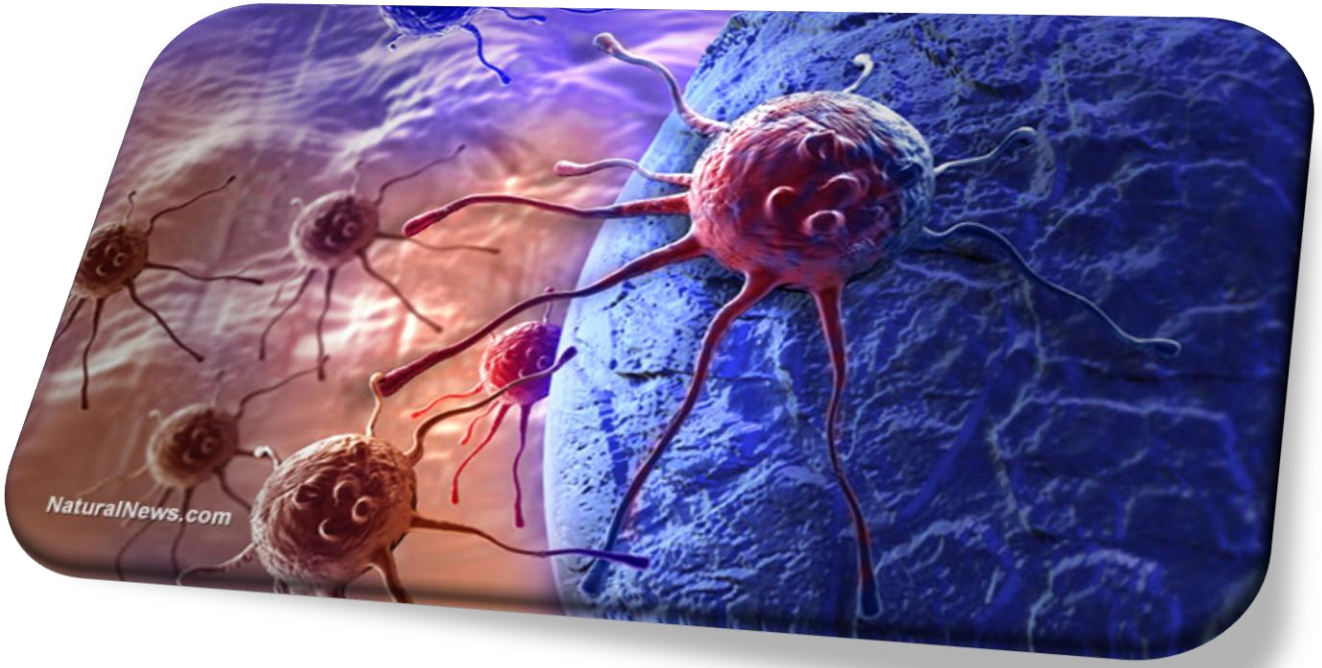
In the name of God

The tumoricidal activity of Salmonella and its role in treating cancers



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Research Paper

Salmonella typhimurium Mediated Delivery of Apoptin in Human Laryngeal Cancer

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A chicken anemia virus derived protein Apoptin

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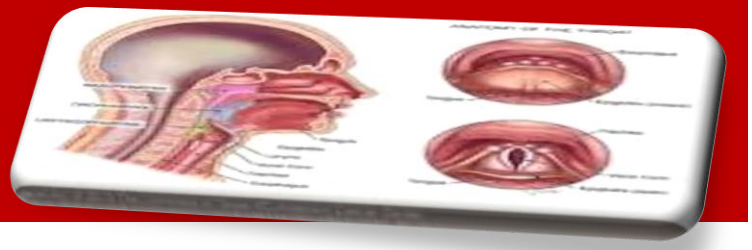
Introduction

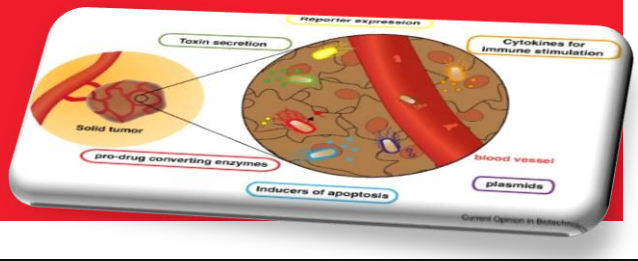


- Head and neck squamous carcinoma is estimated to be the **sixth most** common malignant tumour worldwide, of which laryngeal carcinoma is the second most common type.

Many bacteria have been shown to selectively target tumours and have been used in cancer gene delivery

To produce significant anti-tumour responses, high concentrations and multiple administrations of the bacteria are often required

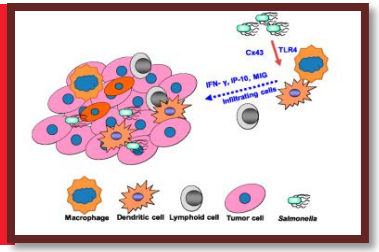
Bacteria in cancer therapy



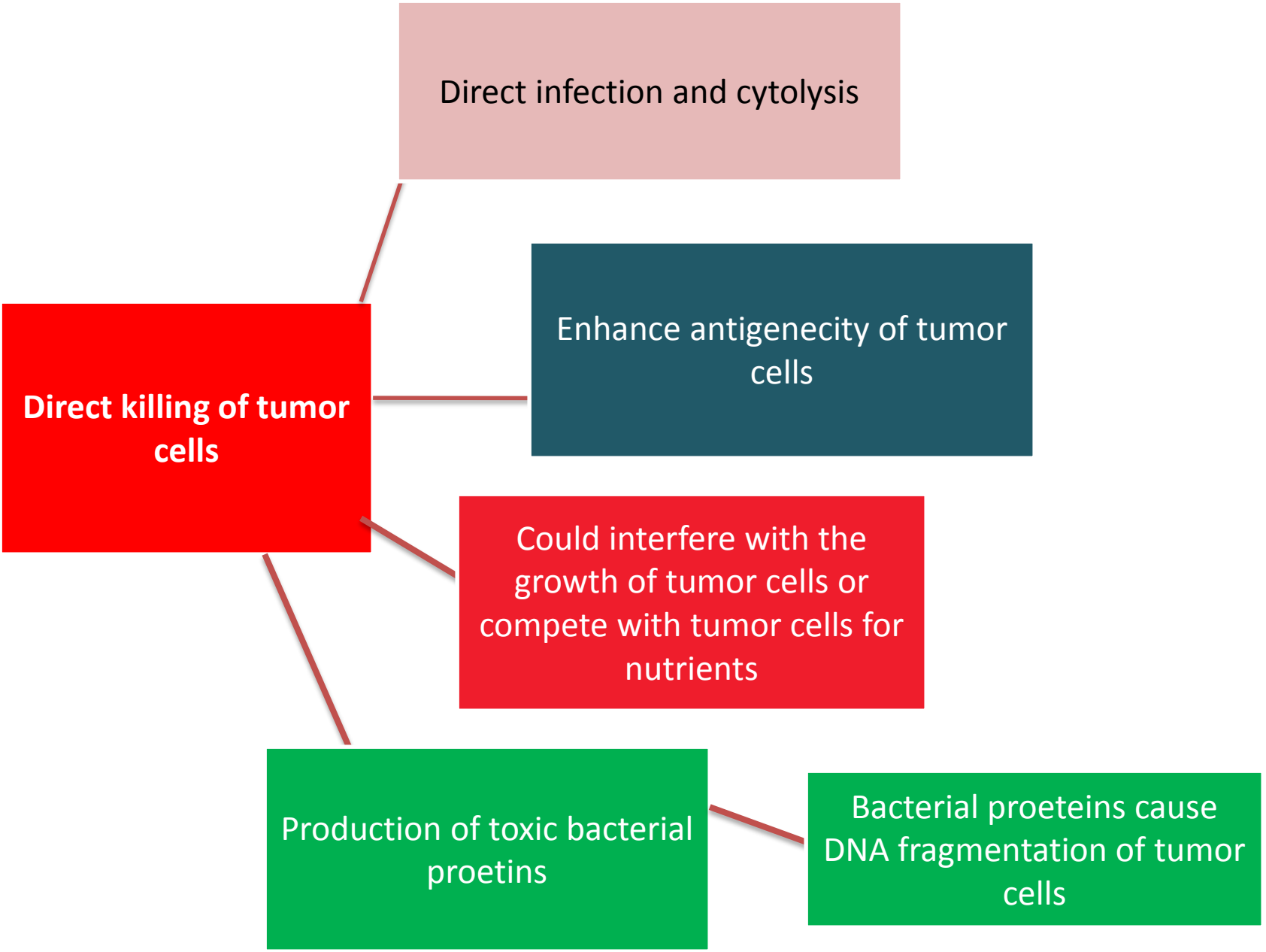
- Bacteria-mediated cancer therapy (BCT) was first introduced in the late 19th century when William B.
- In the past 20 years, bacterial cancer therapy has again become a hot topic, and various kinds of bacteria have been the subject of preclinical and clinical research.

