

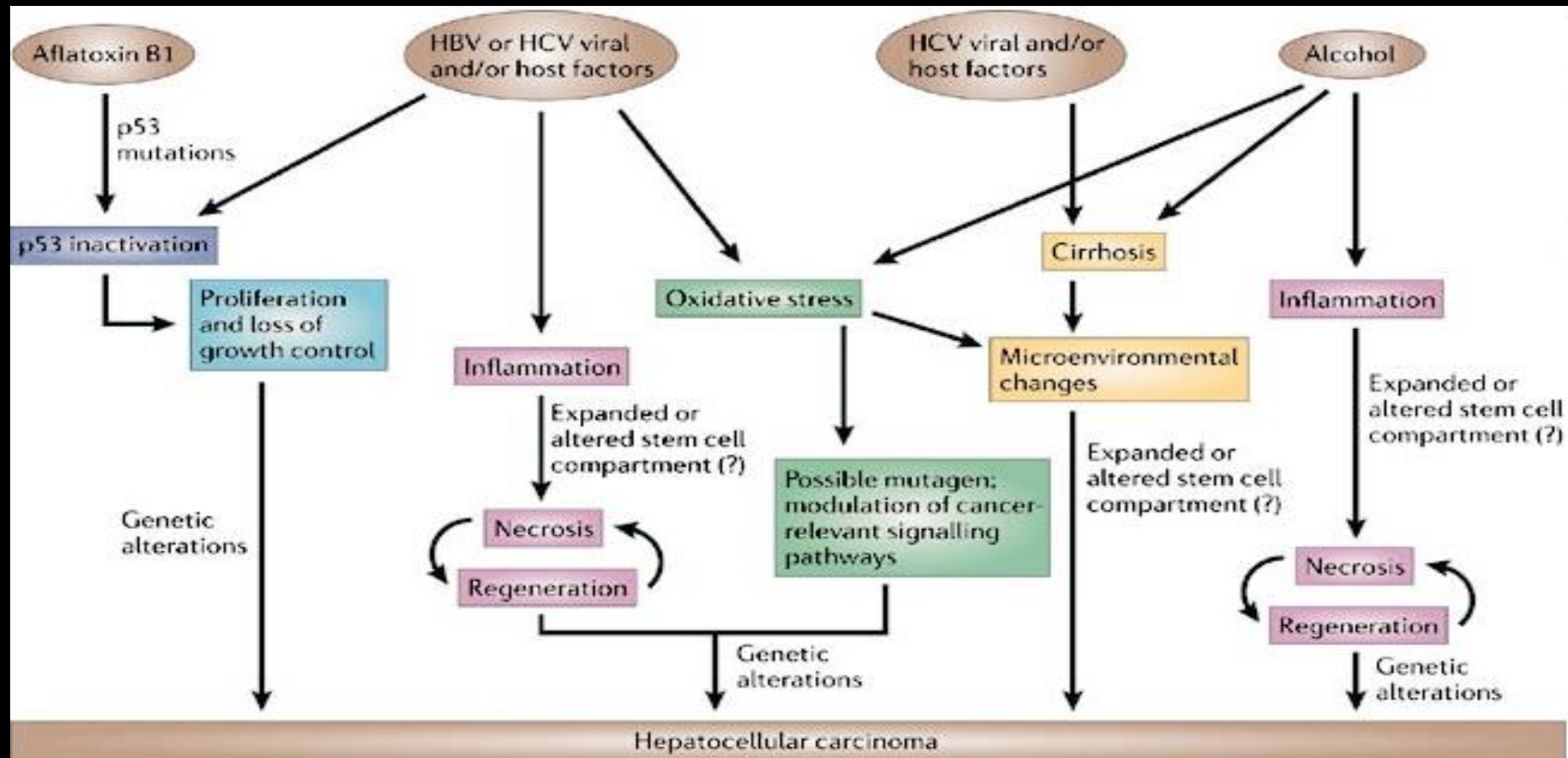
“Transcriptomics: A tool for finding efficient biomarkers for early HBV associated Liver Cancer (HCC)”



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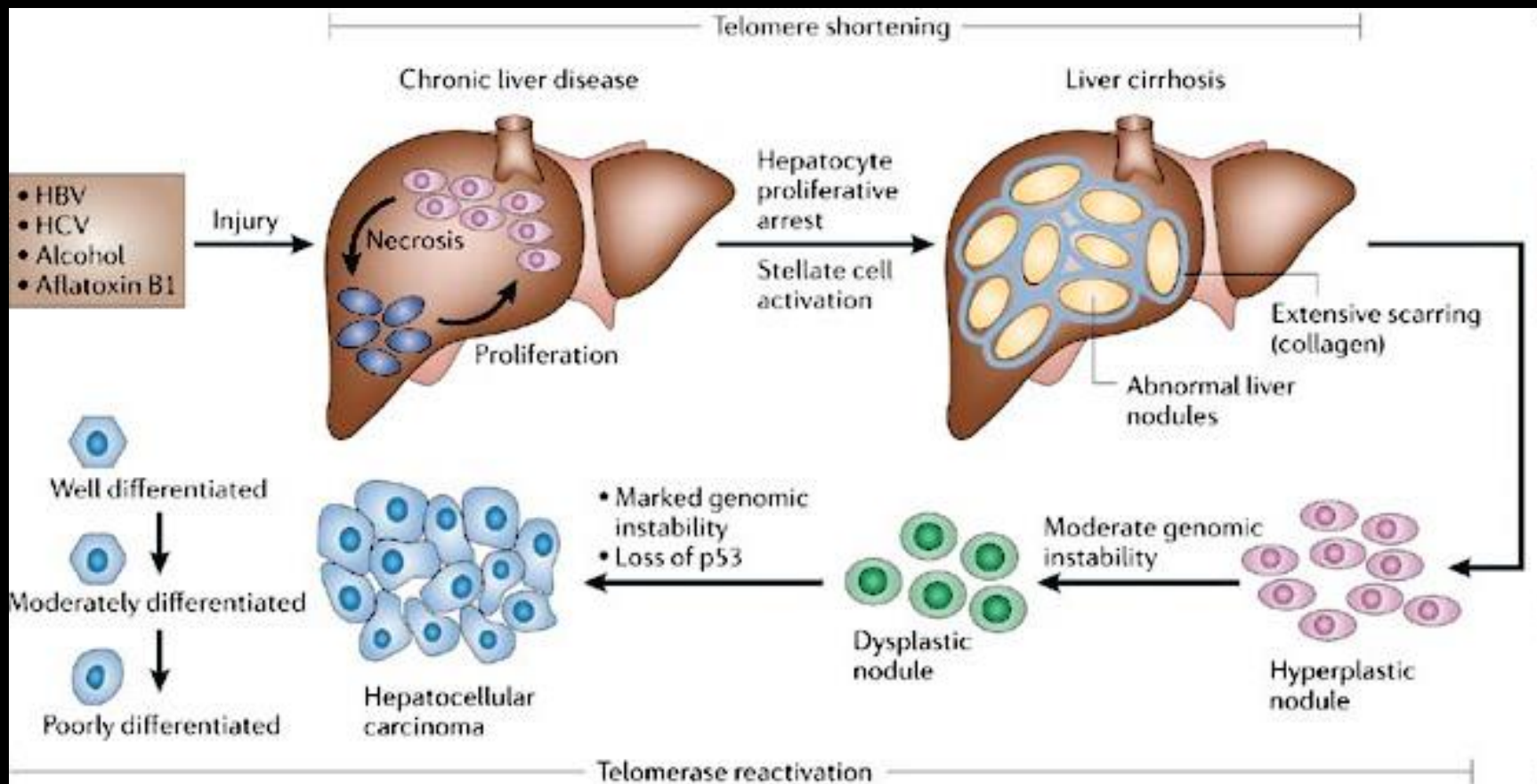
Introduction

- **Primary liver cancer - HCC is the most common type, accounting for 70%-85% of cases**
- **Peak in East-Asia, Mediteranean region and sub-Saharan Africa**
- **Third most frequent cause of cancer death in men and the sixth in women.**
- **Most of the patients currently identified in clinics are often at advanced stage of disease .**
- **All therapy regimens have provided limited success.**



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➤ Variations in the prevalence- etiological factors mirror the geographical distribution of the incidence of HCC.



