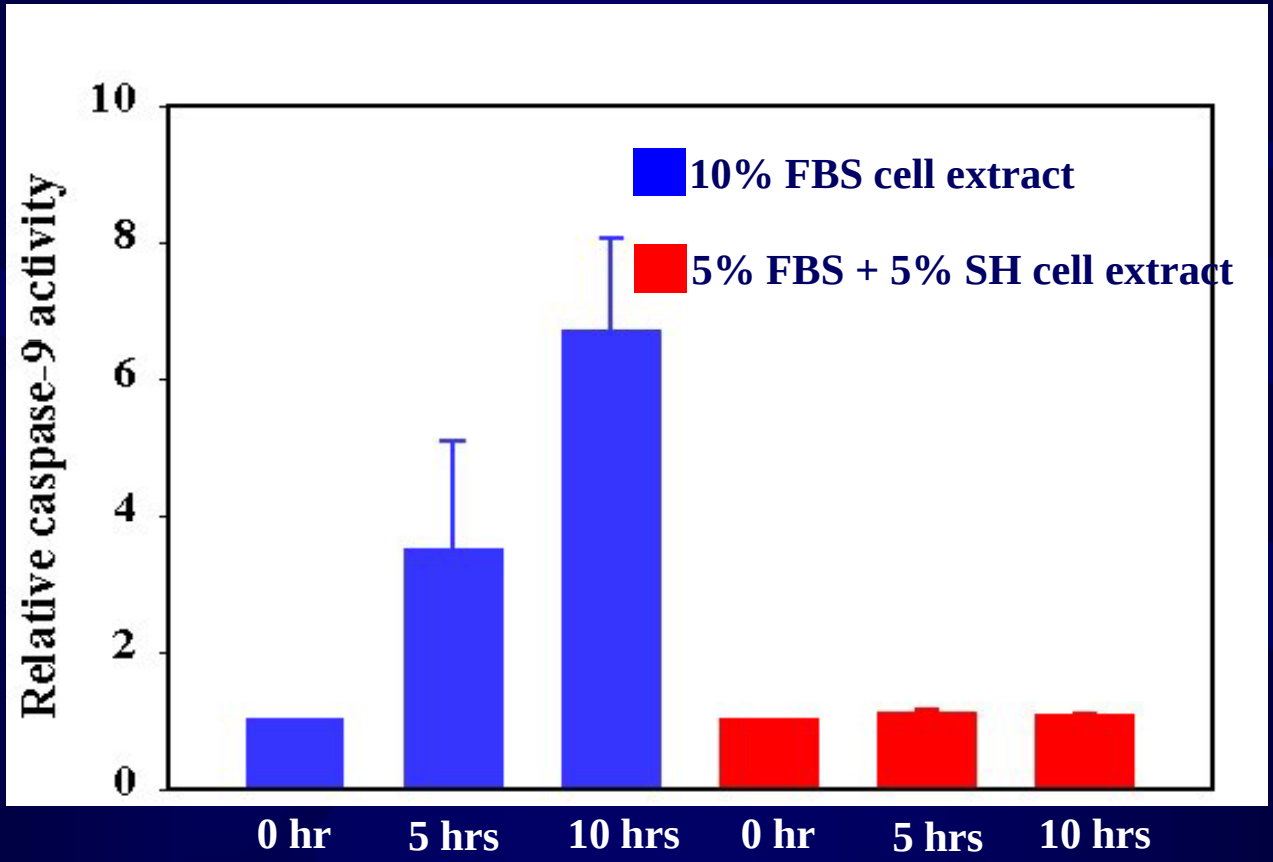
Intracellular Caspase-3 Activity
(staurosporine-induced HeLa cell)