S. typhimurium can grow under both aerobic and anaerobic conditions and so can colonize both large and small tumors.

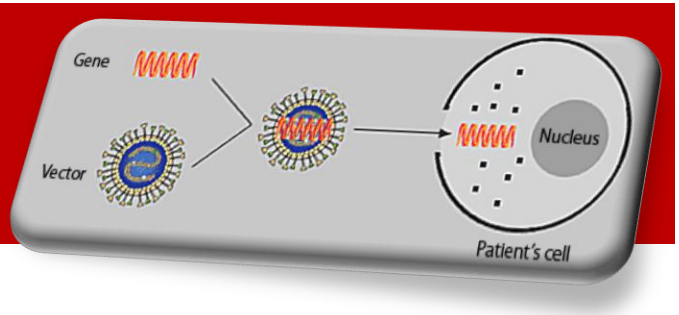
Salmonella typhimurium in cancer therapy



- ✓ Sensing the tumor microenvironment : Hypoxia (＜1% oxygen)
- ✓ Tumor penetration and proliferation: Motility is a key feature
- ✓ Immune stimulation: **IL-1, IL-18, TNF, and INF**
- ✓ Competition for food: **nitrate and nitrite reductase enzymes**


Direct infection and cytolysis
Enhance antigenecity of tumor cells
Direct killing of tumor cells
Could interfere with the growth of tumor cells or compete with tumor cells for nutrients
Production of toxic bacterial proetins
Bacterial proeteins cause DNA fragmentation of tumor cells

Indirect killing of tumor cells



Vector for immunotheraphy	Vector for Gene theraphy	Vector for cytotoxicity
cytokines	DNA vaccine for tumor antigens or anti-apoptotics	Carry cytotoxic drugs

Materials and Methods

- ✓ Cell line, bacteria, plasmids and animals
- ✓ Construction of expression vectors
- ✓ Attenuated S. typhimurium transformation using electroporation
- ✓ Cell infection and western blotting
- ✓ Cell proliferation assay
- ✓ Annexin V and Flow cytometric apoptosis assays
- ✓ Caspase activity assay
- ✓ Immunohistochemistry
- ✓ Tumour growth experiments
- ✓ Analysis of bacterial distribution
- ✓ Statistical analysis

Cell line, bacteria, plasmids and animals

- ✓ The human laryngeal cancer cell line, Hep-2, was purchased from the China Center for Type Culture Collection:

→ maintained at 37°C, 5% CO2 in RPMI 1640 complete medium ⇄ with 10% fetal bovine serum

- ✓ Six to eight week old female BALB/c nude mice were obtained from the Shanghai Institute of Experimental Animals.
- ✓ S. typhimurium phoP/phoQ null strain LH430

Construction of expression vectors

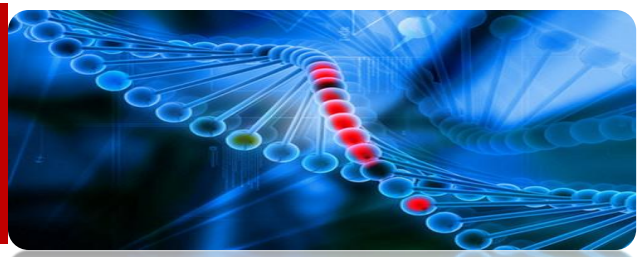
- Prokaryotic expression plasmids were generated that contained Apoptin or enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus (CMV) promoter.

In brief, pcDNA-EGFP, the control plasmid, was constructed by excising the EGFP fragment from pGH-EGFP

using EcoRI and PstI, and sub-cloned into EcoRI and PstI sites on pcDNA3.1.

The appropriate sequences of inserts were confirmed with use of an ABI DNA Sequencer (USA).

Attenuated S. typhimurium transformation using electroporation



- LH430, was harvested at 4°C → washed in ice-cold 10% glycerol.

the bacteria were re-suspended into electrotransfer buffer (10% glycerol, 0.125% yeast extract, 0.25% tryptone)

electro-transferred into 0.2 cm cuvettes after 40 µl competent cells were mixed

with either 25 ng plasmid DNA pcDNA-EGFP ⟷ **Luria-Bertani (LB) media with 50 µg/ml ampicillin**

pcDNA-EGFP or pcDNA-Apoptin plasmid constructs were named ST-rC-EGFP and ST-rC-Apoptin, respectively

Cell infection and western blotting

- Cell infection, cell lysis, protein quantification, and western blots were performed as described previously.
- In brief, Hep-2 cells (2 x105) were co-cultured with recombinant bacteria (1×108 cfu) for 1 hour at 37°C.
- The infected cells were then rinsed and cultured in RPMI 1640 medium containing gentamycin sulfate (20 μg/ml) to eliminate external bacteria and tetracycline (5 μg/ml) to prevent infection with internal bacteria.