ANIMAL MODELS

- **Carcinogen Induced Mouse models of HCC**
- **Implantation models of HCC**
- **Genetically Engineered Mouse (GEM) Models for HCC**
- **Viral Hepatocarcinogenesis-HBV and HCV associated mouse models of HCC**

Transgenic mouse models for HCC

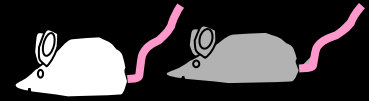
Transgene	Promoter	Mouse strain	Percentage HCCs	Reference
TGF- α	MT	CD1	50% in males > 12 months	1. Jhappan C, <i>et.al.</i> , 1990. 2. Lee GH, <i>et.al.</i> , 1992
c-myc	Alb	C57BL/6 $\times$ CBA/J 65	65% in males at 20 months	Santoni-Rugiu E, <i>et.al.</i> , 1996
c-myc/TGF- α	Alb, MT	C57BL/6 $\times$ CBA/J $\times$ CD1	100% in males at 8 months	Murakami <i>et.al.</i> , 1993 Santoni-Rugiu E, <i>et.al.</i> , 1996
SV40 T-Ag	AT III	C57BL/6 $\times$ DBA2	100% at 8 months	Dubois N, <i>et.al.</i> , 1991
E2F-1	Alb	C57BL/6 $\times$ CBA/J	33%-60% at 12 months	1. Conner EA, <i>et.al.</i> , 2000 2. Calvisi DF, <i>et.al.</i> , 2005
c-myc/E2F-1	Alb	C57BL/6 $\times$ CBA/J	100% at 9 months	Calvisi DF, <i>et.al.</i> , 2005

❖ Alb, albumin; AT III, Antithrombin III; MT, Metallothionein

**Comparative Genomic Analysis
More Humanized Mouse Model**



Human HCCs



Mouse HCCs

Selection of orthologous genes

**Standardization and integration of data sets
& relative expression of both species analysis**



**Identify best-fit mouse
models for each
subclass of human
HCC**

**Identify conserved
gene expression
patterns in mouse and
human HCC**

**Identify potential
therapeutic targets
in human HCC**

Build hypothesis

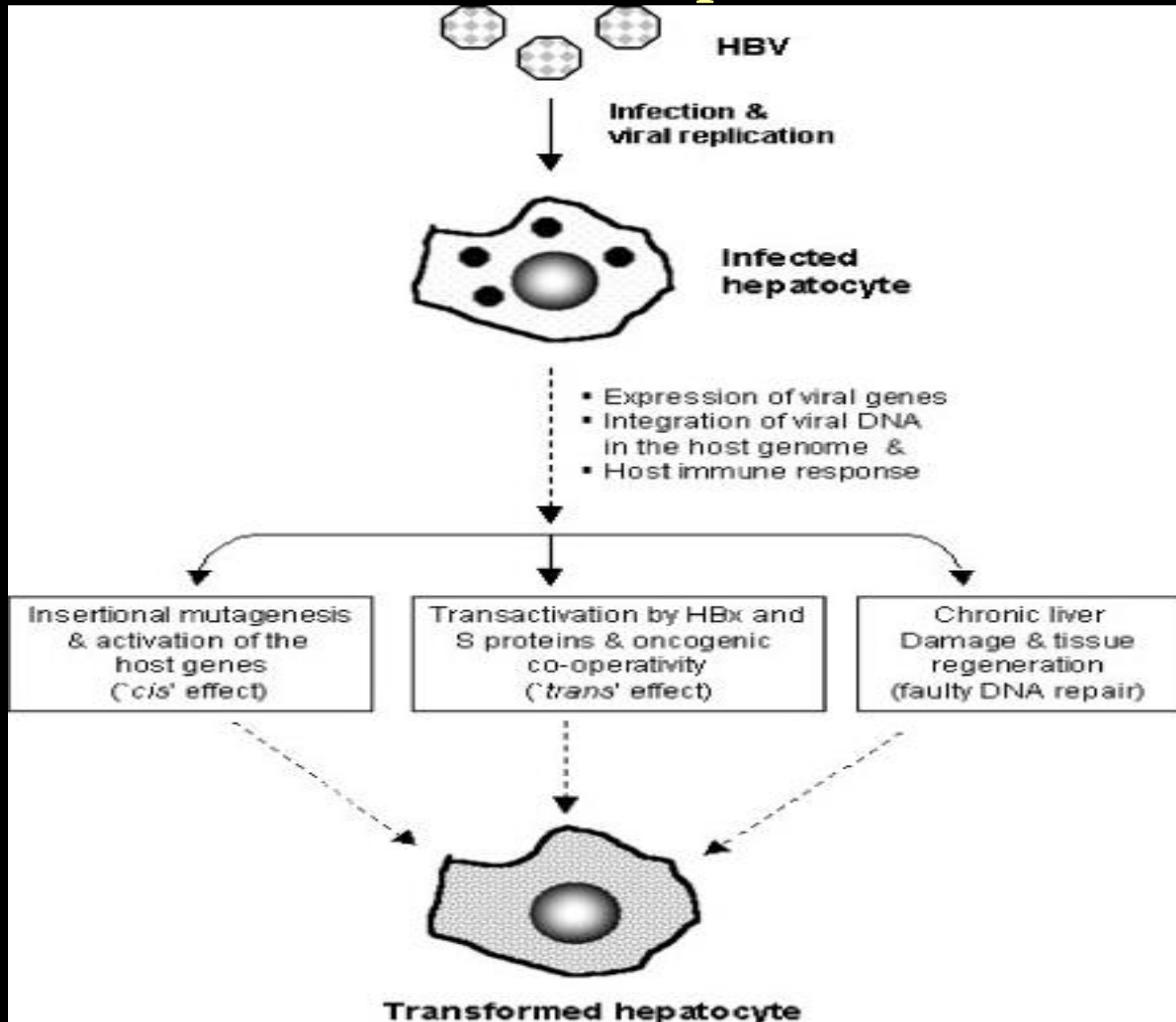
Test hypothesis

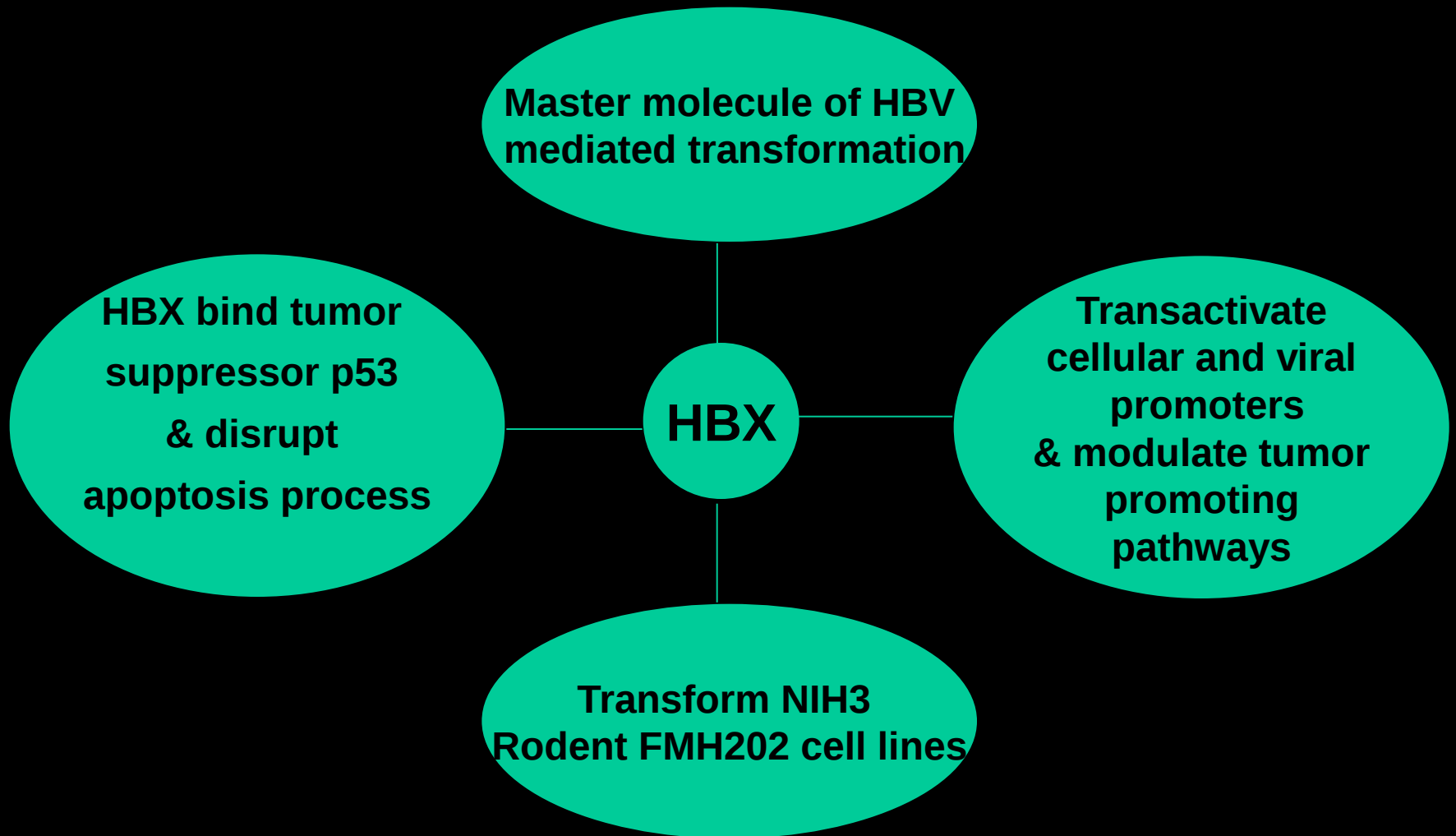
Validation and preclinical trial of targets

