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Forced Expression of Heat Shock Protein 27 (Hsp27) Reverses P-Glycoprotein (ABCB1)-mediated Drug Efflux and MDR1 Gene Expression in Adriamycin-resistant Human Breast Cancer Cells

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Hypothesis

- Chronic exposure of cancer cells to Adriamycin (= Doxorubicin) silences HSF-1 gene

  → Supression of HSPs incl. HSP 27, which plays an important role in homeostasis of p53 and inducement of MDR1 gene in MCF-7/adr cells

- Overexpression of HSP 27 could potentially reverse drug resistance in these cells

Background

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Background

- Breast Cancer
  - Most common malignancy in women
  - Incidence =150/100000 women/a
  - Affects 1.38 million women per year worldwide
  - Statistically 1 out of 8 women will get a mamma-CA
  - Most breast tumors develop between 60 years and 70 years of age

Background

- Adriamycin/Doxorubicin
  - Antibiotic found in Streptomyces peucetius
  - Intercaling between DNA-bases
  - Complexing with Topoisomerase II → Break of DNA strands
  - Generates radicals
  - Causes cumulative cardiomyopathy → max. life-dose
  - Causes cell-cycle arrest in the G2-phase

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Background

- Multidrug Resistance
  - Caused by enhanced antiapoptotic activity in the cancer cells or by upregulated expression of ATP-binding-cassette transporters
  - Different pathways for different therapeutics
  - Obstacle in treating malignancies by causing non-response to chemotherapeutics
  - Cell-selection analog to evolution

  → relapse with resistant tumor cells

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Background

- ABCB1/P-glycoprotein
  - First efflux pump that was identified
  - Broad spectrum of substrates such as clinical used drugs, fluorescent dyes etc.
  - Suggested to operate by an alternating access model (two different conformations)
  - Transmembrane translocation is fueled by ATP-hydrolysis
  - Still many gaps in understanding its function on the molecular level

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Background

- HSP 27
  - Detector and chaperon for misfolded proteins
  - Protector from apoptosis
  - Intracellular acute phase protein
  - Spilled by trauma or inflammatory stress
  - Possibility to differenciate healthy smokers from patients suffering from COPD
  - Higher levels in serum of NSCLC patients in comparism to healthy persons

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Materials & Methods

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Materials & Methods

- Outline
  - MCF-7 cells / MCF-7/adr cells
  - MTT-Assay
  - Preparation of whole cell lysates
  - Western Blot
  - Confocal microscopic flourescence imaging
  - Flow cytometry
  - Electrolytic mobility (super)shift assay
  - Luciferase activity assay

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Materials & Methods

- MCF-7 cells
  - Epithelial adenocarcinoma cells of a 69 years old caucasian women
  - Suitable as a transfection host
  - Drug sensitive
- MCF-7/adr cells
  - Stepwise selected from MCF-7 parental cells by using Adriamycin
  - Adriamycin resistant

Materials & Methods

- MTT-assay
  - MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is added to the cells
  - Crosses cell membrane
  - Metabolised by mitchondrial Dehydrogenases of living/proliferating cells

  → deep blue Formazan-Crystalls
  - Analysis of cell lysate with a multiwell-spectrophotometer
  - Assay to determine cytotoxicity of substances/viability of cells against certain substances

Materials & Methods

- Preparation of whole cell lysates
  - Cells are lysed in radioimmunoprecipitation assay lysis buffer
  - Centrifugation → removal of cell debris
  - Isolation of nuclear and cytosolic proteins by using NE-PER kit
  - Centrifugation → pellet of nuclear proteins
  - Centrifugation of supernatant → cytosolic fraction

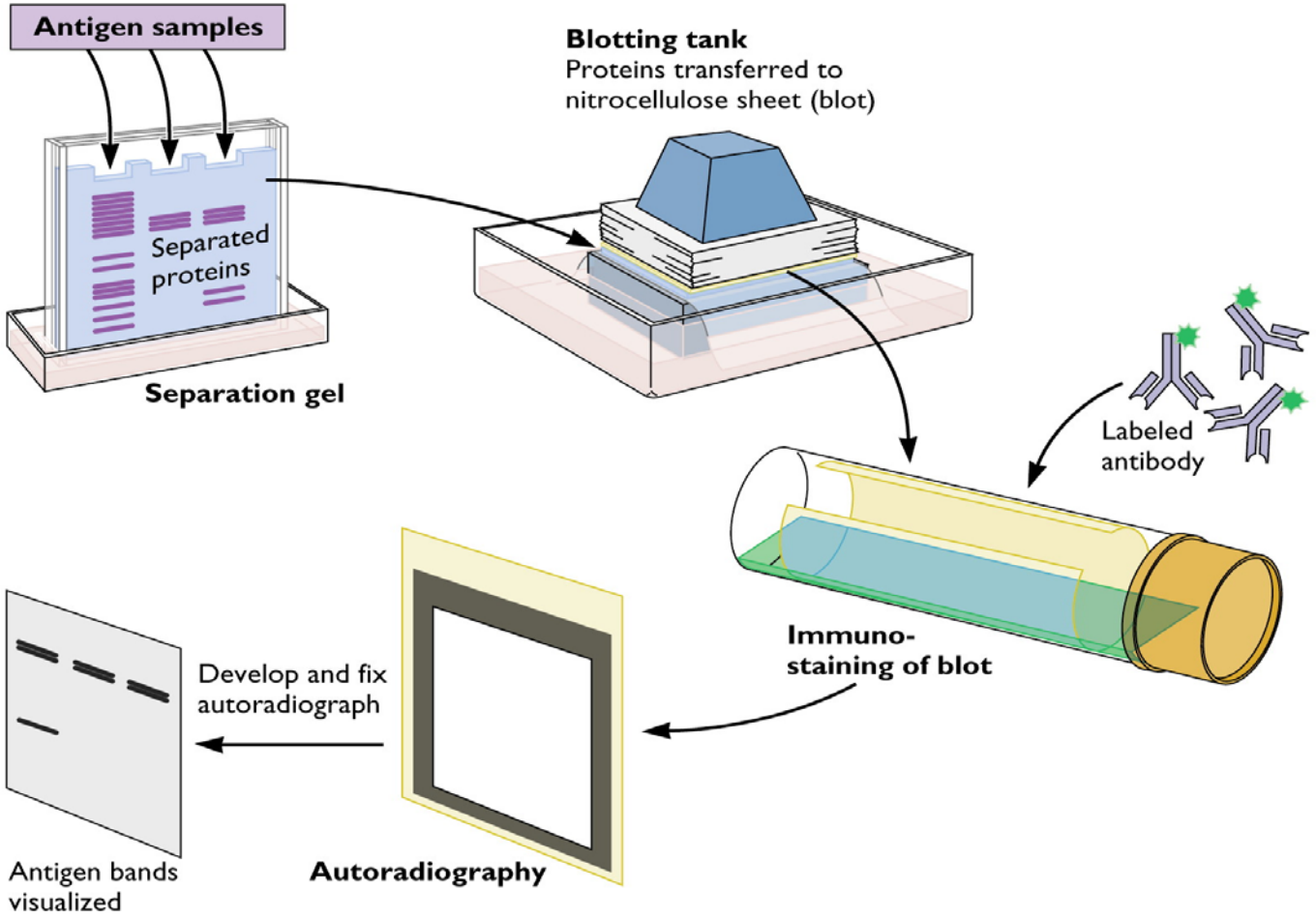
Materials & Methods

- Western Blot
  - Previously the Proteins are separated from each other by gel-electrophoresis, usually by SDS-Page
  - The Proteins are blotted to a nitrocellulose sheet

  → Copy of the gel on the sheet
  - Incubation of the sheet with generic proteins
  - Specific antibody is added
  - Making the antibody visible