Cell infection and western blotting

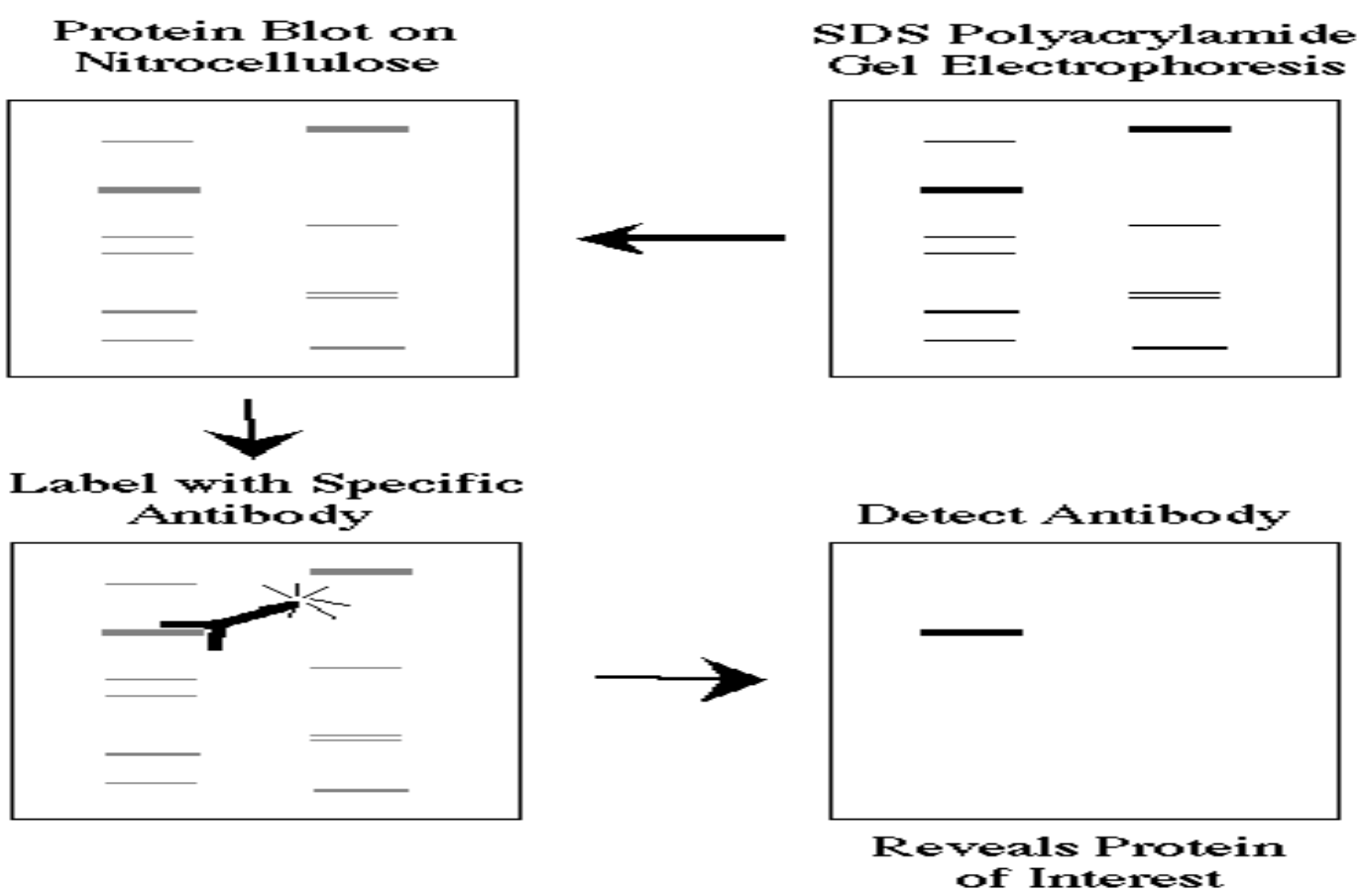
- Twenty-four hours after infections, the cells were collected and lysed.

Protein extracts were prepared and quantified using the BCA method

- With 20 µg of protein per well, 15% SDS-PAGE was performed, and samples were then transferred onto Hybond-C membranes.