Viral Hepatocarcinogenesis

- More than 80% of HCC in human -HBV or HCV or both
- WHO- 400 million people-Chronic HBV-2000
- Infected person may take 20 years to develop HCC
- Undergo multiple steps of genetic alteration
- Human tropism virus
- WHV and GSHV- *in vivo* studies
- Human hepatocyte transplanted model
- Transgenic mice expressing HBV proteins represent the best model

Possible mechanisms for the HBV-associated HCC development





HBV-related transgenic mouse models of HCC

Chisari FV, et.al., 1985 and Babinet C,et.al., 1985

Transgene	expressed Promoters	used Mouse strain	Pathology	Reference
Large and major S	Alb-HBV	C57BL/6 x SJL	HCC in the second half of animals' life span	Chisari FV et.al., 1989
HBx	Xp	CD1	„	Kim CM et.al.,1991
HBx and c-myc in separate lines	Xp for HBx & WHV for c-myc	C57BL/6 x SJL/J	„	Terradillos O,et.al., 1997
HBx	Xp	C57BL/6 x DBA	„	Yu DY et.al., 1999

❖ Alb, albumin; Cp, core gene promoter; S, surface antigen; WHV, woodchuck hepatitis virus; Xp, X gene promoter.

Accelerated HCC - HBX Tg mouse & WHV/c-myc Tg mice hybrid offspring's
But still not fast as the pathogenic studies demanded

Bicistronic DNA construct, X-myc

X15-myc

(58-150aa of X protein)-Exon 2 and 3

Minimal transactivation domain of HBX
(Kumar et.al., 1996)

Murine c-myc protein

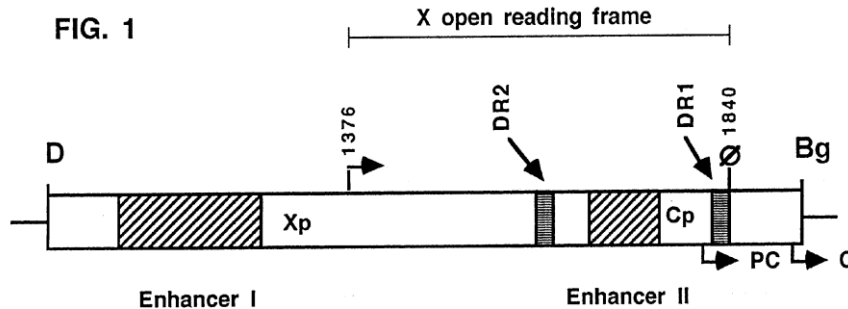
Reasons for choosing myc gene

- Selective amplification of c-myc gene found in HBV related HCC cases (**Peng et.al., 1993**)
- The activation of N-myc and c-myc gene is frequently observed after integration of viral **DNA** (**Moroy et.al 1996** and **Fourel et.al., 1990**)

X15myc-Transgenic construct

Regulatory elements in HBV genome

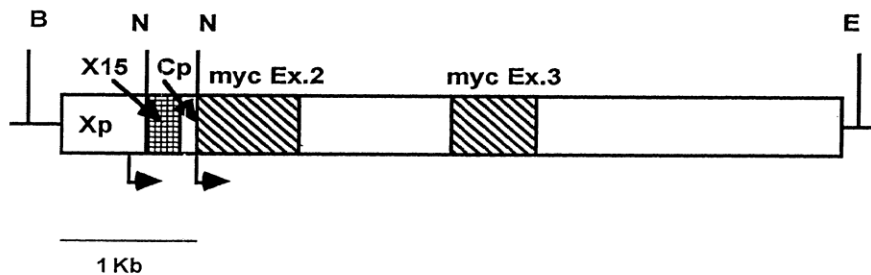
FIG. 1



X15 region positioned to 5' to the murine c-myc gene, and is operatively linked to under the regulatory control of its natural promoter and enhancer I element.

C-myc gene is operatively linked to under the regulatory control of core promoter and enhancer II elements

FIG. 2 X15-myc bicistronic construct

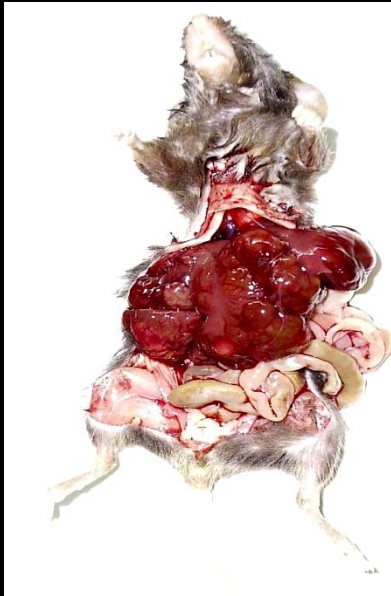


This compact present construct facilitate the the core promoter and and enhancer II regions are embodied in the X gene sequence

5.7Kb EcoR I and Bam HI fragment

D-Dra I, Bg-BglIII, Xp-Natural X promoter, Cp-Core promoter, B-BamhI, N-Nco I, E-EcoR I,

X 15- myc Transgenic Mouse Model for HCC



X15- myc Transgenic Mouse

➤ Recombinant bicistronic construct (Singh *et al*, 2003) (Kumar *et al*, 2001) US patent no: 6274788 B1.

➤ X-15 myc transgenic mice appeared to be an ideal model to study the disease process and also screening drugs.