Materials & Methods

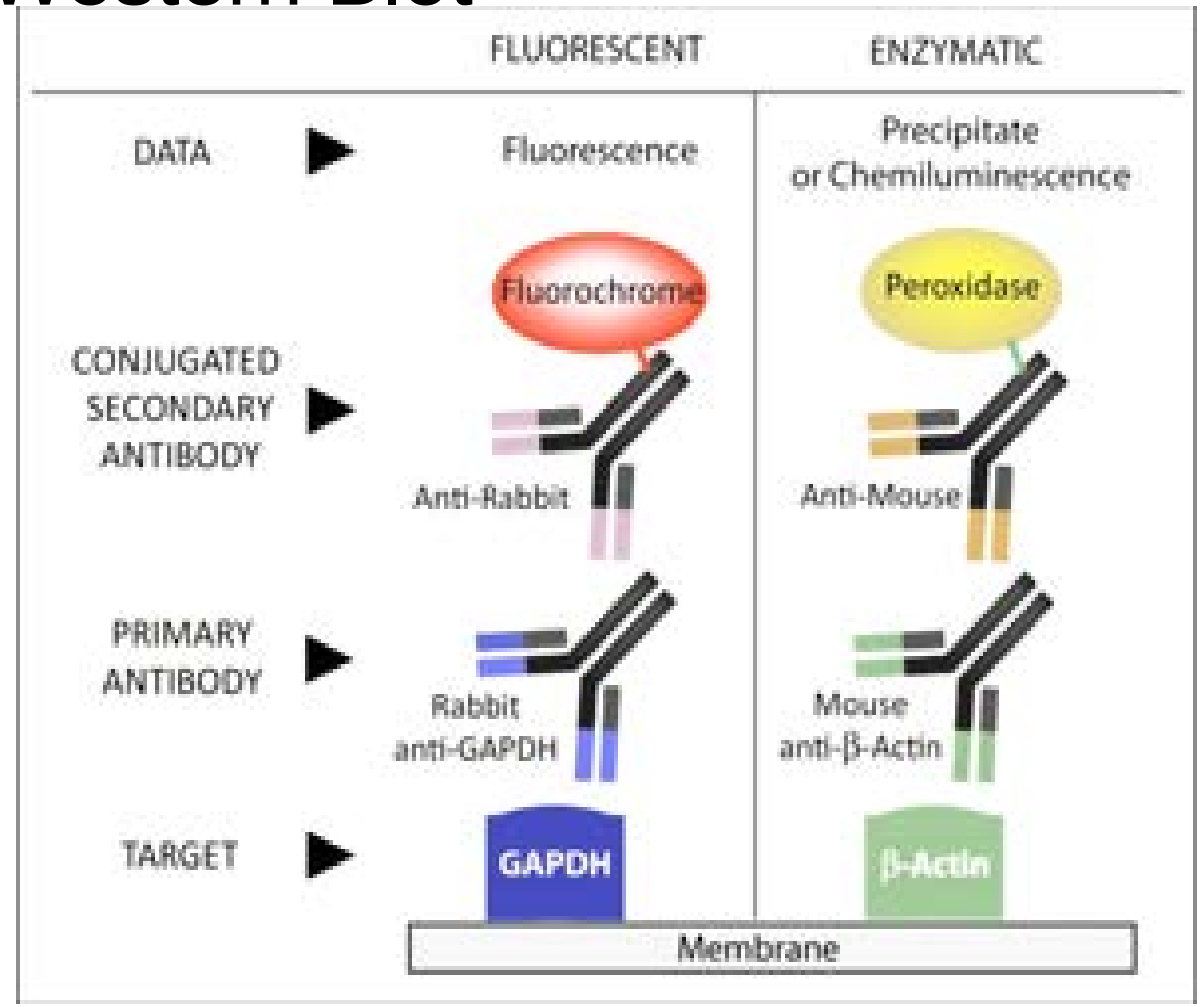
- Western Blot



http://www.virology.ws/2010/07/07/virology-toolbox-the-western-blot/

Materials & Methods

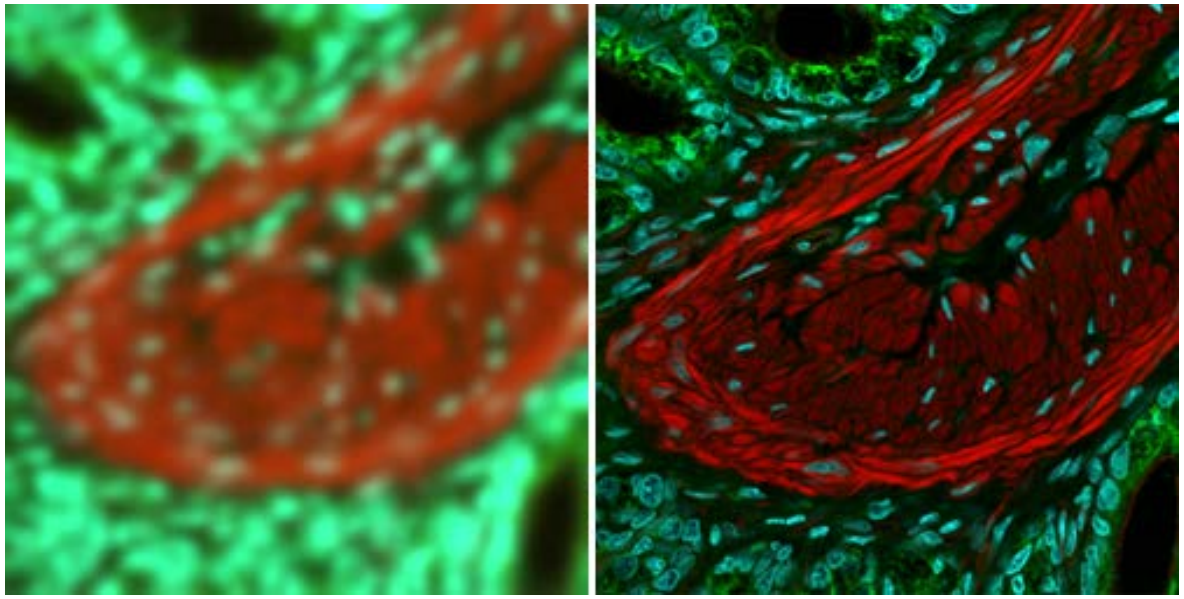
- Western Blot



http://www.rockland-inc.com/western-blot.aspx

Materials & Methods

- Confocal microscopic fluorescence imaging
  - Uses spatial filtering → elimination of out-of-focus light
  - Is able to take high-quality pictures of specimens prepared for conventional optic microscopy



http://azcc.arizona.edu/research/shared-services/ciss/confocal

Materials & Methods

- Flow cytometry
  - Method for determination of different characteristics of cells
  - Tool for separation of cells with different characteristics
  - Cells are put via fluid stream through a laser

  →different emitted wavenlengths depending on characteristics of the cell
  - Cell sorting by detecting the wavelengths

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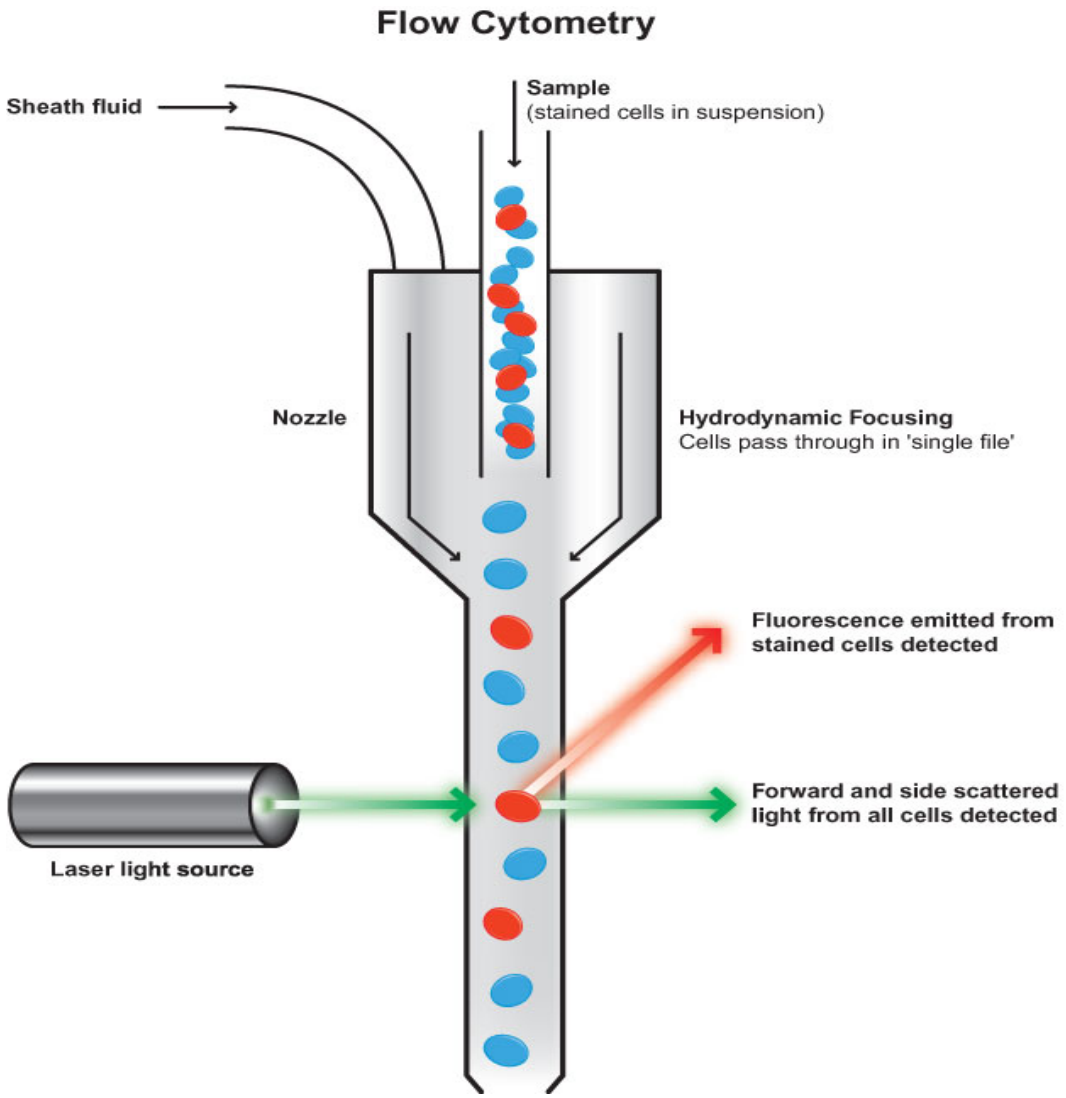
Materials & Methods

- Flow cytometry



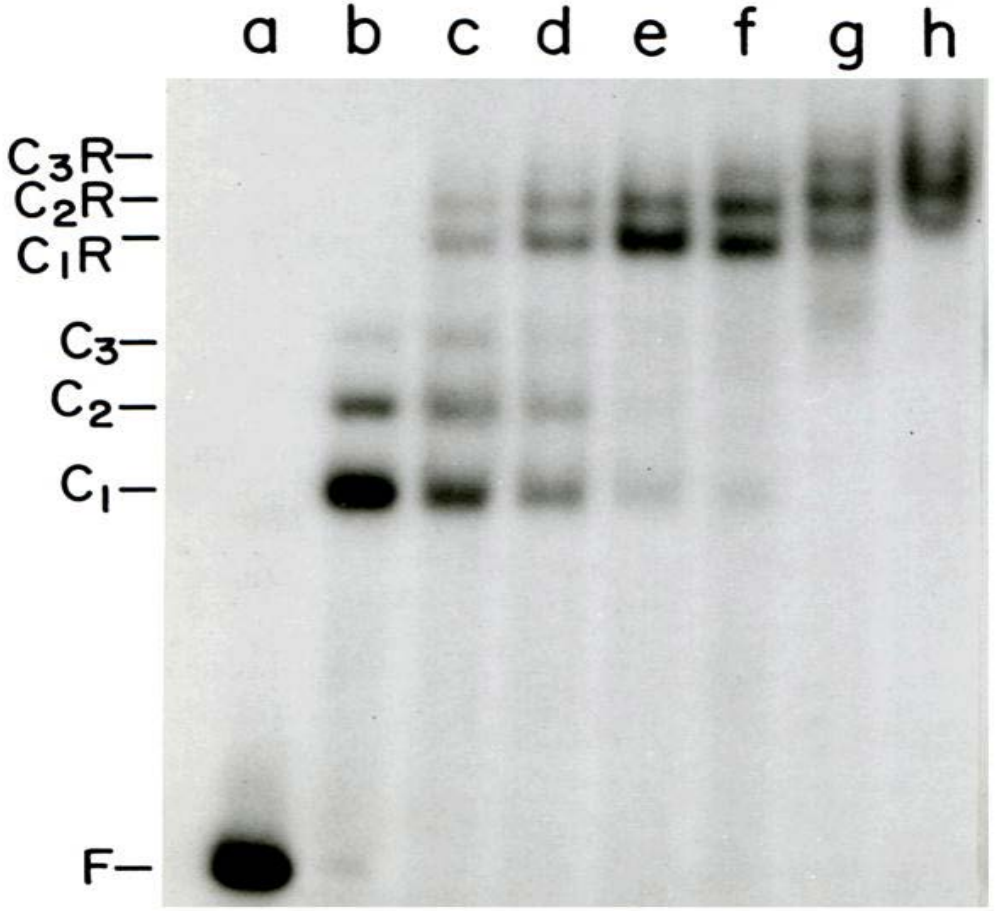
http://docs.abcam.com/pdf/protocols/Introduction_to_flow_cytometry_May_10.pdf

Materials & Methods

- Electrolytic mobility (super)shift assay
  - Used to detect protein complexes with nucleic acids
  - Protein solution and solute marked nucleic acids are combined
  - Mixture undergoes electrophoresis
  - Complexes of proteins and nucleotids are migrating much slowlier than nucleic acids alone

Materials & Methods

- Electrolytic mobility (super)shift assay



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2757439/figure/F4/

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Materials & Methods

- Luciferase activity assay
  - Luciferase is an enzyme that triggers bioluminescence
  - Method for measurement of the binding activity of transcription factors
  - Binding of transcription factors leads to expression of firefly luciferase → bioluminescent reaction
  - Measurement of the emitted light with a luminometer

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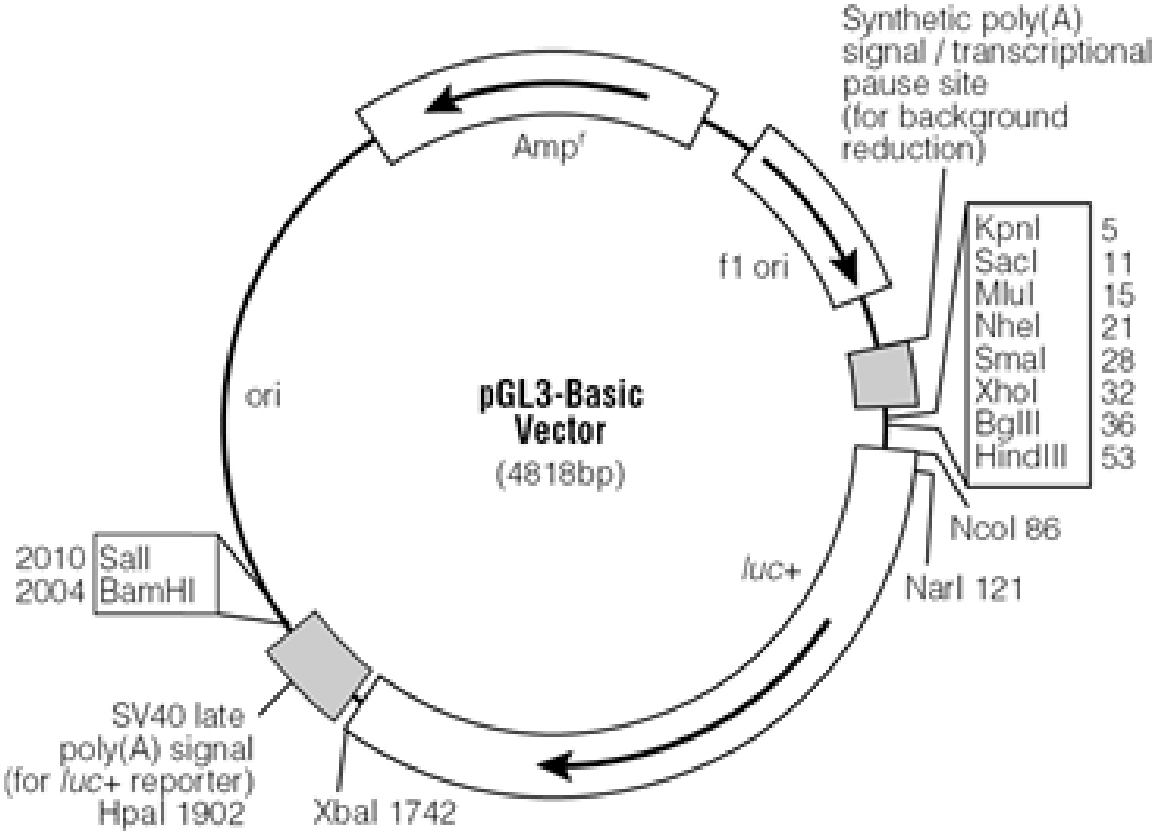
Materials & Methods