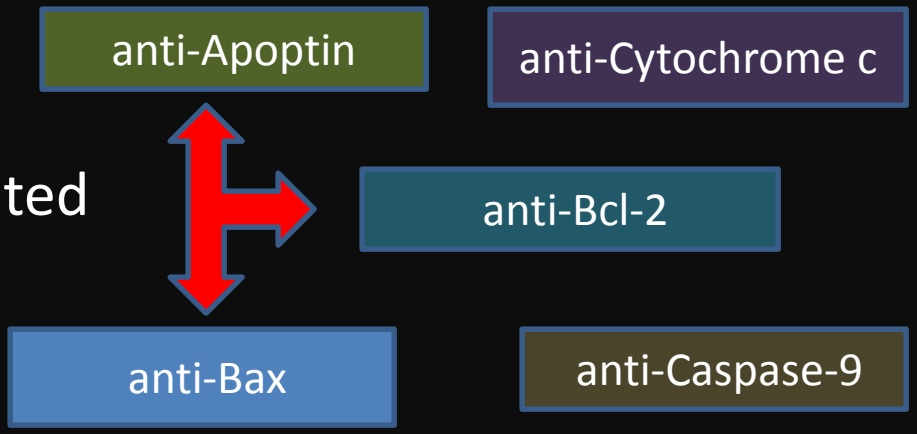
Membranes were blocked in 5% milk

Western Blot



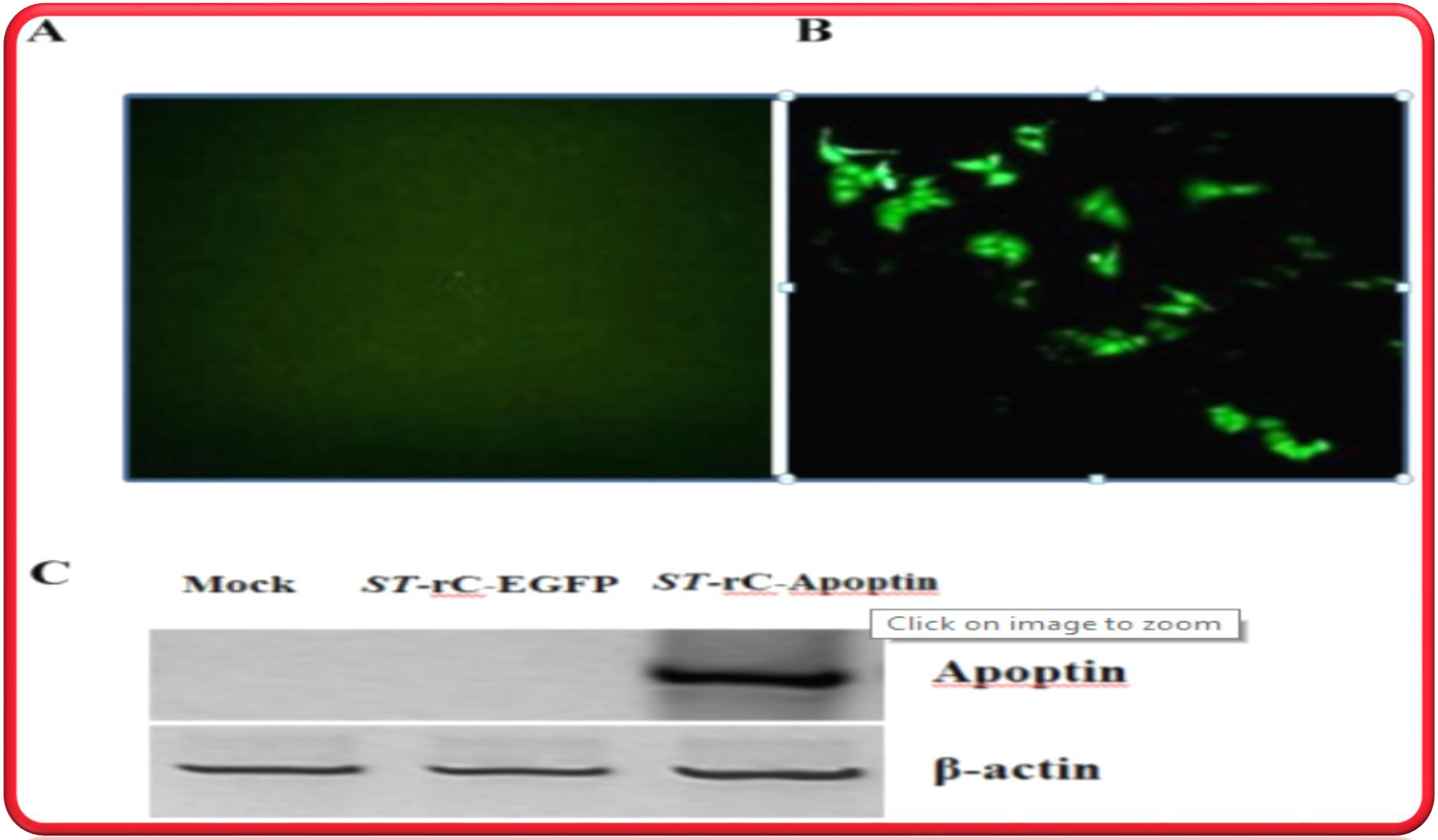
Cell infection and western blotting

- ✓ Blots were incubated



- ✓ for 2 hours followed by incubation with the second antibodies labeled with horseradish peroxidase for 2 hours. Signals were visualized using the ECL Western blotting substrate kit

Expression of attenuated *S. typhimurium*-mediated Apoptin in Hep-2 cells



Cell proliferation assay

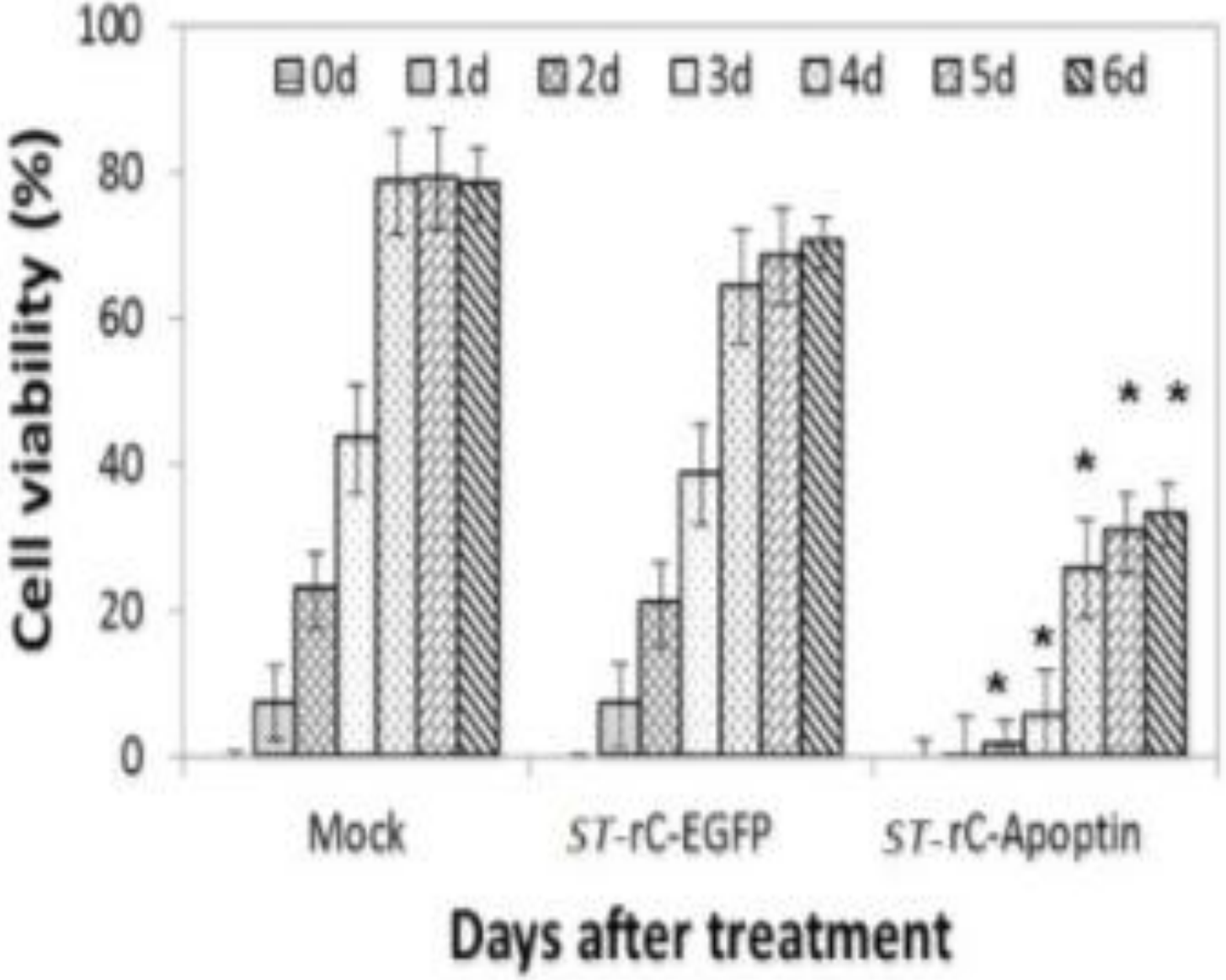
- Hep-2 cells (5×103) were plated in 96-well plates 24 hours before infection with the recombinant bacteria (1×104 cfu per well) as described above.

- Cell viability was assessed using a 3-[4,5-dimethylthiazol-2- yl]-2,5-diphenyltetrazolium bromide (MTT) staining kit (Sigma, USA) every day over a 6 day period according to the manufacturer's protocol. Briefly, cells were incubated with 5 g/L of MTT for 4 hours.

Cell proliferation assay

- the medium was removed, and 100 µl of dimethylsulphoxide were added. The absorbance of the reaction solution was measured at 490 nm. The cell viability was calculated as follows:

A = (1 - absorbance of experimental group/absorbance of control group) × 100%.

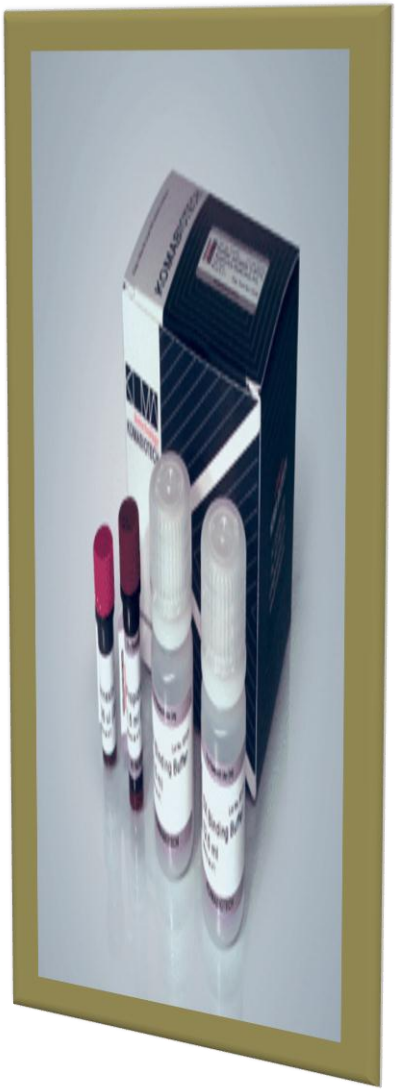

100
80
60
40
20
0
Cell viability (%)
0d
1d
2d
3d
4d
5d
6d
Mock
ST-rC-EGFP
ST-rC-Apoptin
Days after treatment