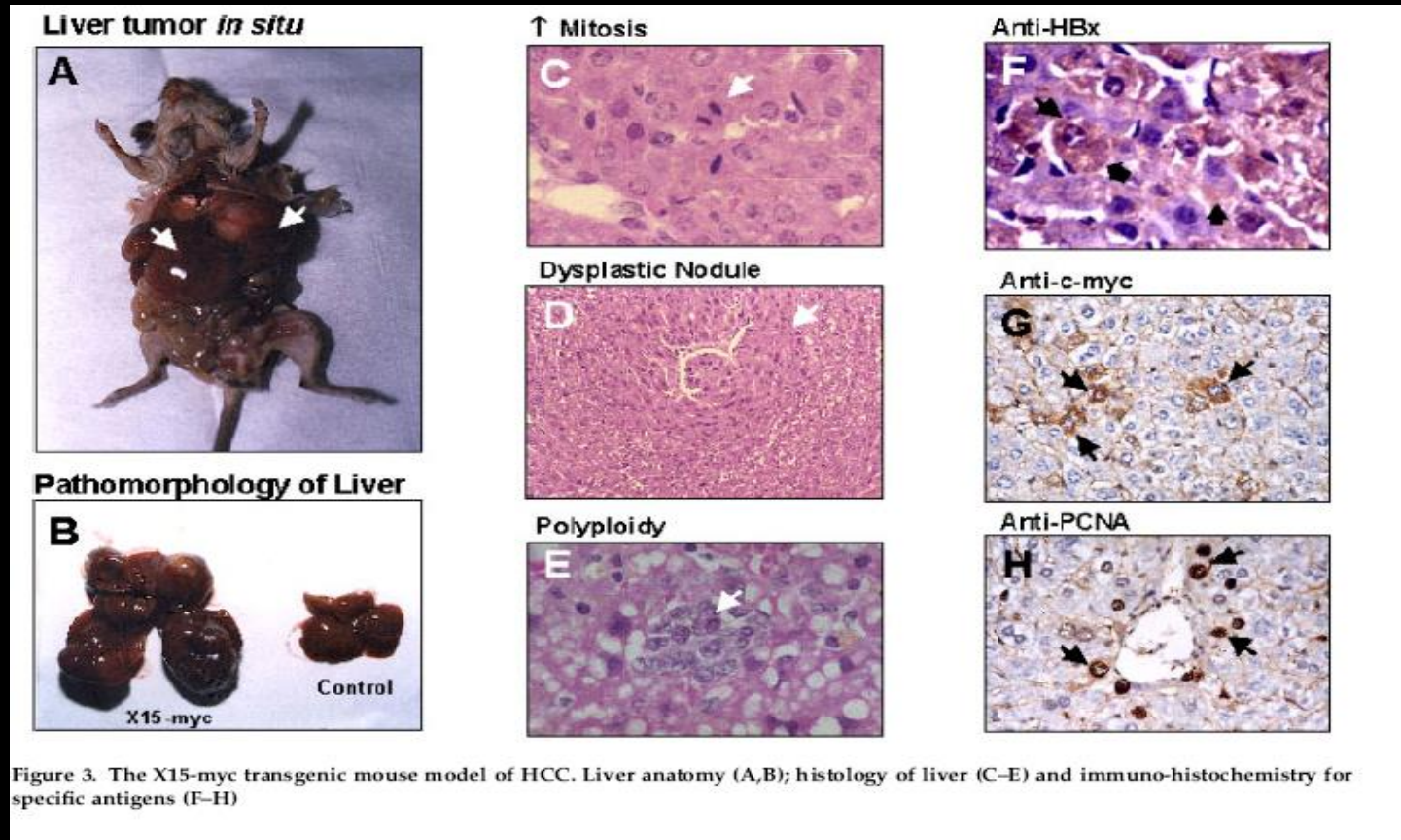


Normal Liver

X15- myc Tg Liver

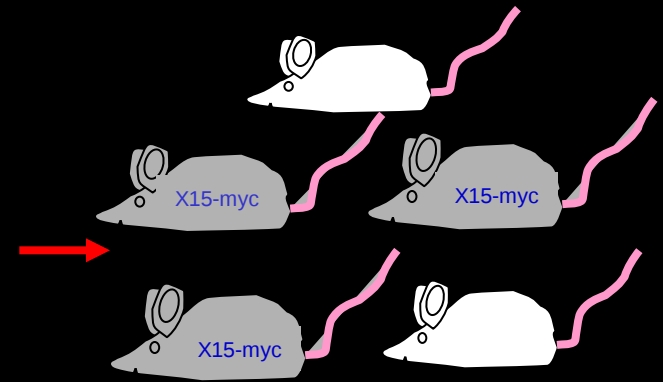
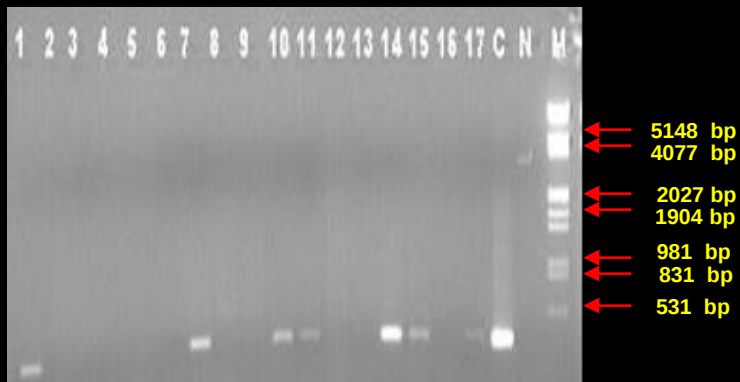
Transgene	expressed Promoters	used Mouse strain	Pathology	Reference
HBx and c-myc	Xp and Cp	C57BL/6 x SJL	HCC in the first half of animals' life span (3 to 5 months)	1. Singh M,et.al., 1998 2. Kumar V,et.al., 2001(us patent)

X15-myc transgenic mouse model

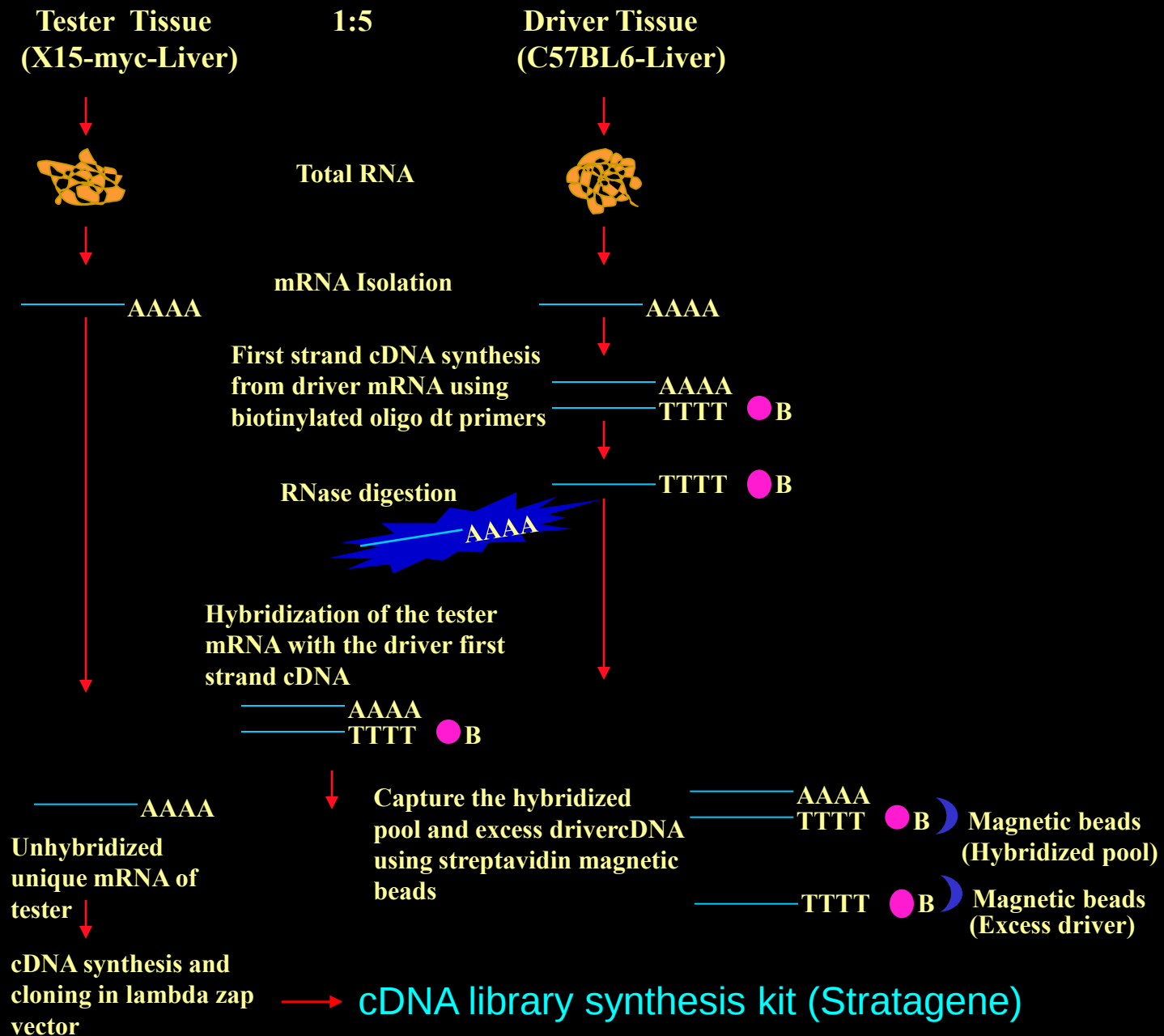


➤ Most of the pathomorphological and microscopic changes were similar to those observed with the HCC patients (Lakhtakia et al, 2003)