- Luciferase activity assay
  - Used Luciferase vector



https://www.promega.de/products/reporter-assays-and-transfection/reporter-vectors-and-cell-lines/pgl3-luciferase-reporter-vectors/?origUrl=http%3a%2f%2fwww.promega.com%3a81%2fproducts%2freporter-assays-and-transfection%2freporter-vectors-and-cell-lines%2fpgl3-luciferase-reporter-vectors%2f

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Results

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Results

Inhibition of HSF-1, Depletion of HSP 27 and accumulation of mutant p53 and NF-κB in MCF-7/adr cells

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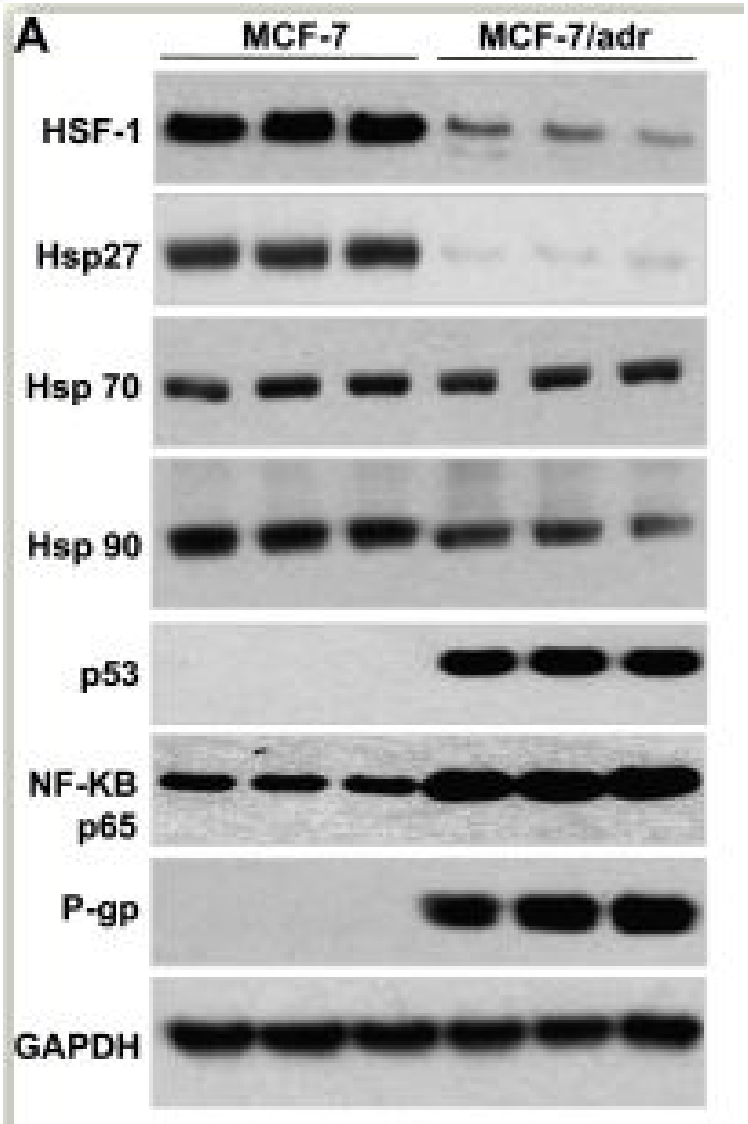
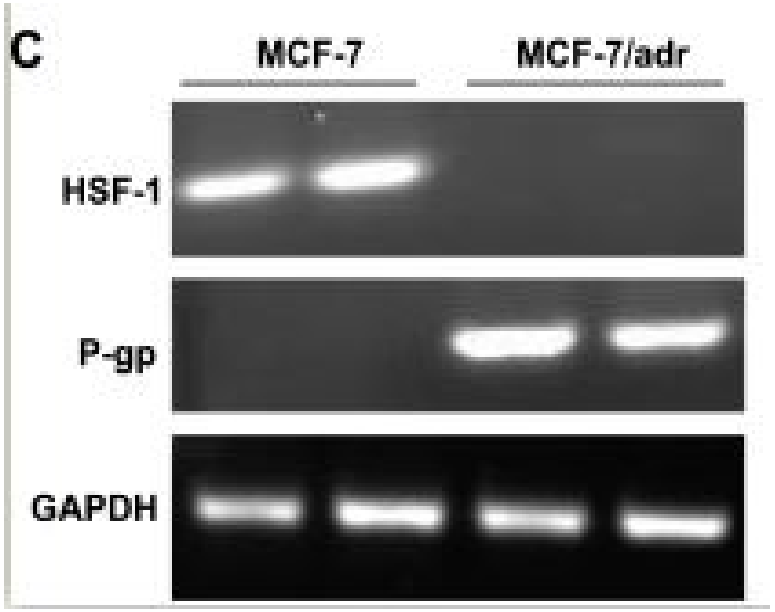
Results



← Westernblot results



↑ Results of RT-PCR concerning mRNA of HSF-1 and P-gp

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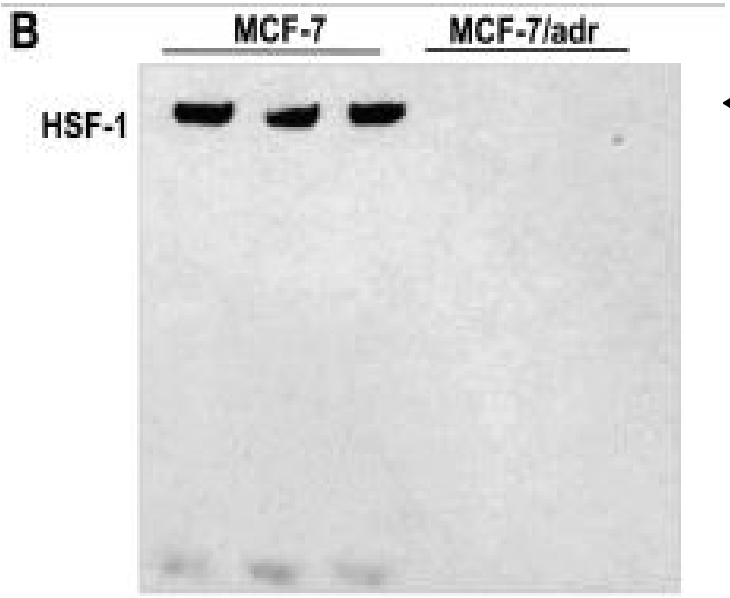
Results



← Electrophoretic mobility shift assay

Results

- **Conclusions**:
  - Inhibition of HSF-1 transcriptional activity is responsible of the abrogation of HSP 27 expression in MCF-7/adr cells
  - Accumulation of mutant p53 supresses constitutive expression of HSF-1 and HSP 27, which leads to higher expression of NF-κB and P-glycoprotein

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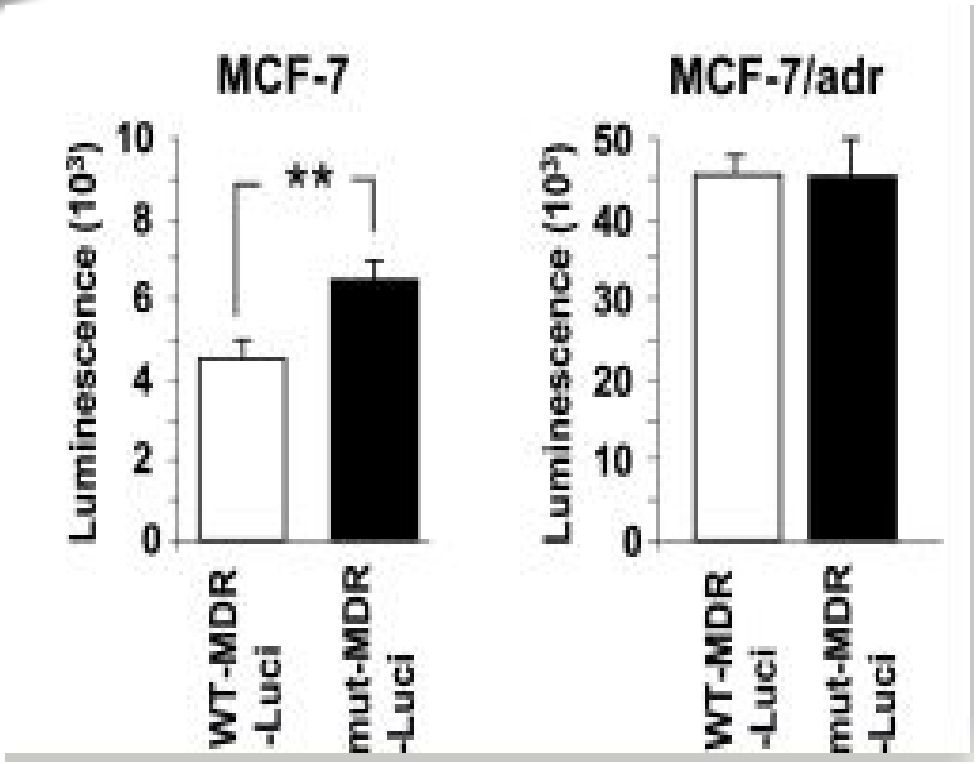
Results



reporter gene assay

- Indication for HSF-1 binding is inhibiting the MDR1 gene expression in MCF-7 cells

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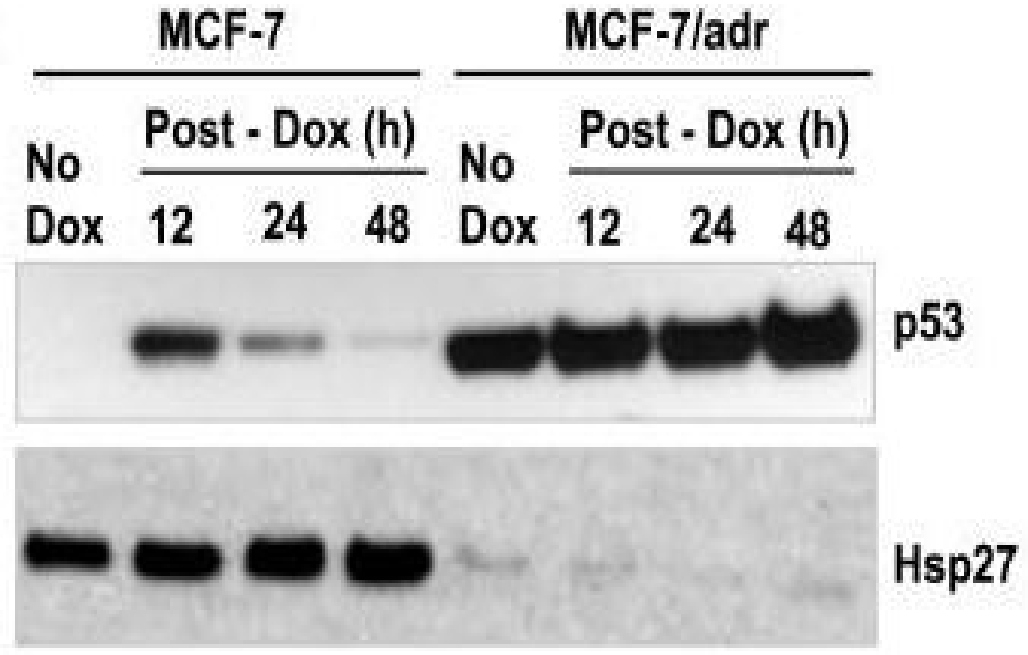
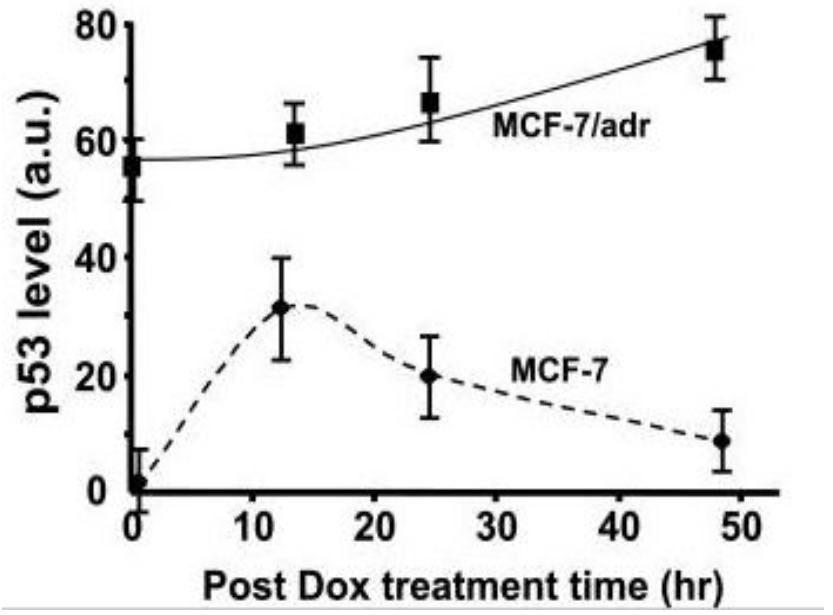
Results