Seoul National University

Intracellular Caspase-9 Activity

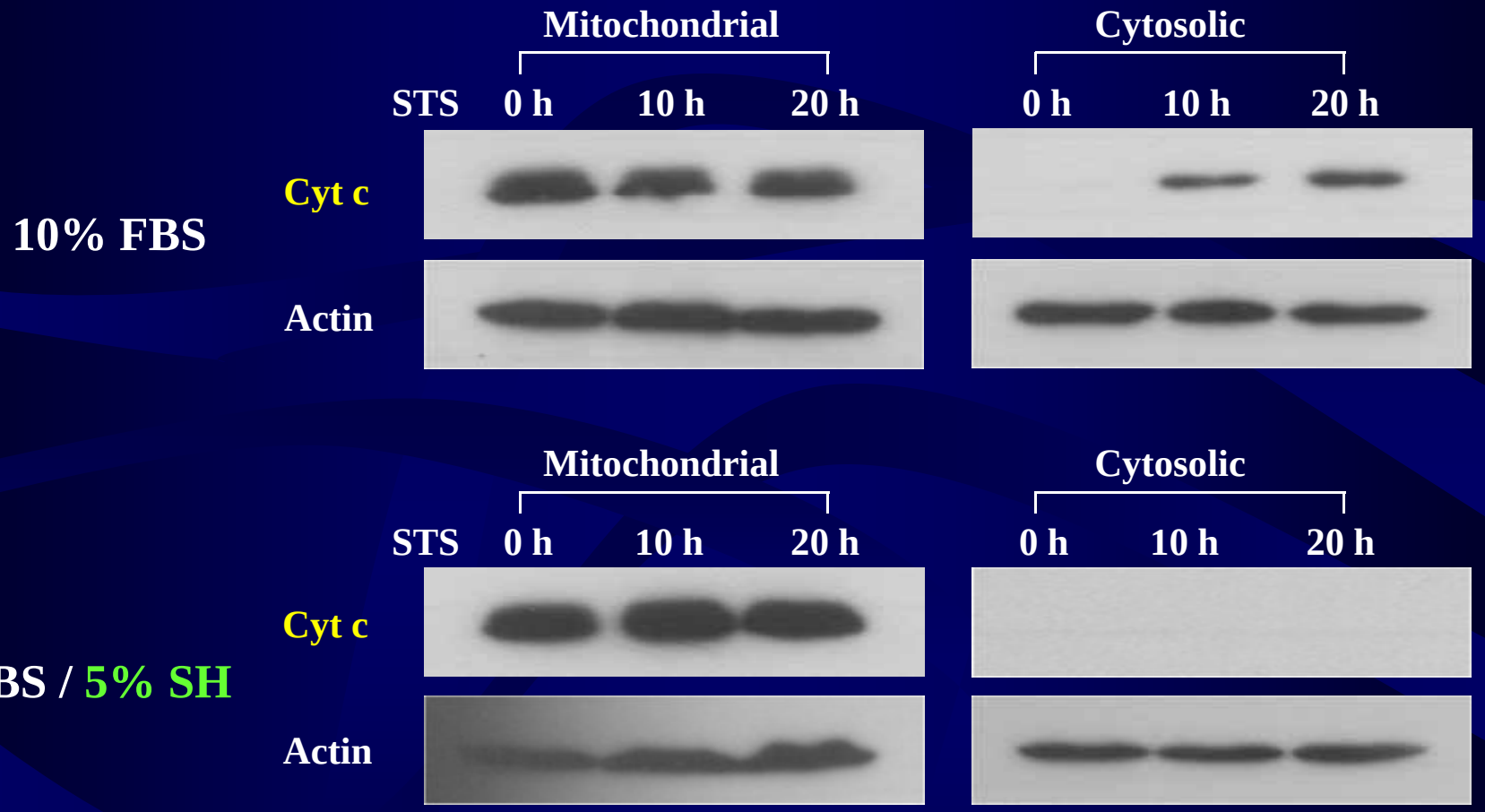
(staurosporine-induced HeLa cell)



Seoul National University

Cytochrome c Release from Mitochondria

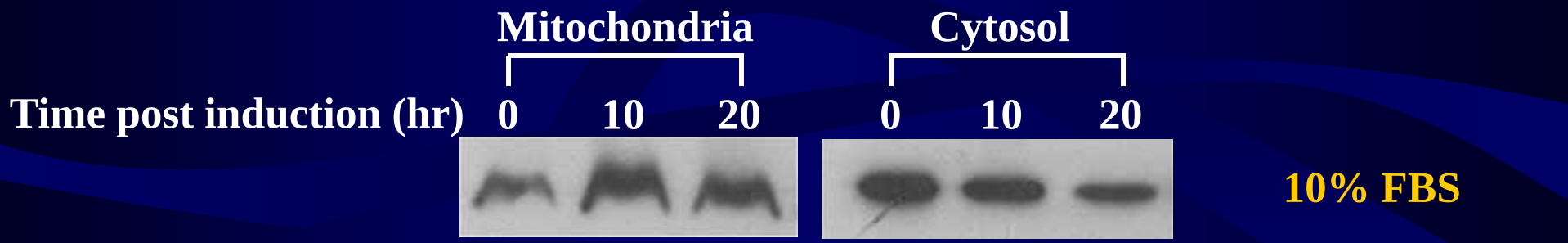
(staurosporine-induced HeLa cell)



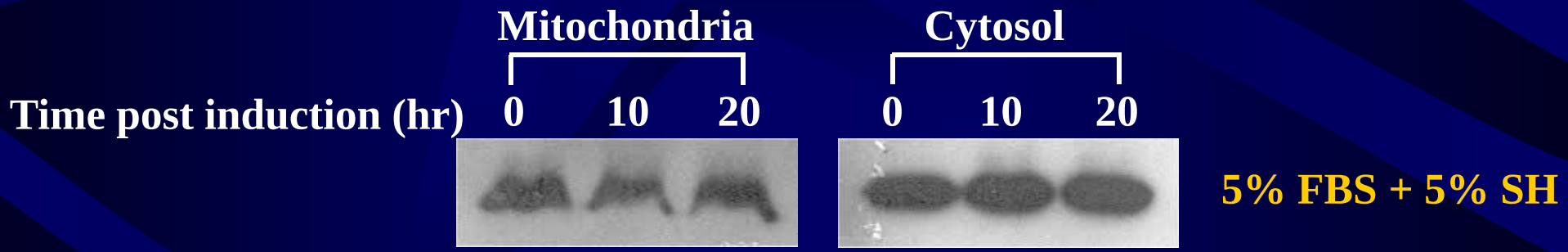
Seoul National University

Bax Translocation to Mitochondria

(staurosporine-induced HeLa cell)