**Analysis of differential gene expression
in the tumors of X15-myc oncomouse.**

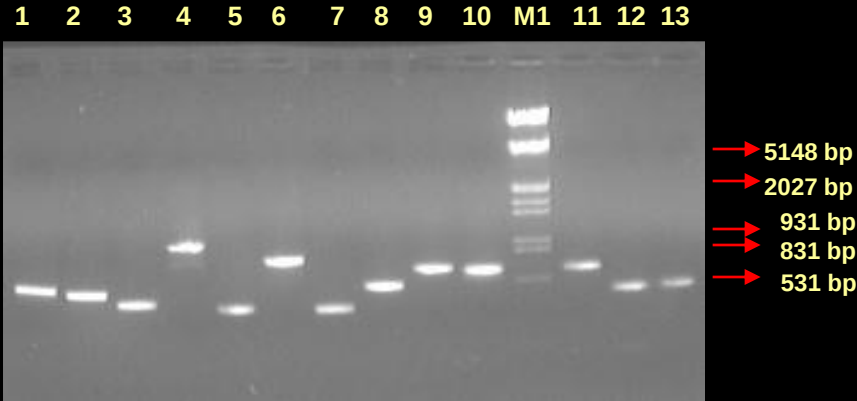


PCR analysis for X15-myc Transgenic positive animals
 “ C “- Positive control; “ N” – Negative control; “ M “- λ DNA Marker

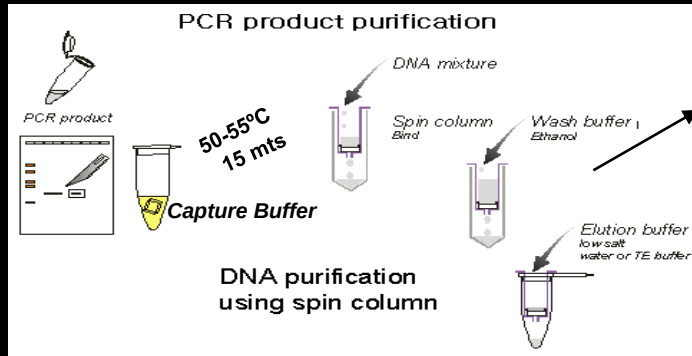


Primers M13 F: 5'-CGTTGTAAAACGACGGCCAGTG -3'
M13 R: 5'-CACAGGAAACAGCTATGACCATG-3'

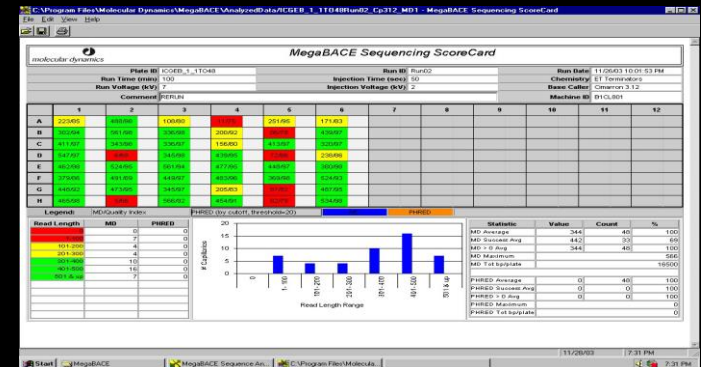
A.



B.



C.



A. Agarose gel analysis of PCR amplified cDNA inserts from phagemids. M1, DNA Marker, B. DNA band purification steps by GFX PCR, C. Chromatogram of MegaBACE sequencing score card.

Vector Screened & Changed in to FASTA format sequences



BLAST Analysis

<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>

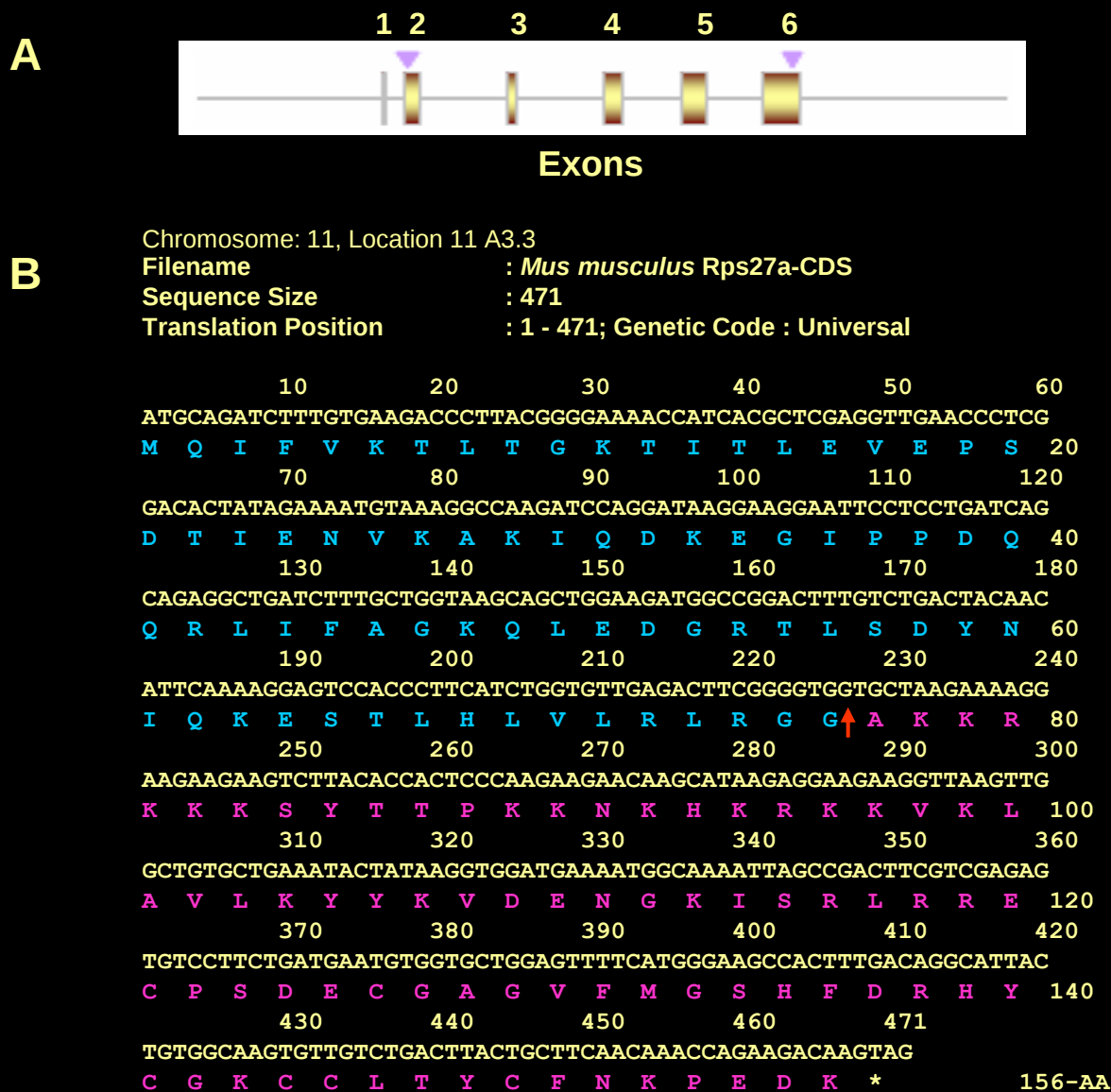
List of differentially expressed genes.

Sequence analysis of transcripts revealed 19 discrete categories

Protein biosynthesis genes	100
Electron transport genes	81
Metabolism related genes	75
Transport molecule genes	36
Ubiquitin proteasome pathway genes	25
Protein metabolism genes	23
Signal transduction genes	22
RNA processing genes	18
Calcium/sugar/carbohydrate/copper binding genes	14
Cytoskeletal organisation/ cell adhesion genes	17
Endocytosis /protein transport genes	13
Oxidative stress genes	13
Transcription genes	13
Cell cycle and growth differentiation genes	12
Immune response genes	11
Apoptosis and anti apoptosis genes	9
Blood coagulation genes	9
Molecular chaperone genes	8
Replication gene	1

List of top five differentially expressed genes obtained from X15-myc Transgenic mouse liver cDNA subtraction library.

<u>SYMBOL</u>	<u>GENE NAME</u>	<u>FREQUENCY</u>
Rps27a	Ribosomal Protein s27a	20
COX3	Cytochrome C Oxidase, Subunit III	16
ATP6	ATP Synthase F0 Subunit 6	14
COX2	Cytochrome C Oxidase, Subunit II	11
ND1	NADH dehydragenase, Subunit, type 1	11



Structure of the Rps27a gene of *Mus musculus* and cDNAs. **A**, Organization and chromosomal location. In total, 6 exons are shown in boxes. Closed boxes designate the coding sequences. **B**, ORF of the Rps27a gene showing 156 amino acids in single letter code (An red arrowmark indicates the site of proteolytic cleavage that would be required to generate mature ubiquitin from the primary translation product).