Western blot



p53 trends

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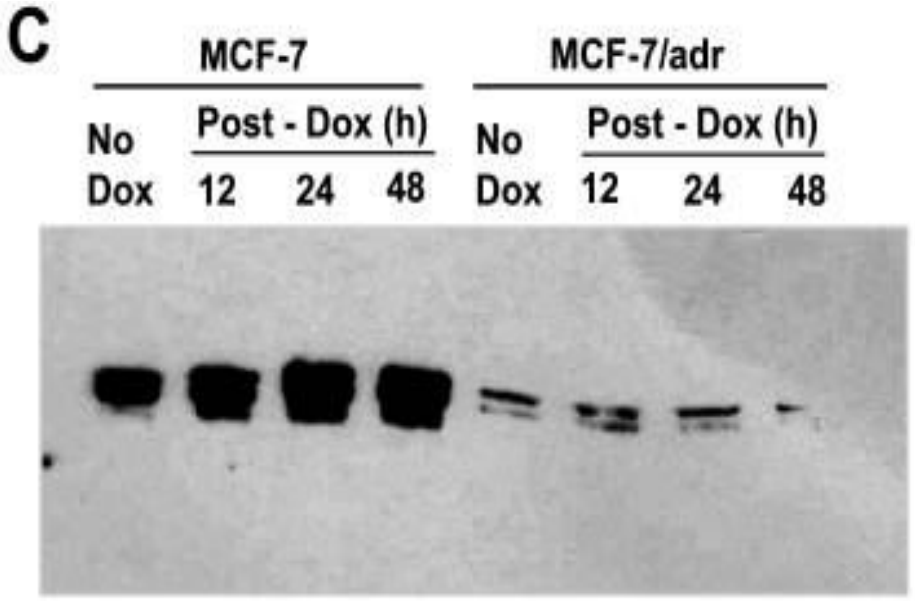
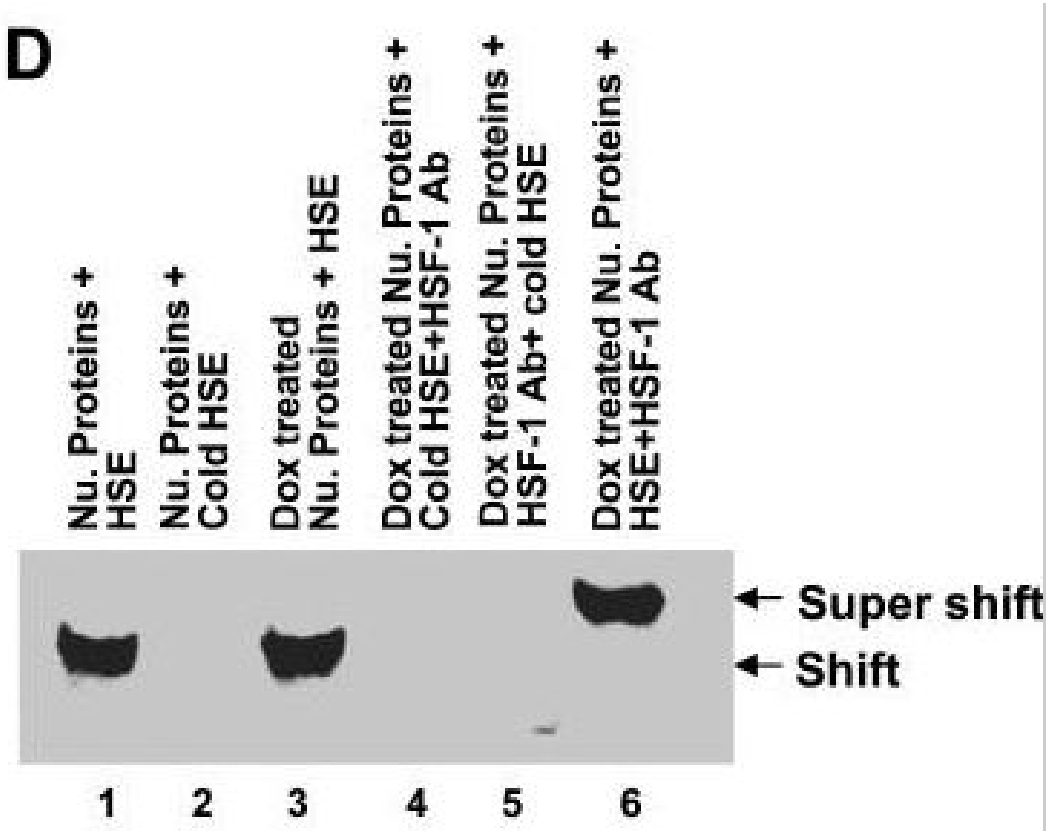
Results



Electrophoretic mobility shift assay



Electrophoretic mobility shift and super shift assay

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Results

- **Confirmations**:
  - HSF-1 is not activated in MCF-7/adr cells during Adriamycin treatment
  - Suppression of HSF-1 is correlative to the mutant p53 accumulation and leads to P-glycoprotein establishment in MCF-7/adr cells

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Results

HSP 27 overexpression depletes mutant p53, sensitizes MCF-7 cells to Adriamycin, and enhances cell death

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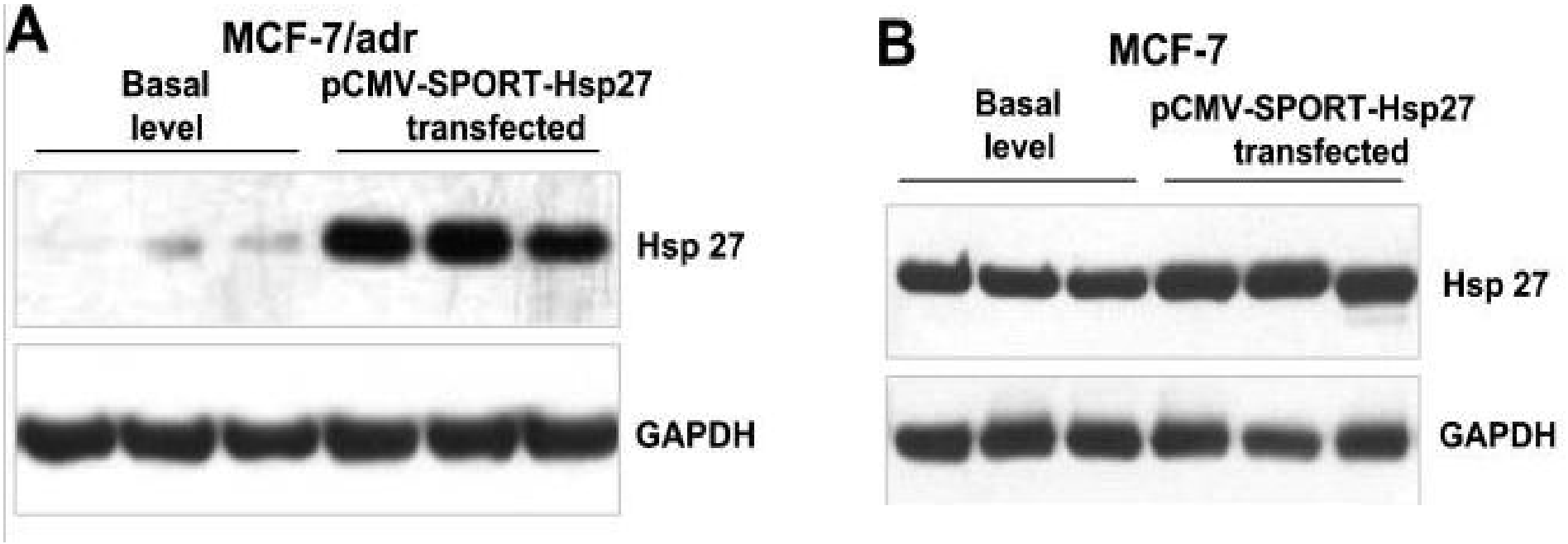
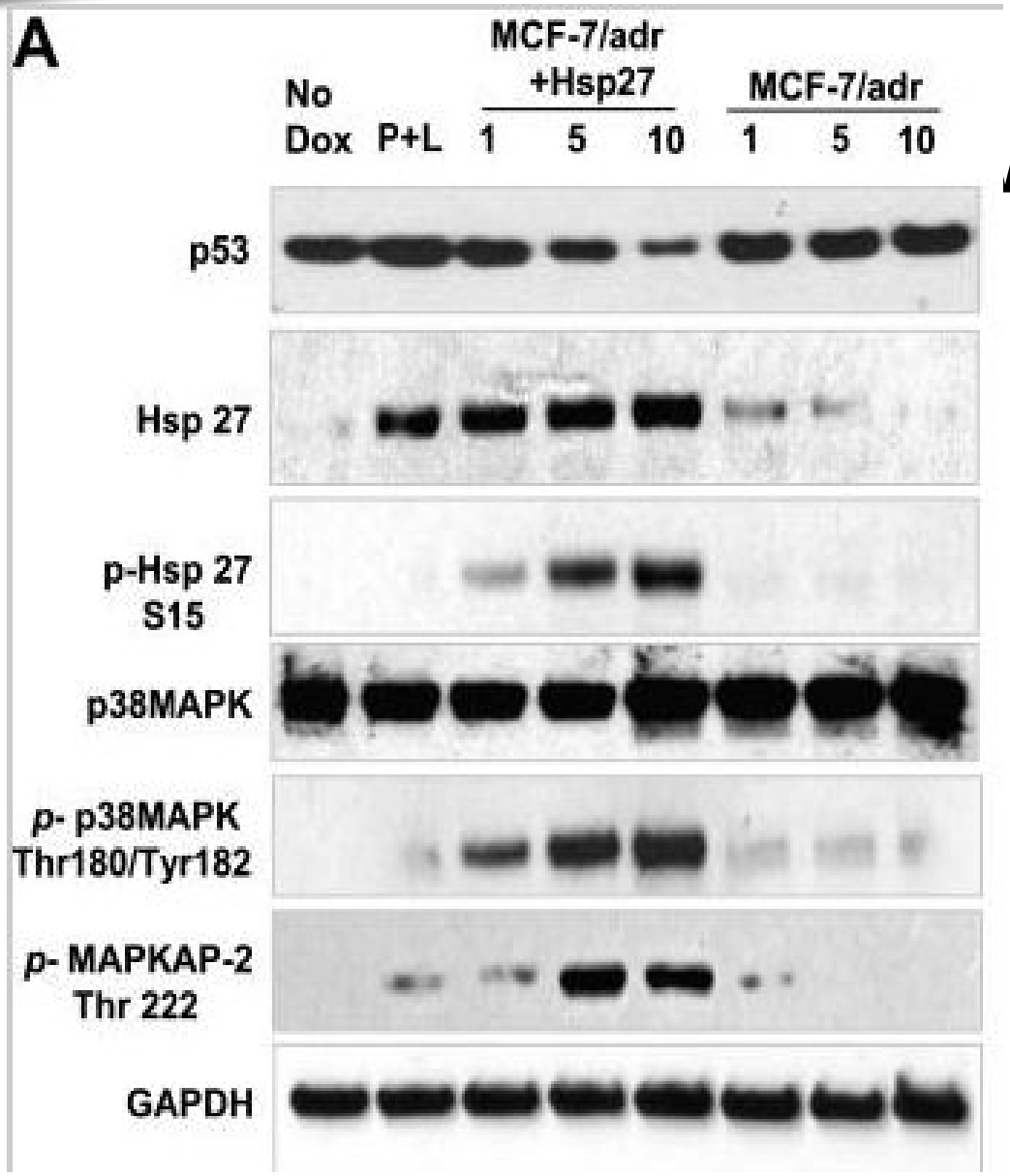
Results