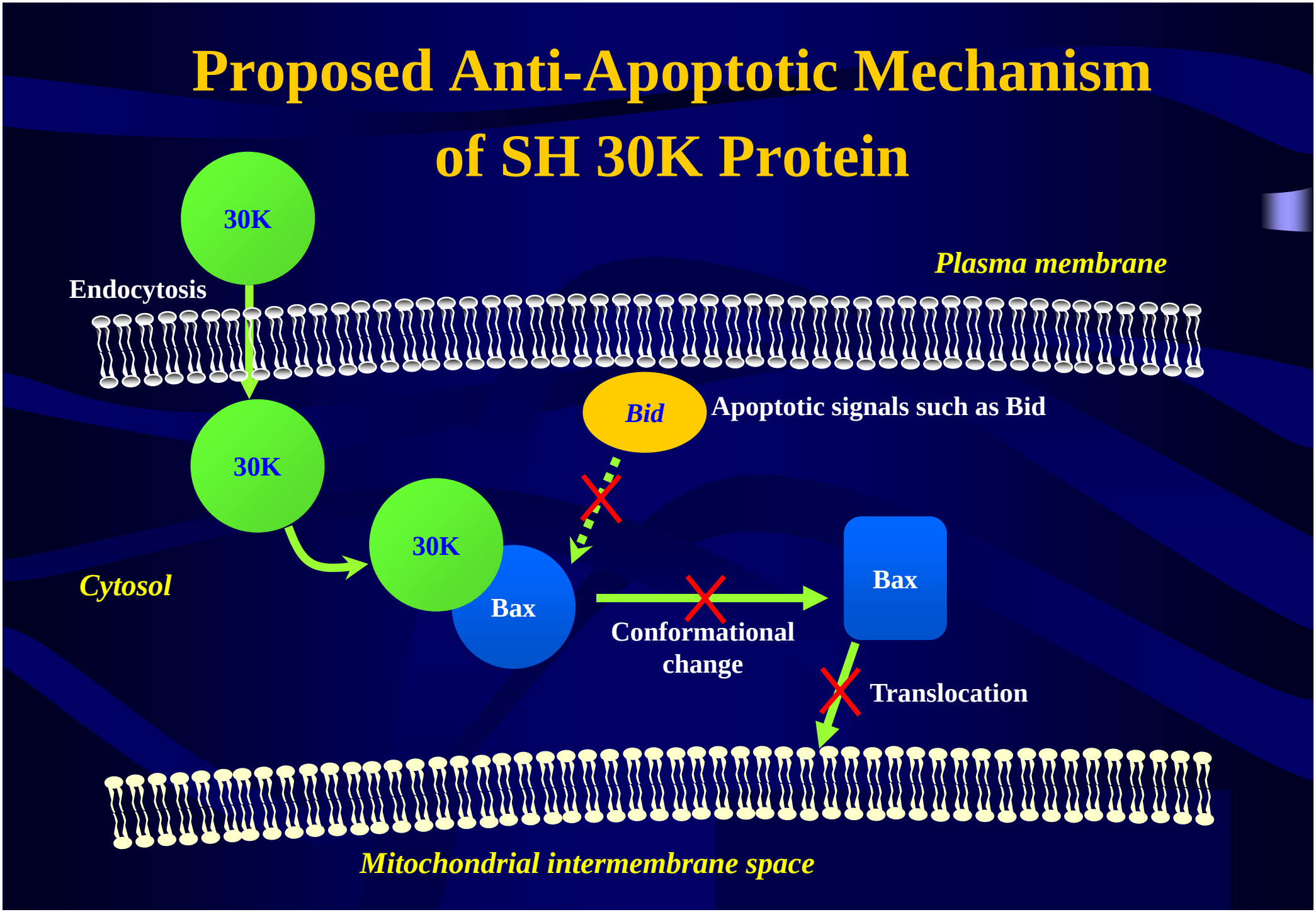
10% FBS



5% FBS + 5% SH

Seoul National University

Proposed Anti-Apoptotic Mechanism of SH 30K Protein



Summary

- **Silkworm hemolymph** inhibits the apoptosis.
- The most effective component for the apoptosis inhibition is the **30K protein**.
- Intracellular expression of the **30K gene** in mammalian cells
  - Inhibition of **apoptosis**
  - Increase of recombinant protein **production**
- This novel anti-apoptotic strategy can be used for **industrial applications** of animal cell cultures.