Annexin V and Flow cytometric apoptosis assays

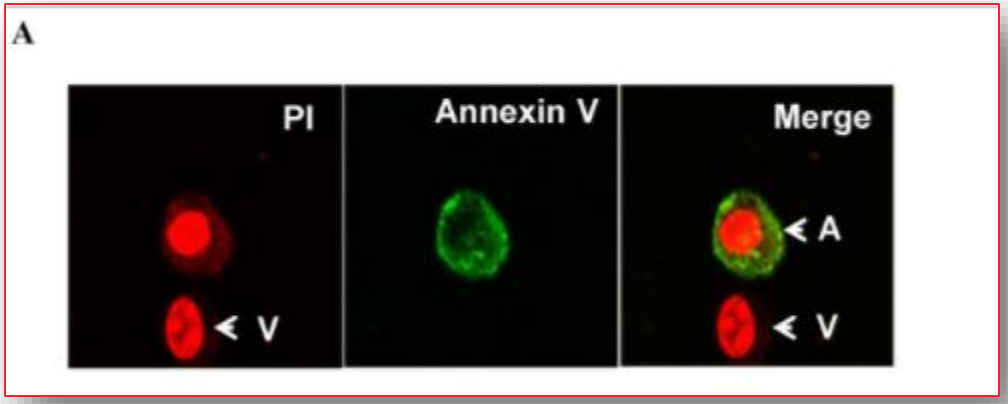
- Apoptosis was first visualized using an Annexin detection kit according to the manufacturer's protocol. In brief, Hep-2 cells (1 x 105) were infected with the recombinant bacteria for 48 hours.

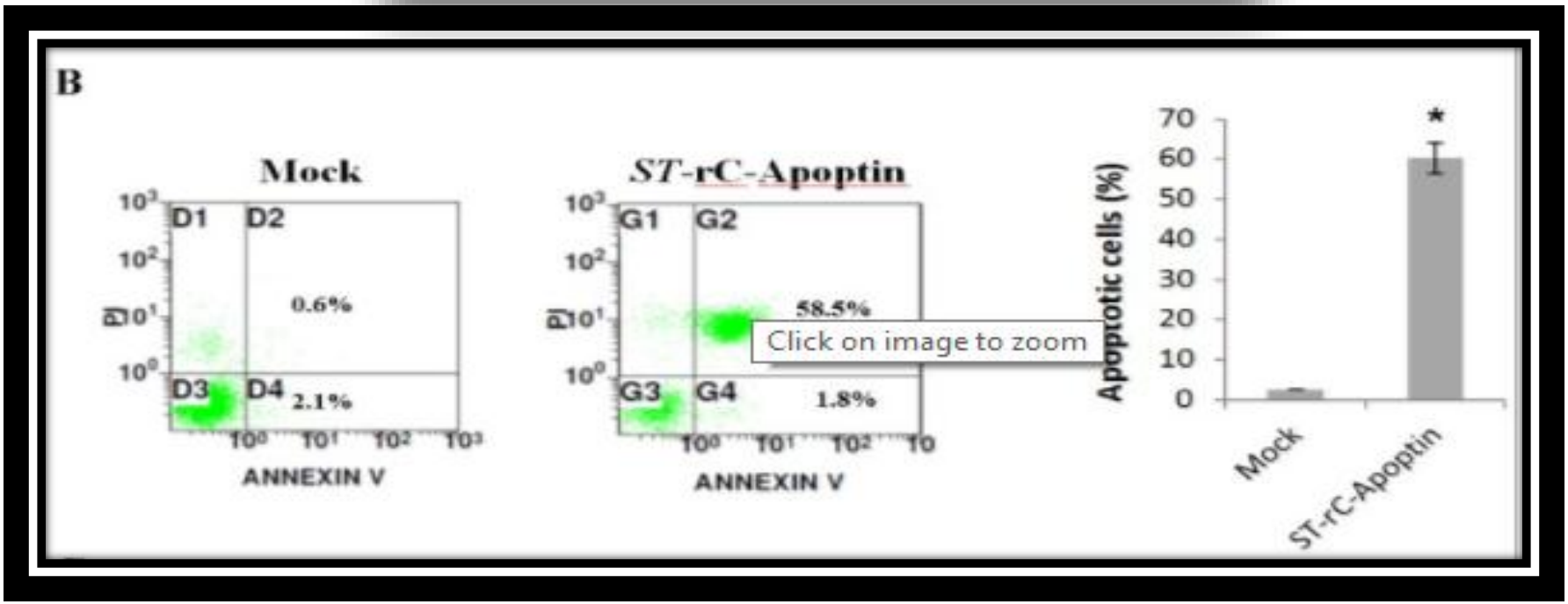
- The cells were then collected and re-suspended in binding buffer, followed by incubation with Annexin V labeled with fluorescein isothiocyanate (FITC) and propidium iodide (PI).

The stained cells were visualized with a TCS SP5 laser scanning confocal microscope