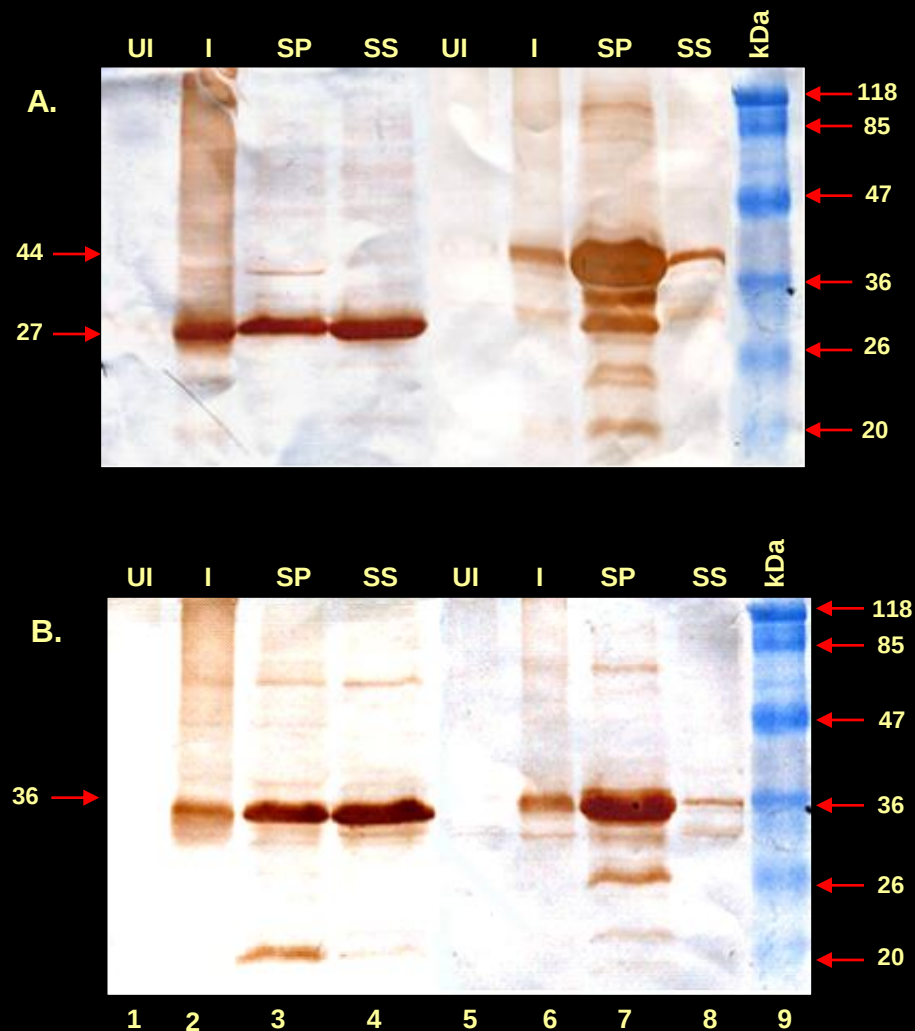
Identities = 155/156 (99.4%), Positives = 155/156 (99%)
 Gap frequency = 0/156 (0%) , PI= 9.68

mRps27a 1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ	40
hRps27a 1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQ	40
UBQ		
mRps27a 41	QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGGAKKR	80
hRps27a 41	QRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGGAKKR	80
UBQ		
mRps27a 81	KKKSYTTPKKNKHKRKKVKLAVLKYYKVDENGKISRLRRE	120
hRps27a 81	KKKSYTTPKKNKHKRKKVKLAVLKYYKVDENGKISRLRRE	120
Ribosomal_s27		
mRps27a 121	CPSDECGAGVFMSGHFD RHYCGKCCLTYCFNKPEDK	156
hRps27a 121	CPSDECGAGVFMA SHFD RHYCGKCCLTYCFNKPEDK	156
Ribosomal_s27		

Alignment of the primary sequences of human and mouse Rps27a. Identical residues are shown in same color, while the non-identical regions are marked in different colors. Blue and rose boxes denote the regions of ubiquitin and Ribosomal_s27 respectively.



- The Rps27a Highly conserved very basic protein due to presence of 80 basic AA in the Carboxyl terminal (CEP) of Ubiquitin.
 - UBQ-76 AA conserved eukaryotic protein- Diverse cellular functions
 - UBQ-Poly ubiquitin chain (UBb, UBc) or Fused to unrelated protein (UBA)
 - UBA —————→ UBA80 (Rps27a) or UBA52 (RpL40). Conserved in man, yeast and plants
- [Salvensan et al, Nucleic Acid Research (1987), Ozkaynak et al, EMBO J (1987), Lund et al, J. Bio. Chem (1985)]
- CEP analysis shows strong homology at the AA level with Zinc finger protein.
 - CEP can associate with ribosome binding specifically ribosome or messenger RNA.
 - Function of UBQ-CEP fusion genes has not been fully elucidated

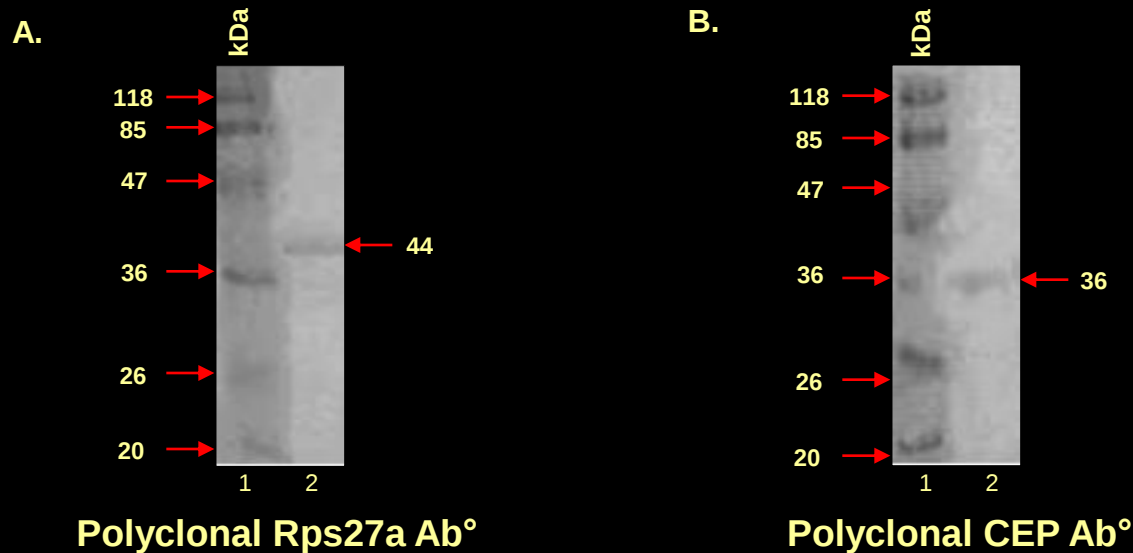


Western blot analysis of GST-Rps27a and GST-CEP recombinant protein expression in *E.coli*.

(A) IPTG (1mM) induced both crude and sonicated GST (27 kDa) and recombinant GST-Rps27a (44 kDa) protein

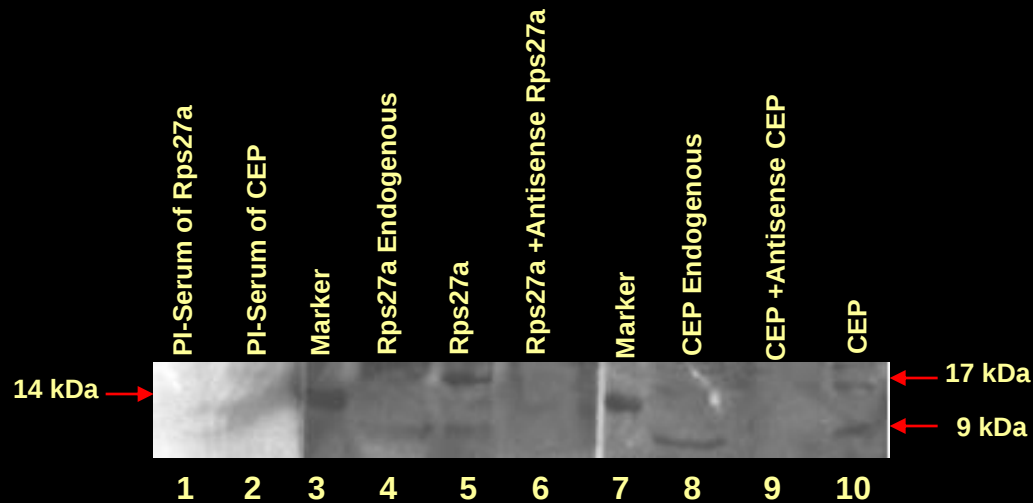
(B) GST-CEP1 (36 kDa) protein extracts were probed with GST antibody. UI, Un-induced; I, Induced; SP, Sonicated Pellet; SS, Sonicated Supernatant.