Westernblots



Results



Westernblot

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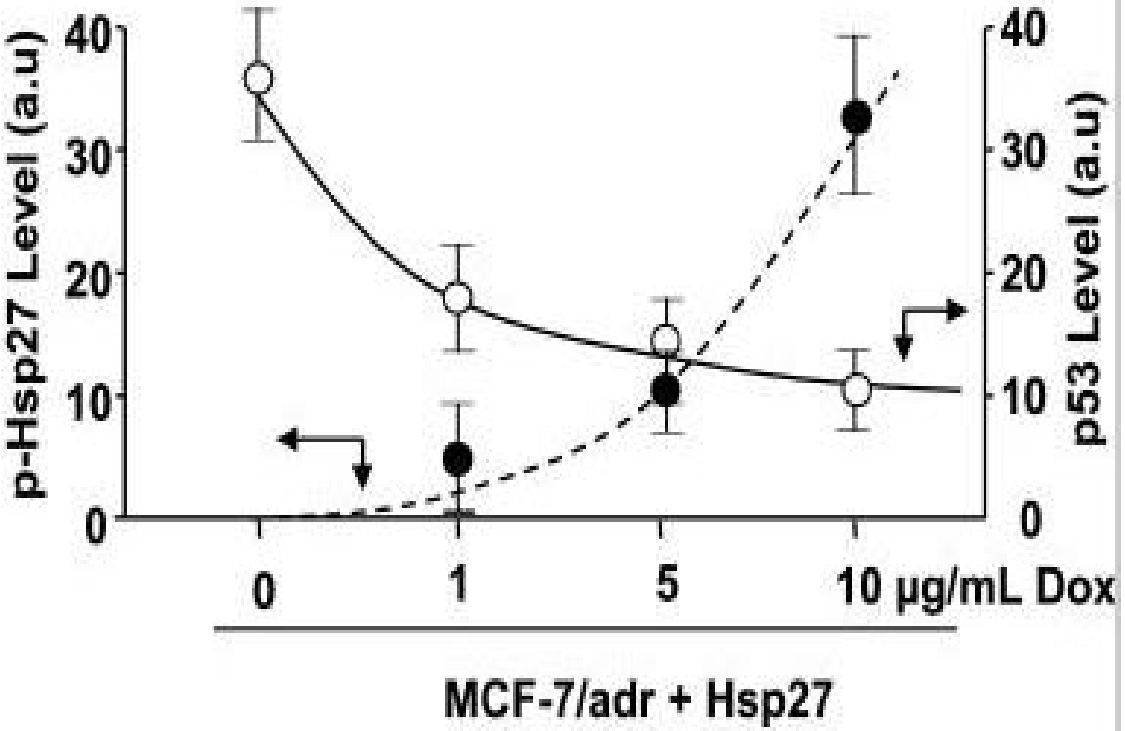
Results



Trends of phosphorylated HSP 27 and p53 in MCF-7/adr cells with HSP 27 overexpression

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Results

- **Confirmations:**
  - Forced HSP 27 overexpression enhances degradation of p53 in MCF-7/adr cells upon Adriamycin treatment
  - Forced HSP 27 overexpression enhances p38 MAPK activity in MCF-7/adr cells upon Adriamycin treatment

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Results

HSP 27 overexpression Represses the NF-κB activity in MCF-7/adr cells

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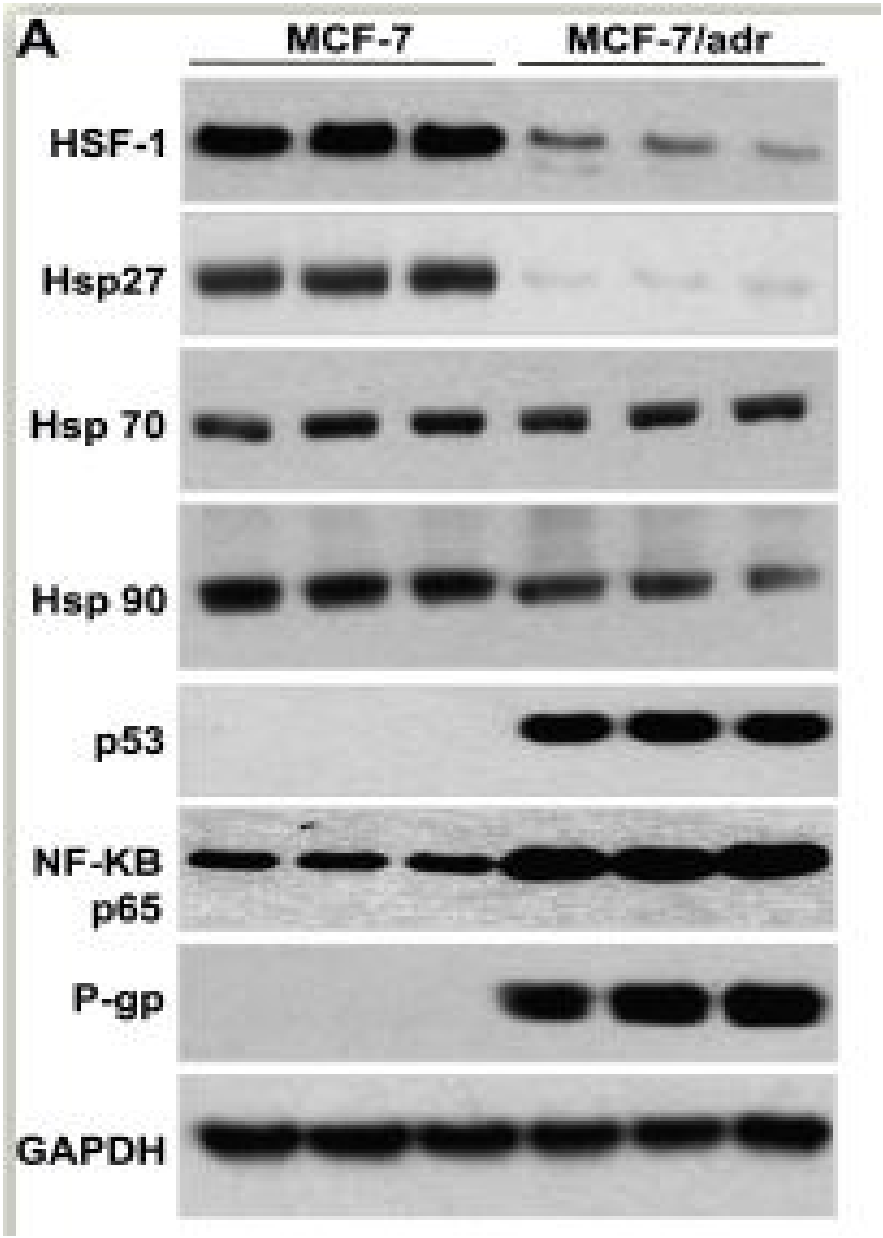
Results



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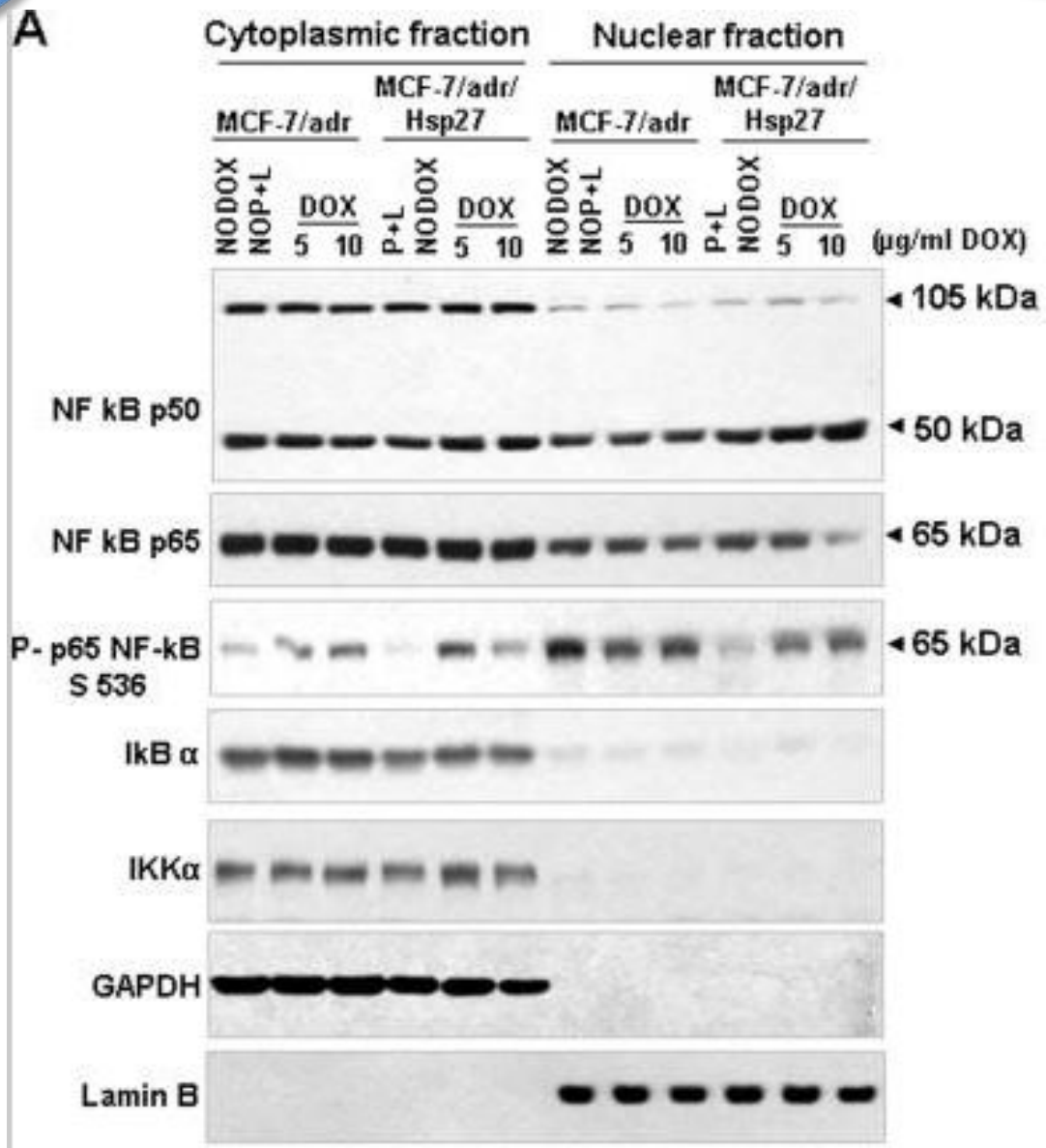
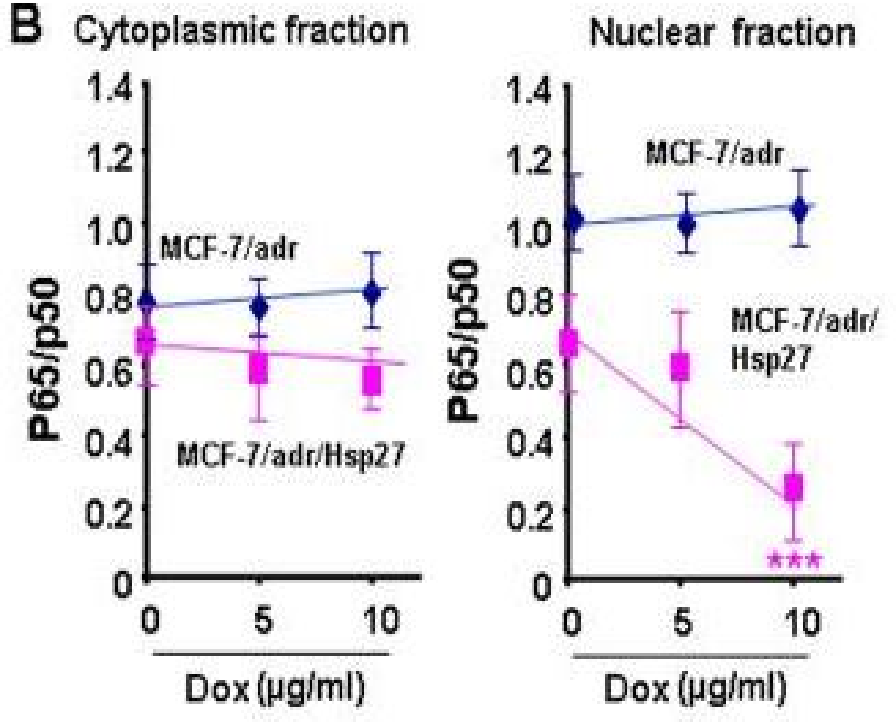
Results