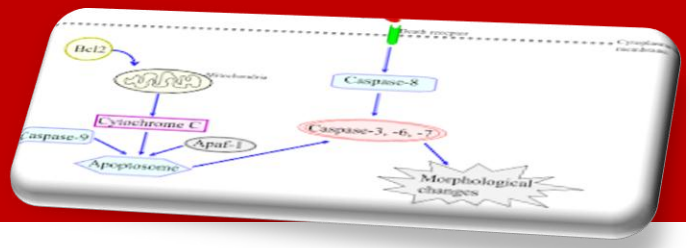


S. typhimurium-mediated Apoptin induces apoptosis and effectively reduces viability of Hep-2 cells



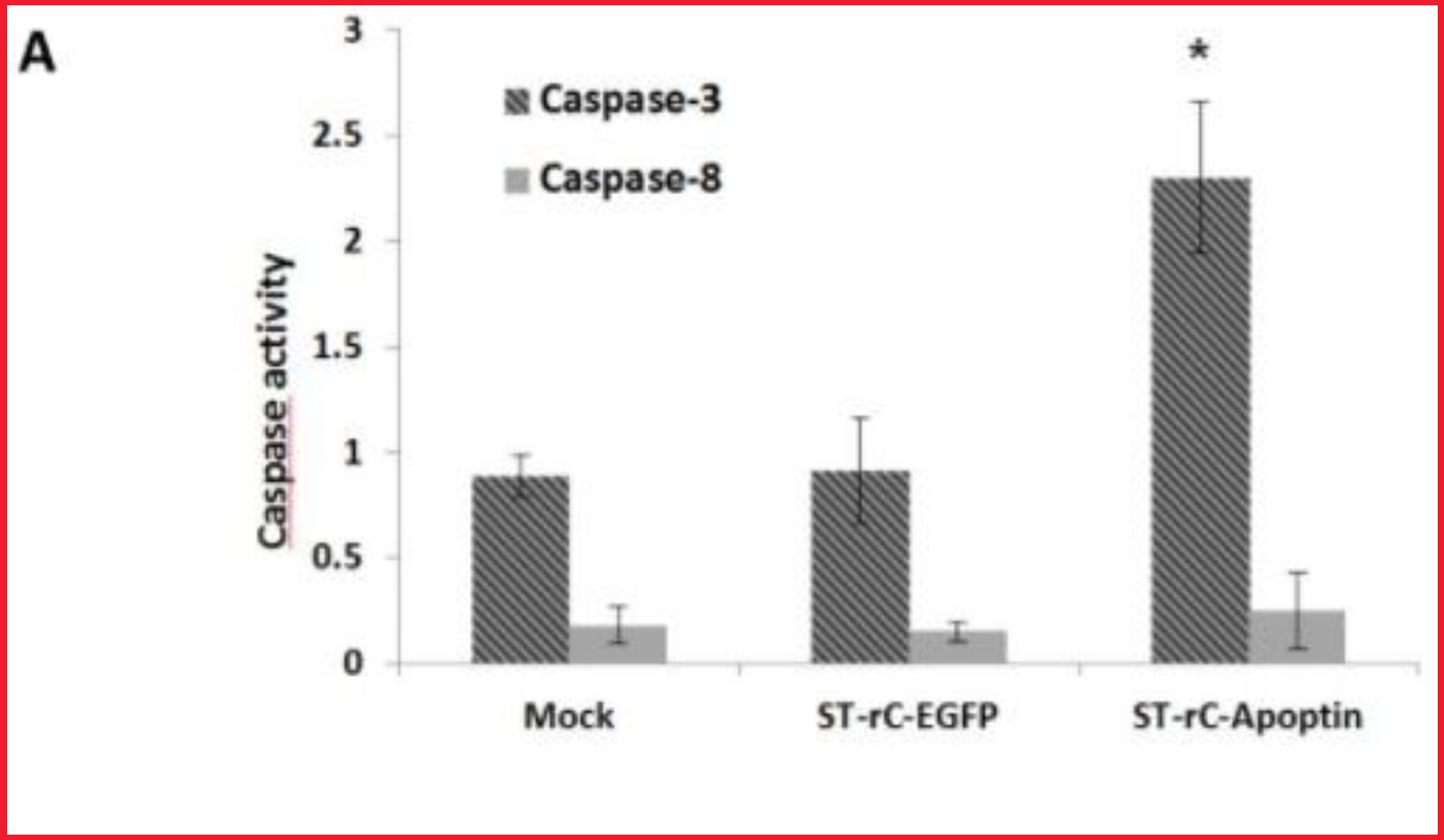


Caspase activity assay



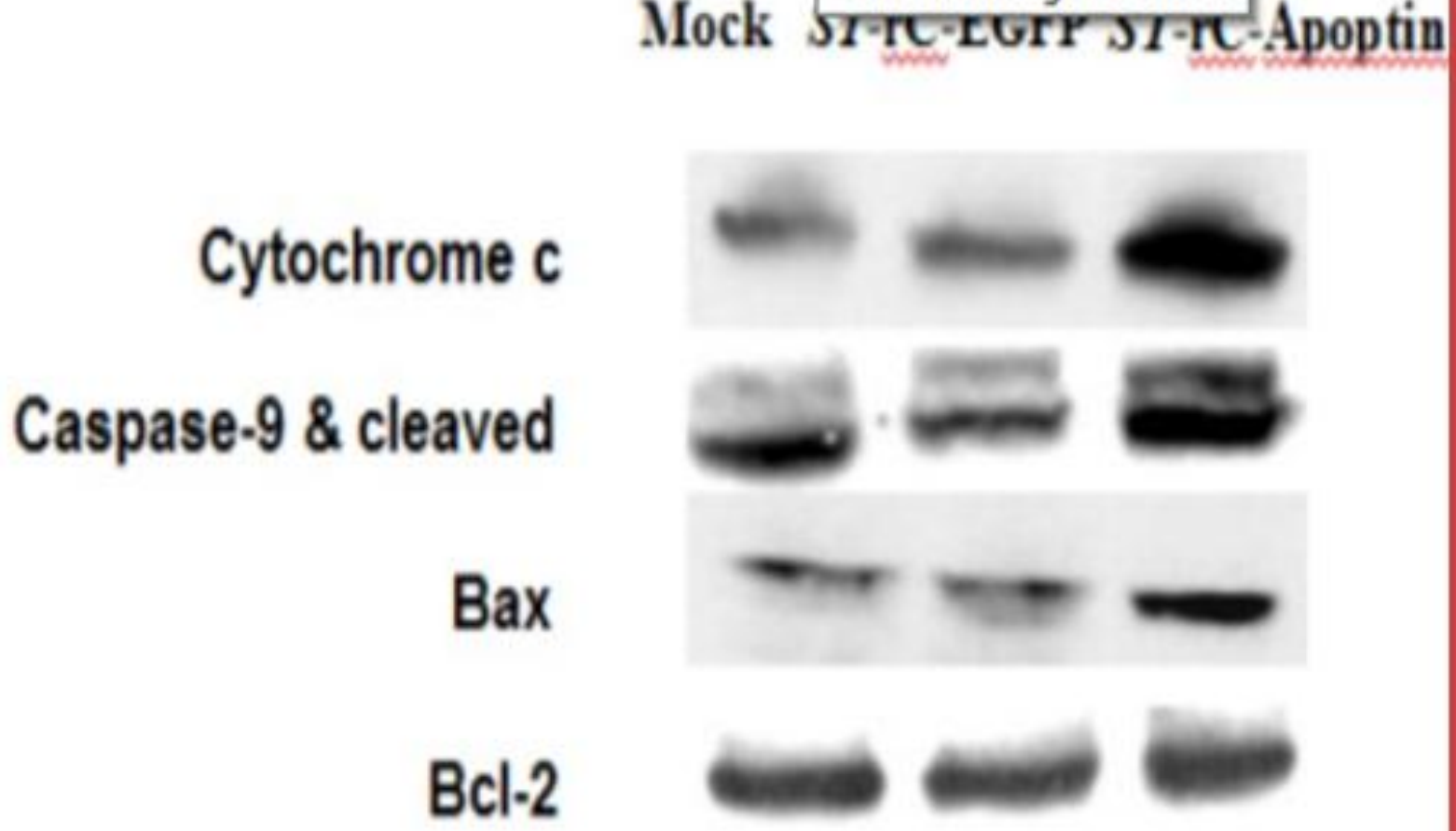
- Caspase activity assays were performed on Hep-2 cells (1 x 105) after infection with the recombinant bacteria for 48 hours using the Caspase 3/8 assay kit .
- according to the manufacturer's protocol. Briefly, cell lysates were obtained from 2×105 infected cells and incubated in caspase-3 substrate (DEVD-pNA) or caspase-8 substrate.
- plates were read at 450 nm and the data was analyzed.