Authentication of Recombinant Rps27a and CEP proteins

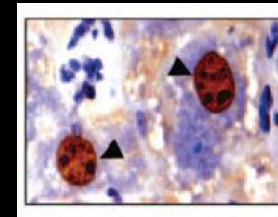
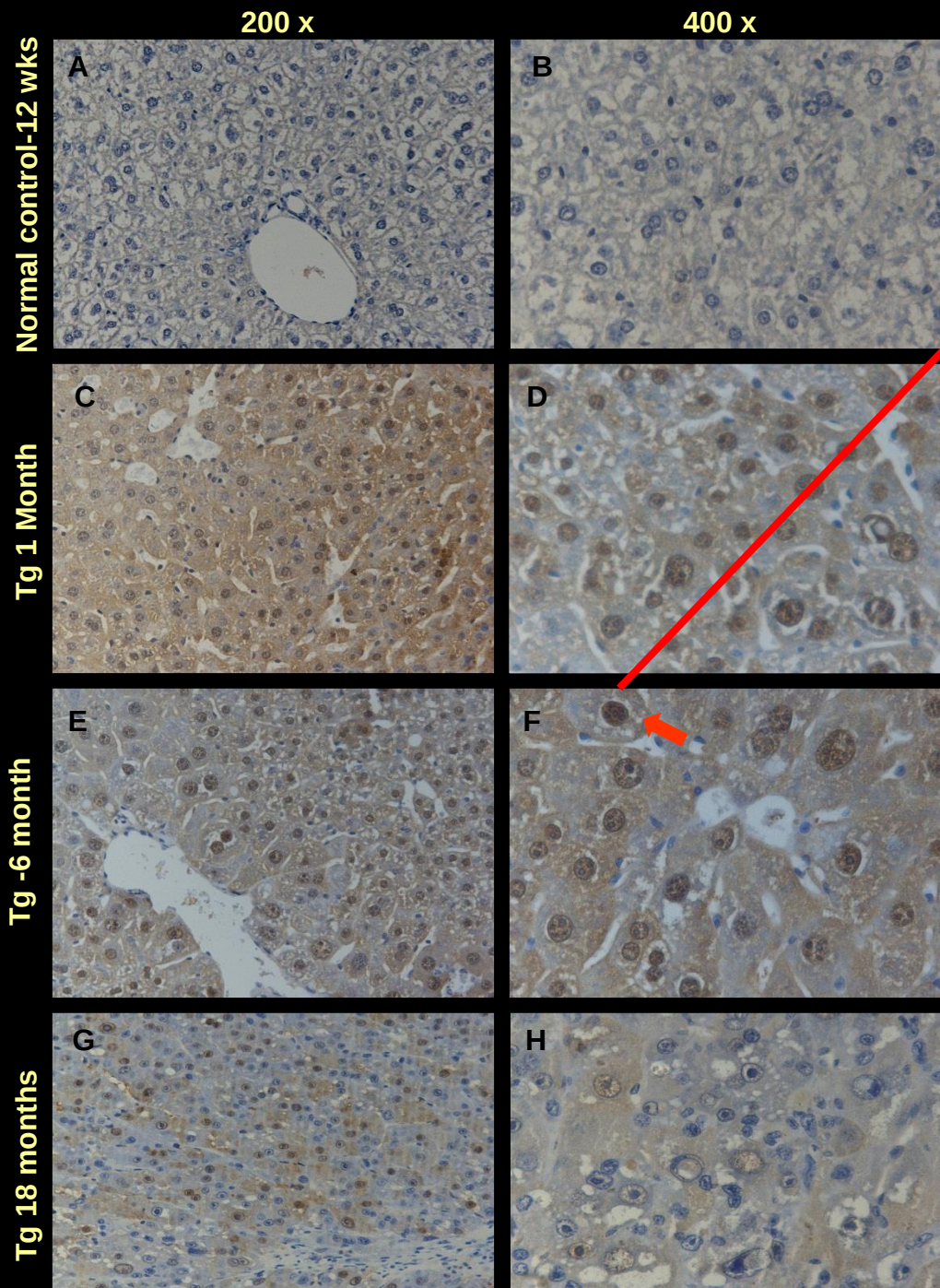


Western blot analysis GST-Rps27a and GST-CEP

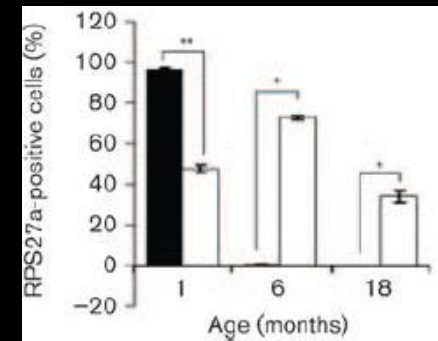
E.coli expression of recombinant GST-Rps27a (A) and GST-CEP (B) proteins were transferred on to nitrocellulose strips and probed with anti-Rps27a (1:200) (A. Lane 2) and anti-CEP polyclonal sera (B. Lane 2). Antigen- antibody reaction was shown on RH side arrow mark. kDa, Prestain marker.



Detection of Rps27a and CEP expression in Huh7 cell lines.
Total cells were lysed after 48hr post transfection with Rps27a (lanes 5), anti-Rps27a (lanes 6), CEP (lanes 10) and anti-CEP (lanes 9).



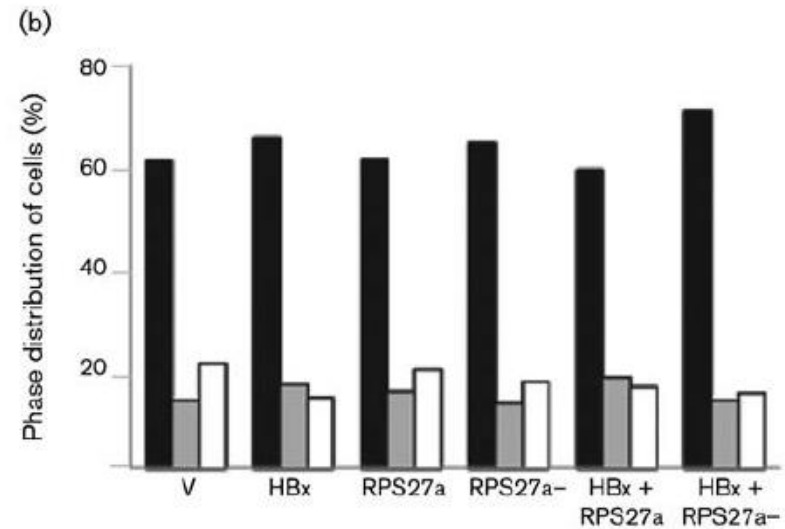
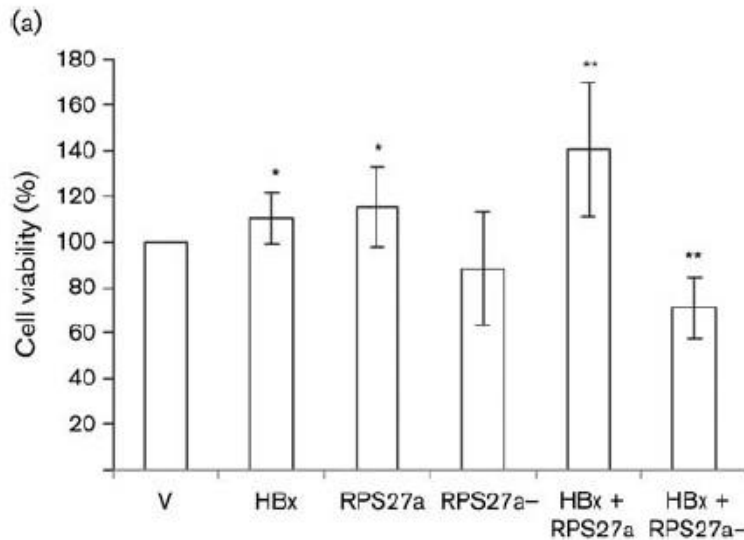
Positive staining is noted more in nuclear and perinuclear (red arrow)



transgenic mice (white bars). Cells ($n=500$) were counted randomly from 10 different areas. * $P<0.01$; ** $P<0.05$.