Results



Trends of the p65/p50 ratio in different Adriamycin concentrations

Suggestion that transcriptional activation by canonical activation of NF-κB is increasingly inhibited in MCF-7/adr/HSP27 cells with increasing concentrations of Adriamycin

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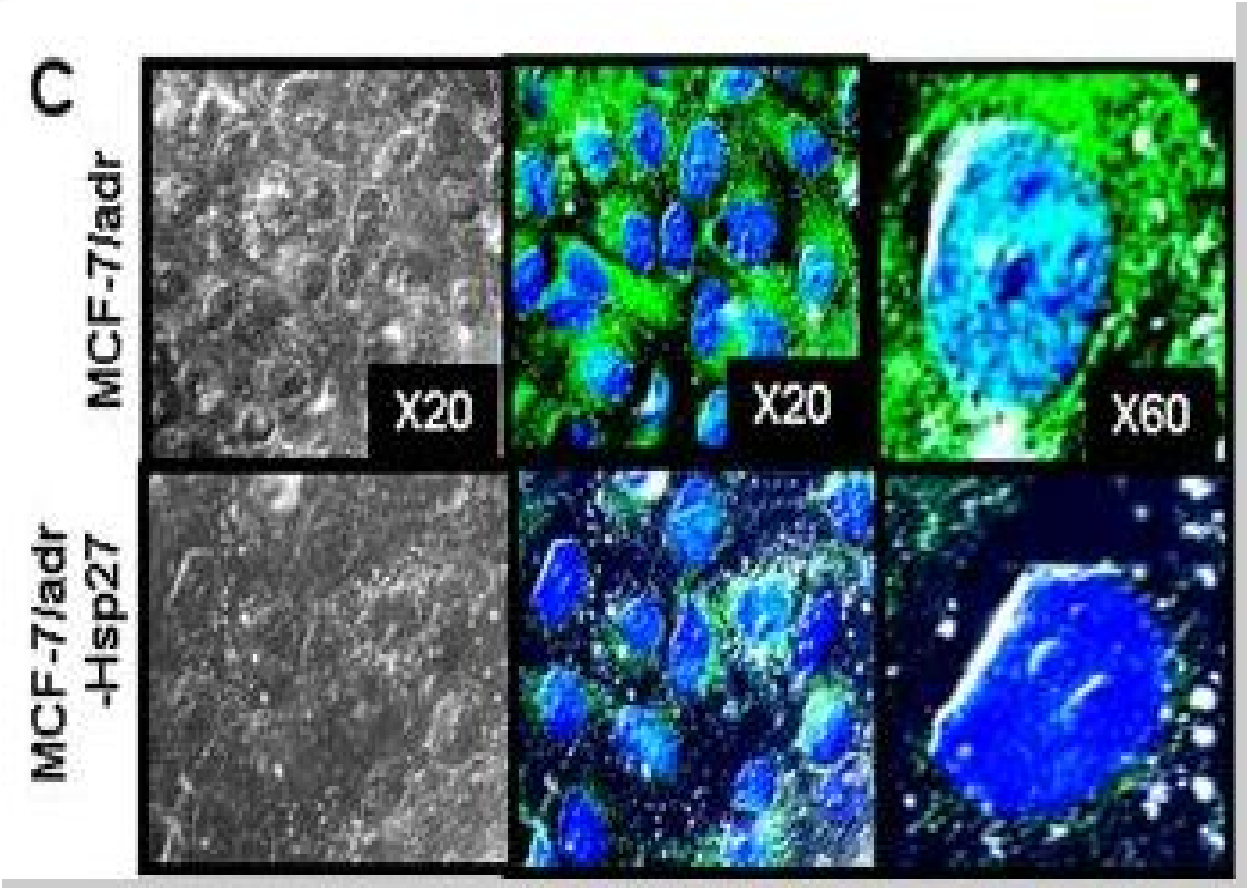
Results



- Confocal microscopy
  - p65 = green
  - Nucleus = blue

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Results

HSP 27 overexpression attenuates P-gp expression and retains higher intracellular Adriamycin concentrations in Mcf-7/adr cells

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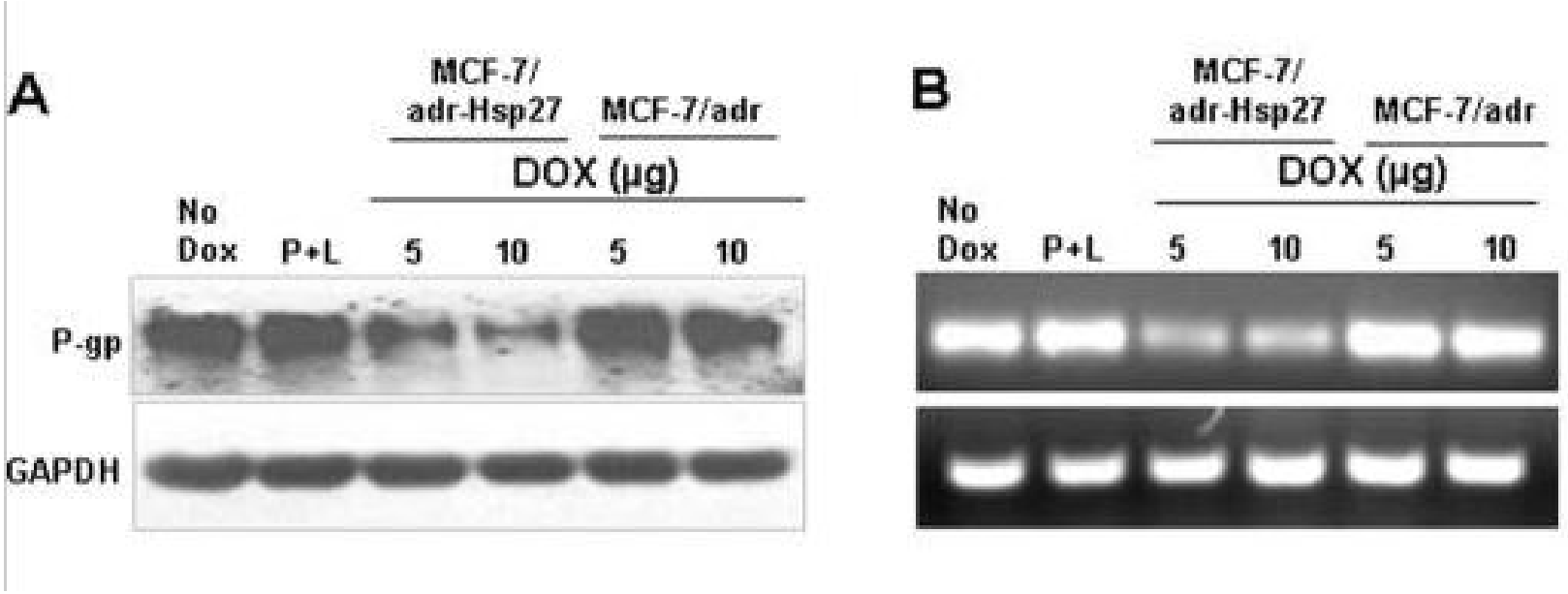
Results



Westernblot

RT-PCR

Suggestion: MDR1/P-glycoprotein expression is susceptible to downregulation by HSP 27 overexpression + Adriamycin treatment

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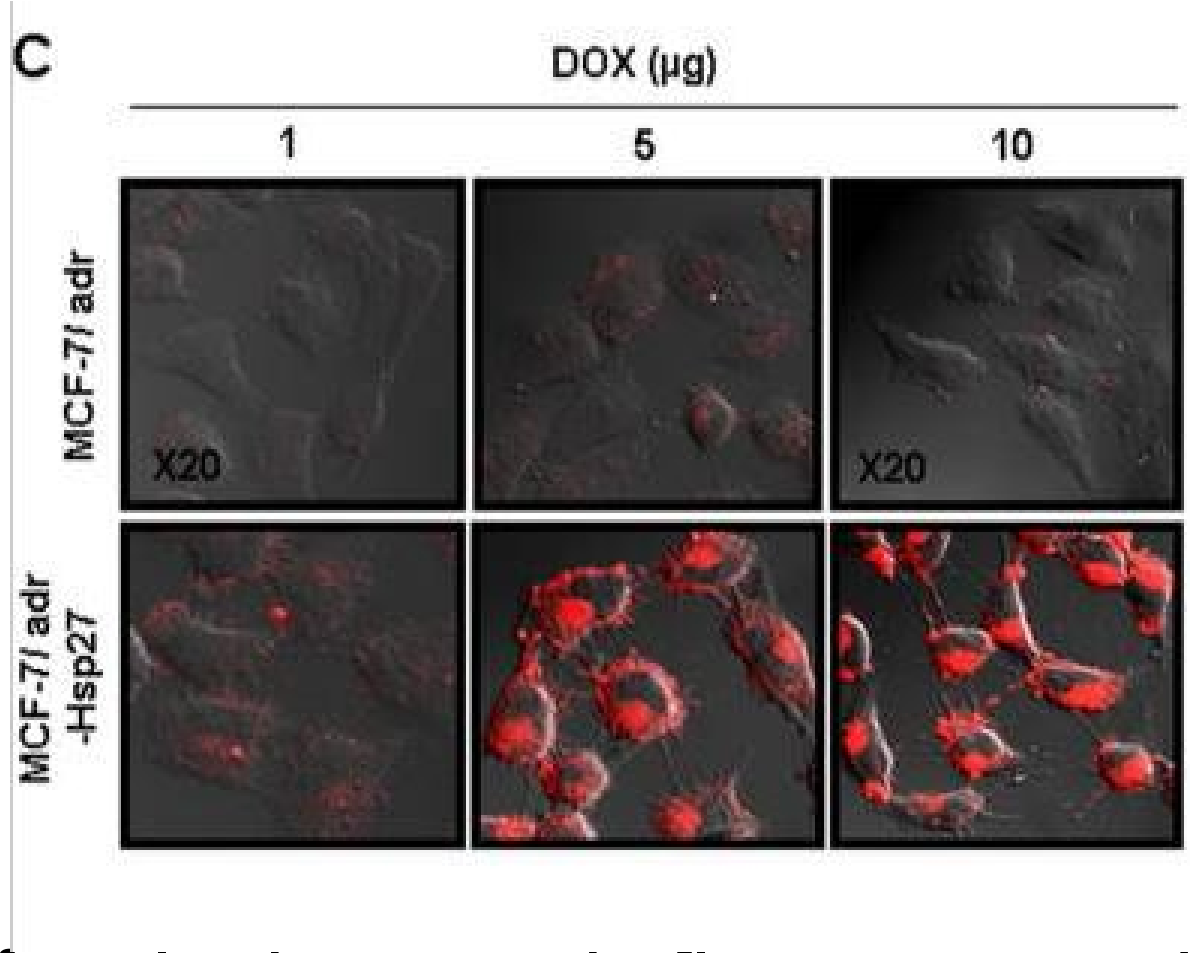
Results