S. typhimurium-mediated Apoptin increases the activity of caspase-3 and involves Bax, caspase-9 and Cytochrome c



Click on image to zoom

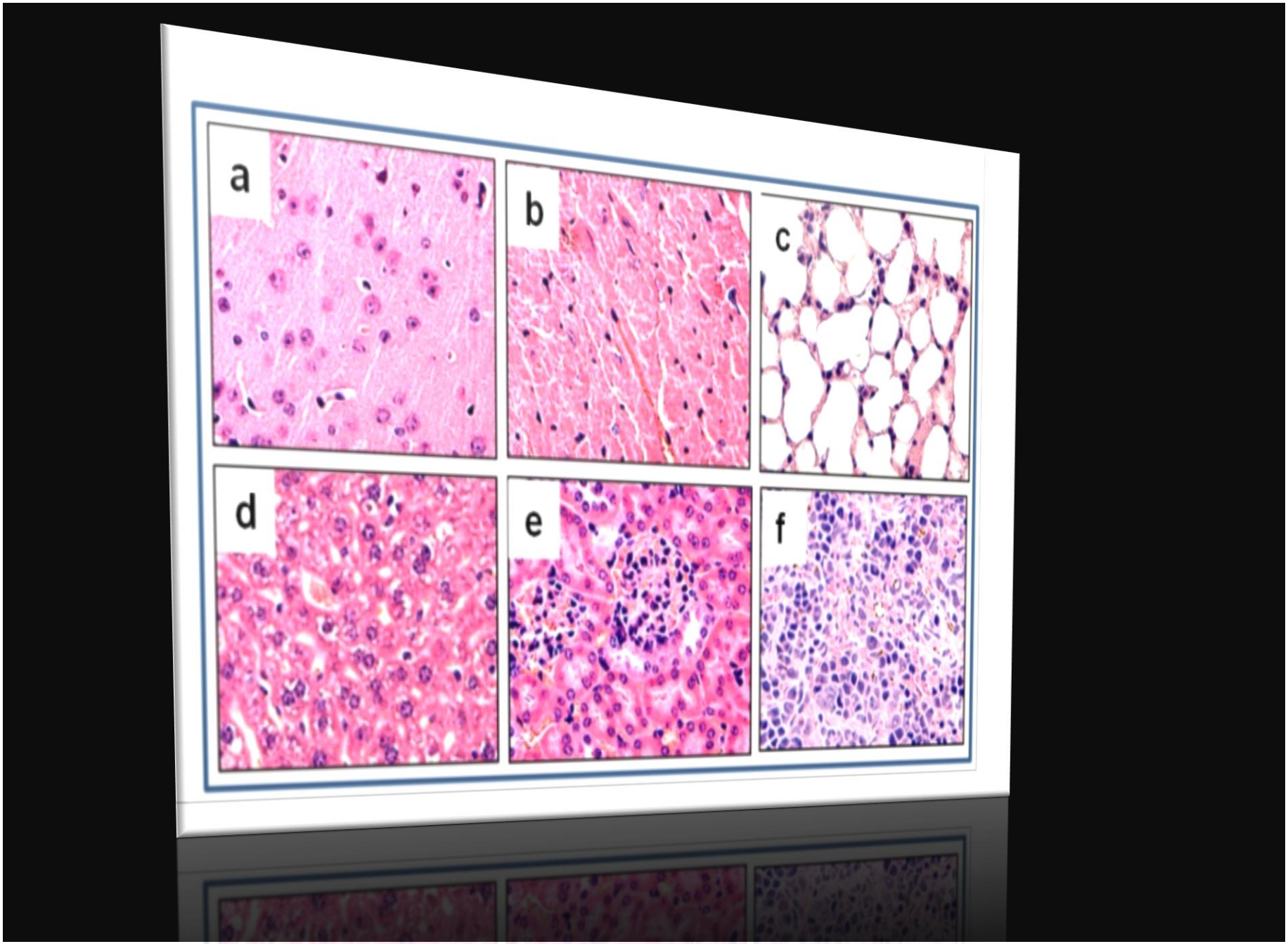
B



Immunohistochemistry

- Tumours were removed and immediately fixed in 10% buffered-formalin for paraffin embedded.
- Immunohistochemistry was performed using a purified rat anti-mouse CD31 antibody and evaluated using the Level-2 Ultra Streptavidin System with microwave antigen retrieval.
- Five randomly selected areas for each tumour were evaluated at 200x magnification.

Positive endothelial cell clusters were counted as an individual vessel.


a
b
c
d
e
f

Tumour growth experiments

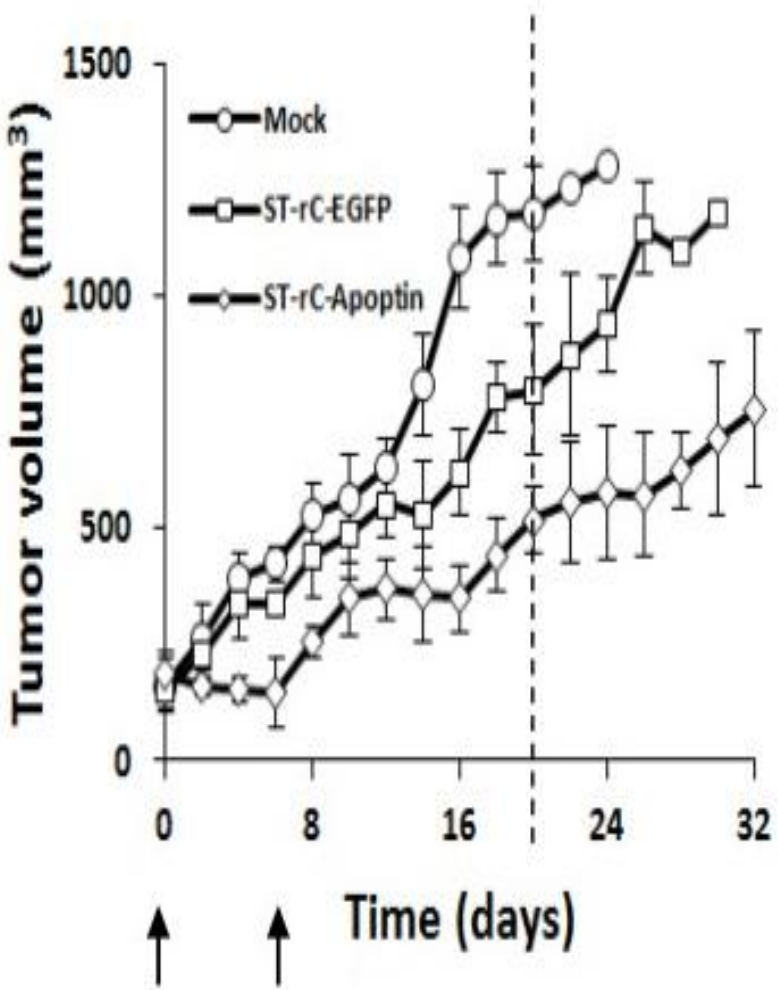
- At 8 weeks age, BALB/c nude mice received a subcutaneous injection of 2×106 Hep-2 cells. After 3 weeks of tumour growth, mice with tumours < 75 mm3 at 21 days were excluded from the analysis and only the rest were randomized into three groups:

A) Experimental group which further divided into following three sub-groups

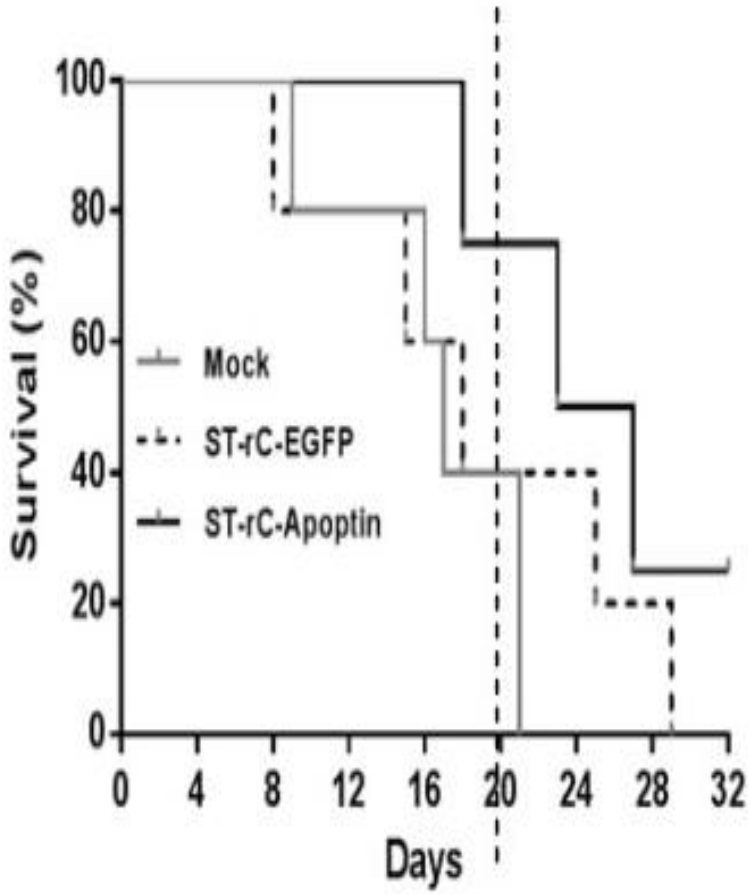
B) Repeat treatment groups;

C) Bacterial distribution and histologic analysis group

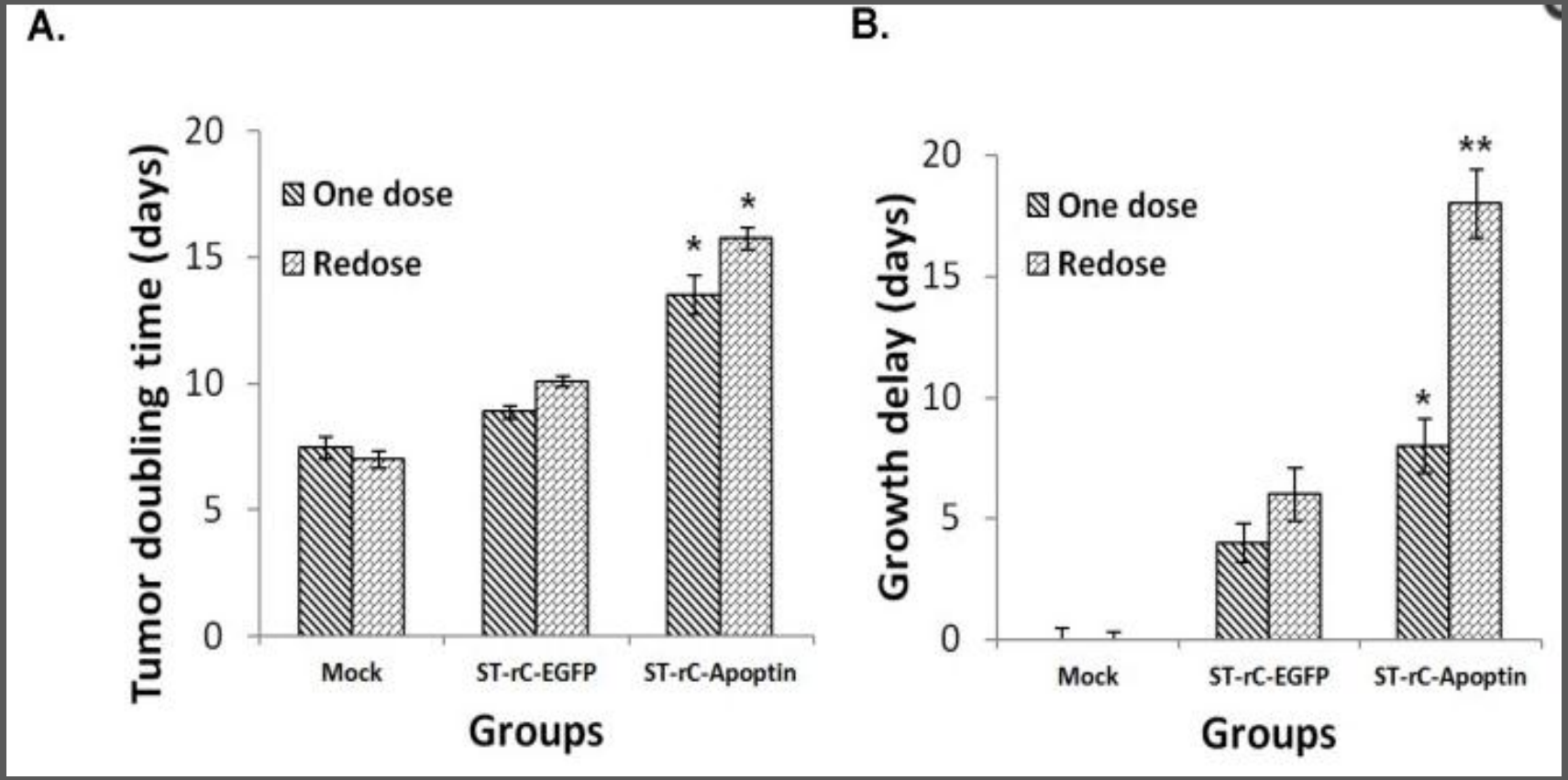
C.


1500
1000
500
0
Tumor volume (mm³)
Mock
ST-rC-EGFP
ST-rC-Apoptin
0
8
16
24
32
Time (days)

D.


100
80
60
40
20
0
Survival (%)
Mock
ST-rC-EGFP
ST-rC-Apoptin
0
4
8
12
16
20
24
28
32
Days

Tumour growth delay and microvessel density involvement.



Analysis of bacterial distribution

- Tissue samples were weighed and minced thoroughly, followed by homogenization.

- The tissue homogenates were plated onto LB agar containing ampicillin in triplicate, and the colony count was determined on the next day

Discussion

- ✓ This is the first report utilizing a recombinant attenuated Salmonella typhimurium to express tumour-specific apoptosis-inducing gene, Apoptin, in human laryngeal cancer.
- ✓ Salmonella typhimurium has been used before as a therapeutic delivery vector because of its natural tropism and immune privilege.
- ✓ Particularly, Salmonella typhimurium itself can secrete and inject more than 20 effector proteins into host cytosol/lysosome, which can inhibit phagosome-lysosome fusion and interact with the host machinery to allow the bacteria to survive and replicate in host cells.

Discussion

- The accurate mechanism of Apoptin-induced cell death has not yet been fully elucidated.
- A fundamental apoptotic pathway for Apoptin is independent of p53 and requires caspase activation.

The intrinsic pathway is involved in the release of cytochrome c from the damaged mitochondrion to the cytosol, which further leads to the activation of caspase-9.

Caspase-9 then triggers a cascade of caspases, e.g. caspase-3 and caspase-7, which in turn results in morphological and biochemical changes associated with apoptosis

Discussion

- The pro- and anti-apoptotic Bcl-2 protein family plays an important role in the presence of Apoptin.

reported that Bcl-2 and Bcl-xL inhibit Apoptin-induced cell death, whereas Bax expression sensitizes cells to apoptosis

- It is known that Bcl-2 binds to and inactivates Bax and other pro-apoptotic proteins, thereby inhibiting apoptosis

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