Confocal microscopic fluorescence image of autofluorescence of Adriamycin

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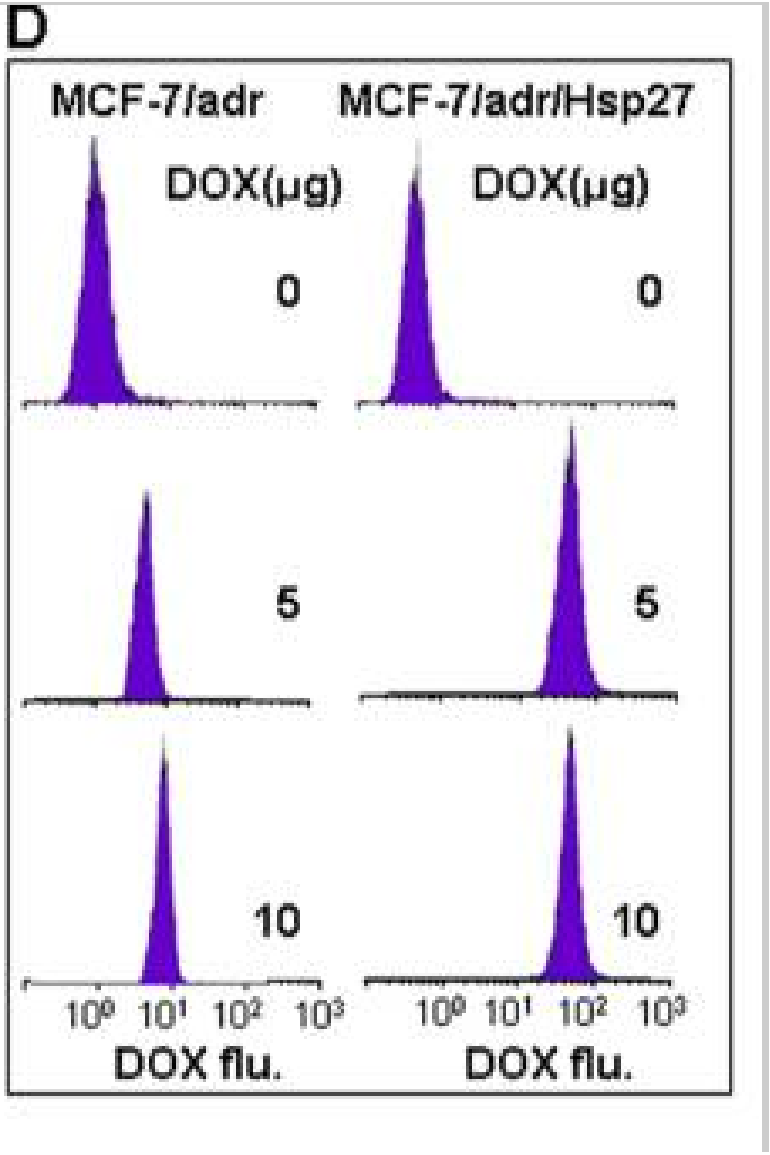
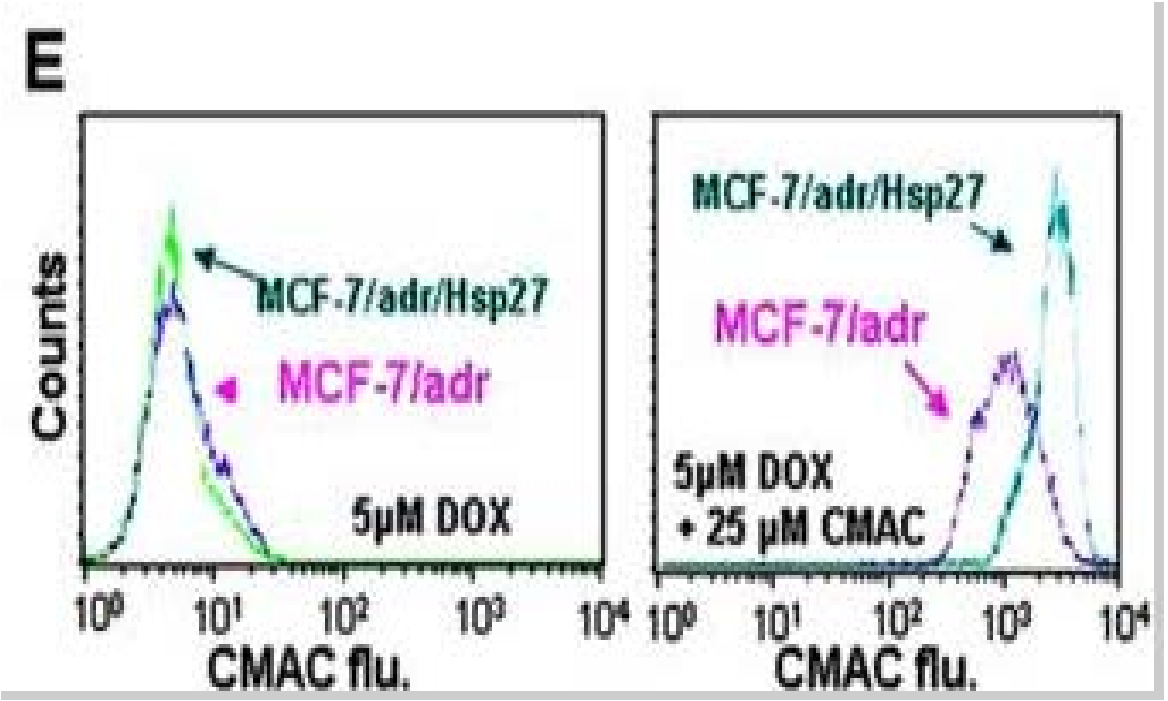
Results



- Flow cytometry of cells by using autofluorescence of Adriamycin
- Higher Adriamycin concentrations in MCF-7/adr/HSP27 cells in comparism to MCF-7/adr cells

Results



- Flow cytometry of MCF-7/adr and MCF-7/adr/HSP27 cells with Adriamycin and Adriamycin + CMAC
- Confirmation that P-glycoprotein activity is reduced

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Results

HSP 27 overexpression reestablishes Adriamycin-induced cytotoxicity and apoptotic pathway in MCF-7/adr cells

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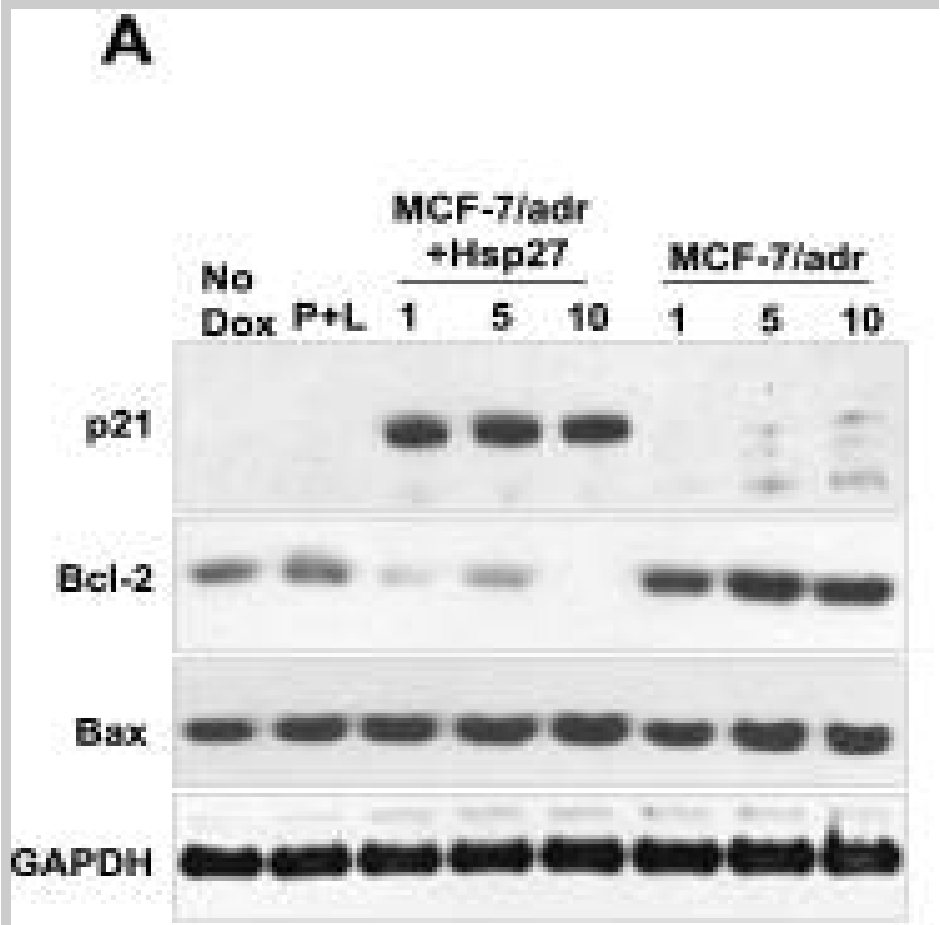
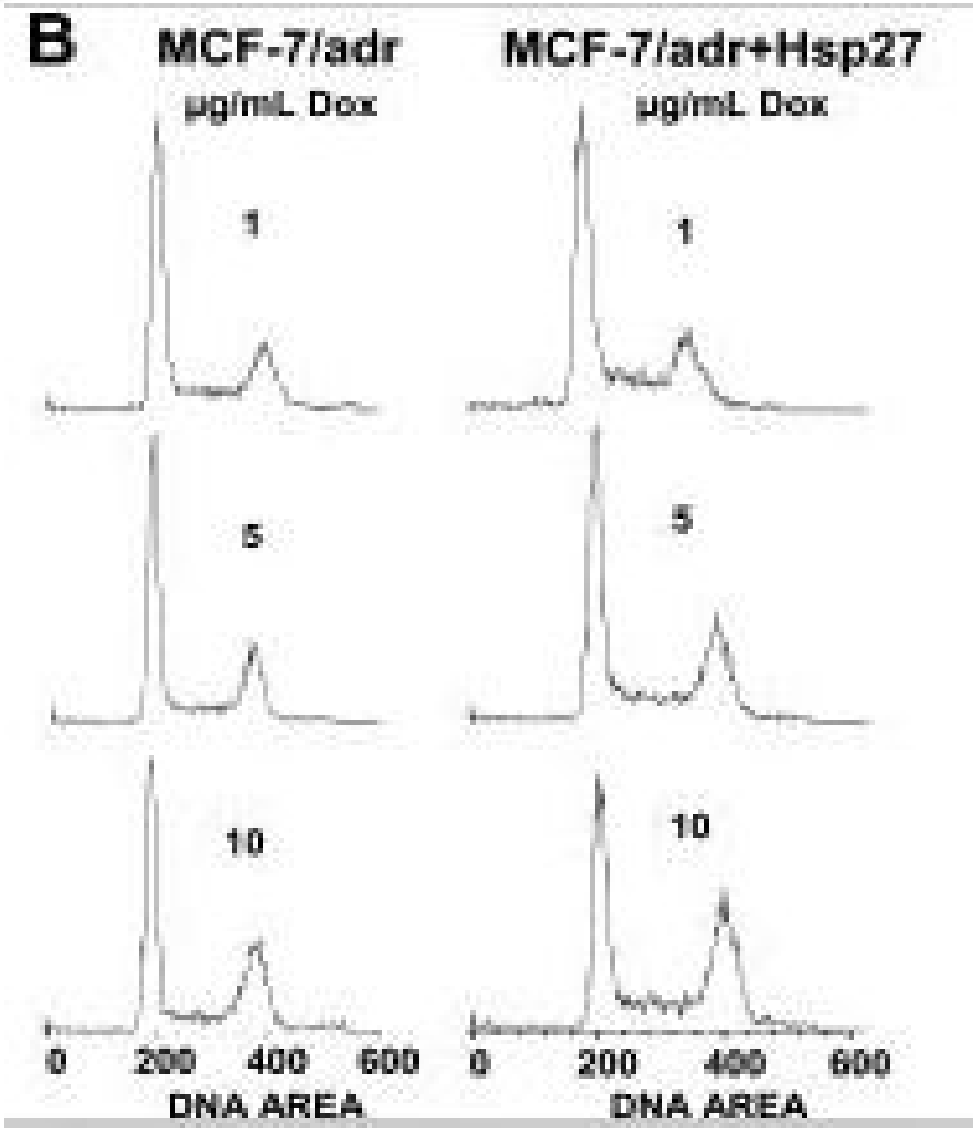
Results



Western Blot

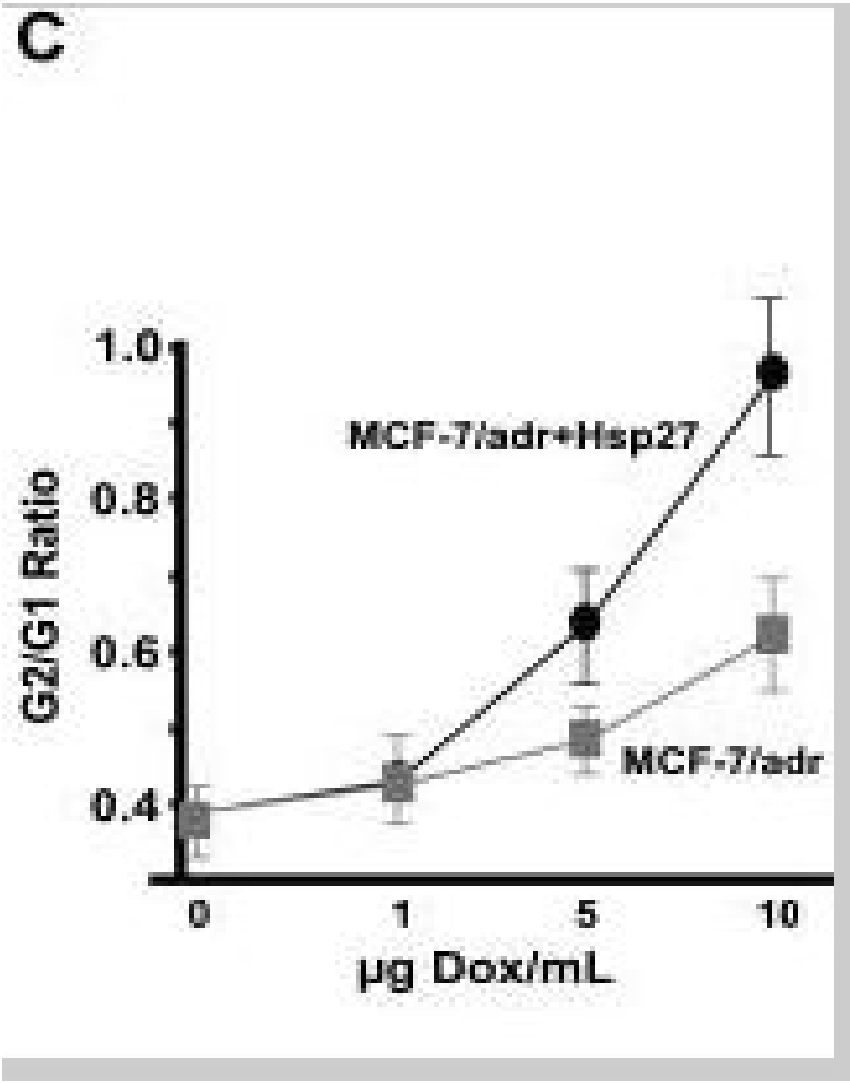
- HSP 27 overexpression leads to:
  - Increase of p21 → Cdc2 kinase inhibition ↑
  - Decrease of Bcl-2 protein
  - Bax stays stable →Bax/BCl-2 ratio↑ =apoptotic ratio

Results



- Flow cytometry analysis of cell cycle arrest
  - First peak = G1
  - Second peak = G2
  - MCF-7/adr cells show no significant change of distribution of the cells with increasing Adriamycin concentrations
  - MCF-7/adr/HSP27 cells show increase of G2-arrests with increasing Adriamycin concentrations

Results



←Quantitative blots of G2/G1 ratio

Conclusion: MCF-7/adr/HSP27 cells rather exhibit an apoptotic pathway than DNA repair and survival

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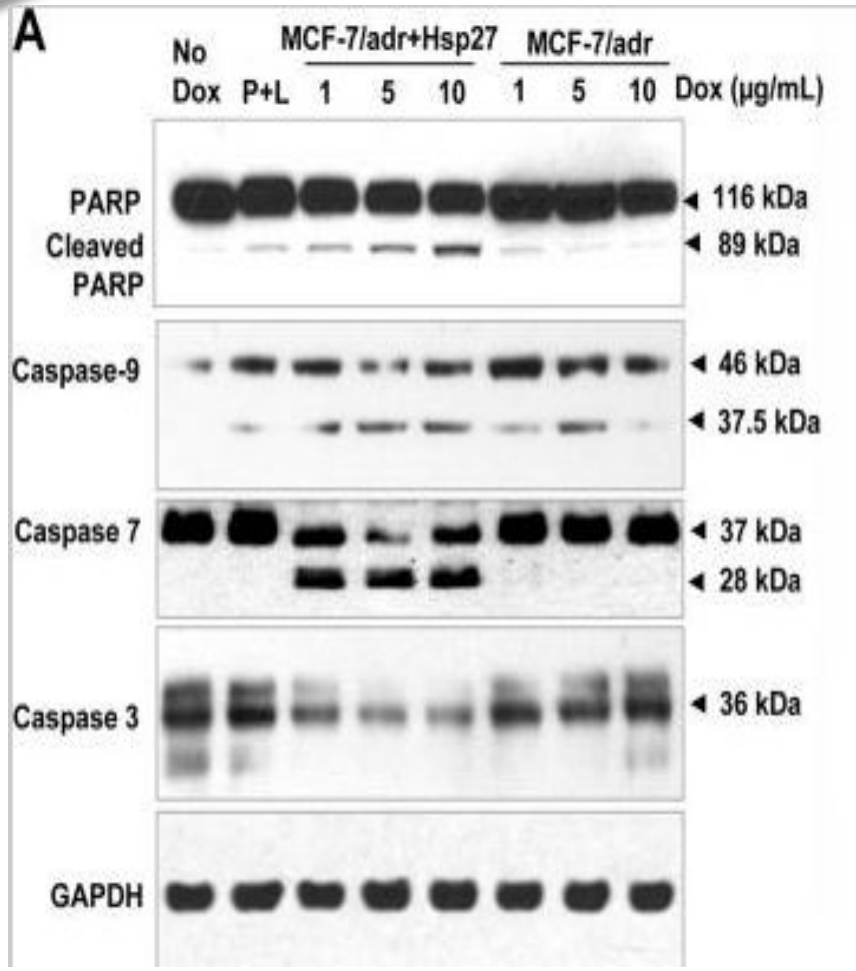

- Western Blot
  - Cleavage of PARP-1 in MCF-7/adr/HSP27 cells
  - Cleavag of Caspase 7 in MCF-7/adr/HSP27 cells
  - Indication that PARP-1 cleavage depending apoptosis in MCF-7/adr/HSP27 cells is mediated by the capase9/7 dependent pathway

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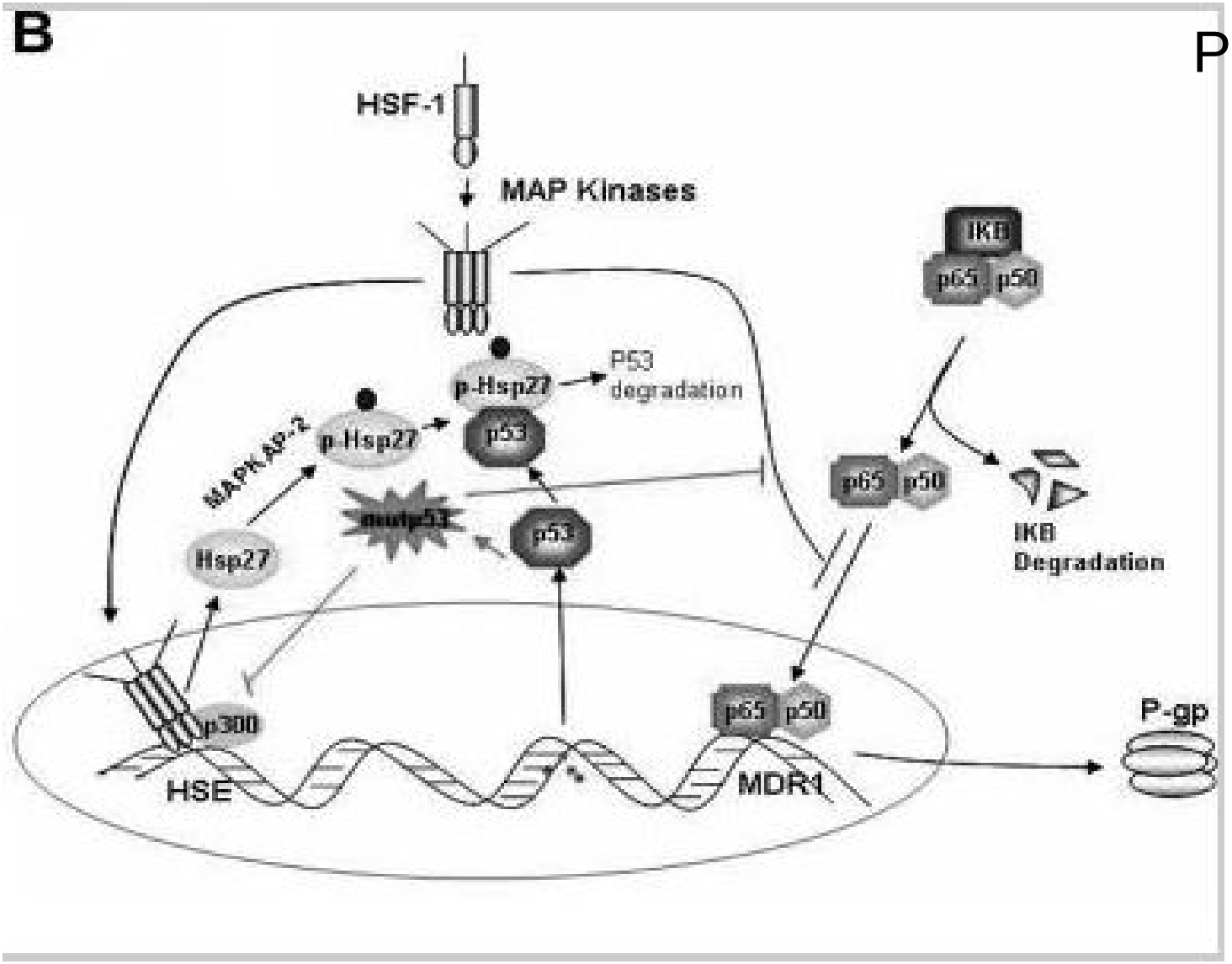
Results

Proposed mechanism



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Discussion

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Discussion

- First study reporting endogenous silencing of HSF-1 by mutant p53 and inhibition of HSP 27 protein expression plays a key role in MDR-1 expression and in establishing P-glycoprotein
- Contrary to general consensus that HSP 27 overexpression is responsible of the induction of drug resistance in many cancer cells

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Discussion

- Apparently inhibition of transcriptional activation of HSF-1 and HSP 27 is responsible at least in part for expression of MDR1/P-glycoprotein in MCF-7/adr cells
- Apparently loss of HSP 27 and mutation of p53 work synergistically toward the adaption of these cells to Adriamycin

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Discussion

- The study suggests that HSP 27 overexpression dictates cell fate decisions not only at the level of cell cycle arrest but also at the level of transcriptional activities of various other factors
- Modulators of HSF-1 and HSP 27 could be used to circumvent multidrug resistance in cancer cells

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References

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