Rps27a expression in X15-myc Tg mice liver tissues.

Co-expression of Rps27a and HBx leads to improved survival and proliferation of cells

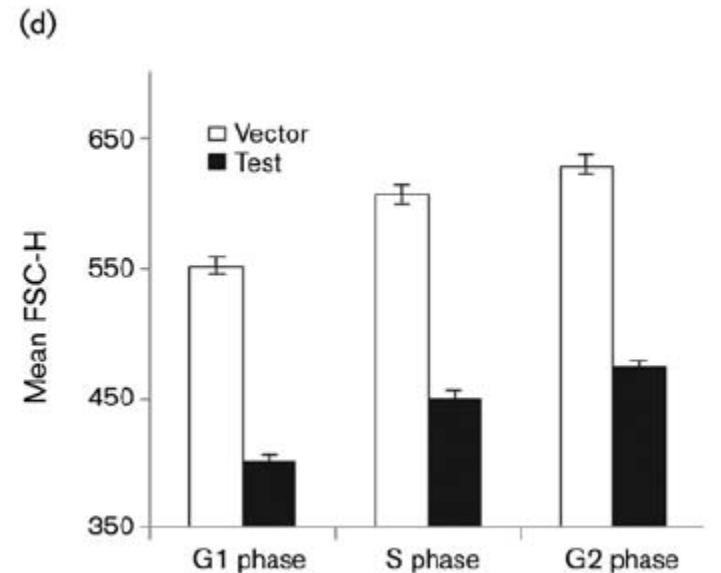
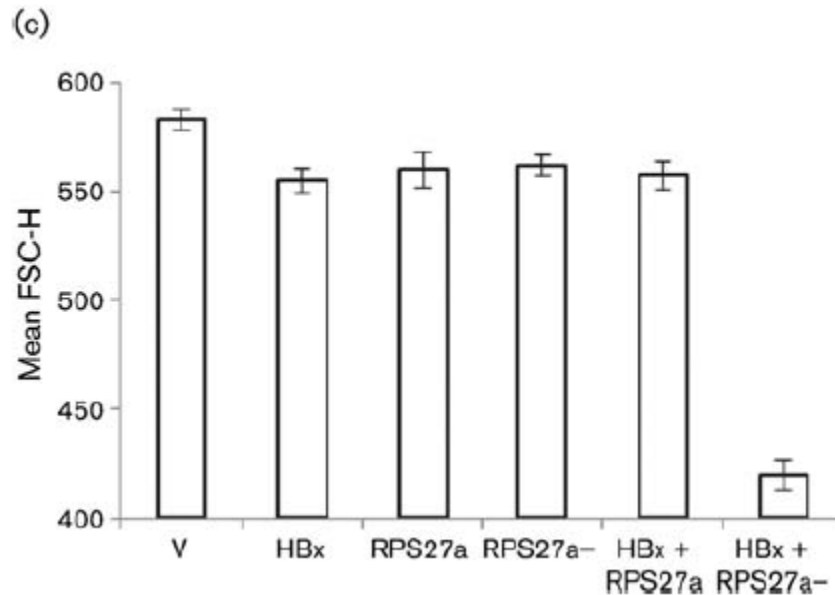


G0/G1	61.0	65.6	61.3	64.4	59.4	71.0
S	13.4	16.7	15.2	12.8	18.1	13.3
G2/M	20.6	14.0	19.7	17.2	16.2	15.0

* $P < 0.01$ ** $P < 0.001$, compared with controls.

Fig. 3. Cooperation between RPS27a and HBx for cell survival, proliferation and size. Huh7 cells were transfected with expression vectors for HBx, RPS27a or RPS27a-, either alone or in combinations as indicated, and their viability, size and cell-phase distribution were monitored. (a) Cell viability by MTT assay. The bar graph shows means $\pm$ SD of ten independent observations. (b) Flow cytometry of asynchronous cells to show percentage distribution of cells in different phases of the cell cycle (black bars, G0/G1; grey bars, S; white bars, G2/M). Results are means of three independent observations. (c, d) The

Rps27a regulates the cell size checkpoint in the presence of HBx

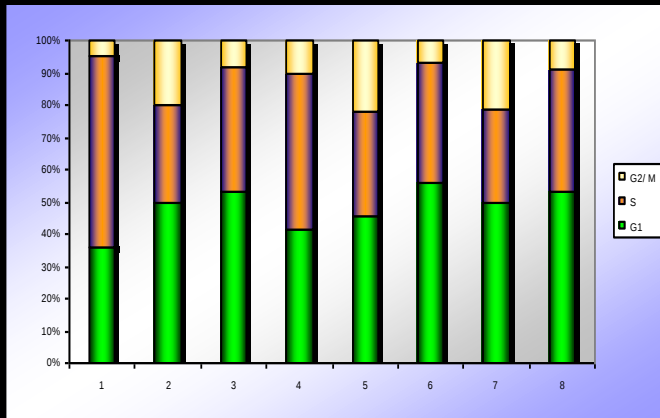


* $P < 0.01$ ** $P < 0.001$, compared with controls.

Need for Rps27a in an HBx micro environment primarily for maintaining cell size, and thus regulating proliferation of cells

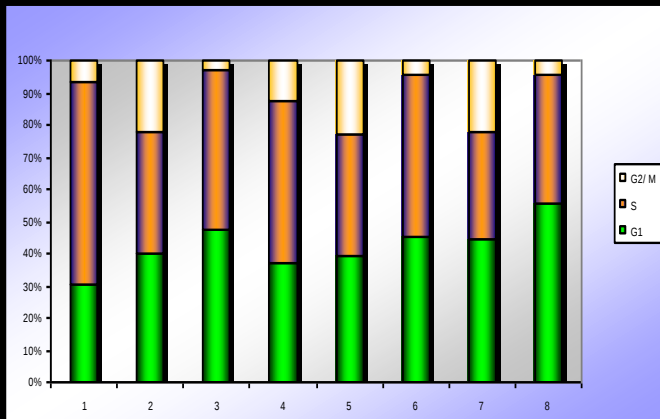
12hrs

A



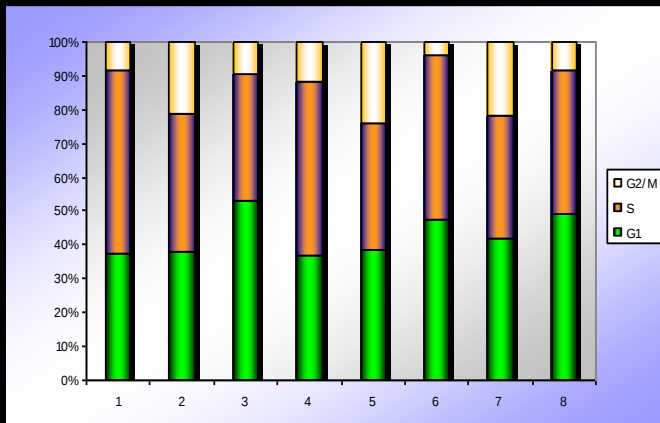
24hrs

B



42 hrs

C



Constructs

1. Rps27a
2. Antisense Rps27a
3. Rps27a + Antisense Rps27a
4. CEP
5. Antisense CEP
6. CEP + Antisense CEP
7. Rps27a + Antisense Rps27a + Antisense CEP
8. pSG5

Effect of Rps27a and CEP on cell cycle. Huh7 cells were transfected with Rps27a, CEP, anti-Rps27a and anti-CEP constructs at 60-70 % confluency and cells were serum synchronized and collected at various time points of 12hrs (A) , 24hrs (B) and 42 hrs (C). The percentages of cells at different phases of the cell cycle were analysed by FACS and values are shown on the left (Y-axis) bar diagram.

Report-Deletion Rps6 (40s RS)
(Rps27a & CEP) ↓

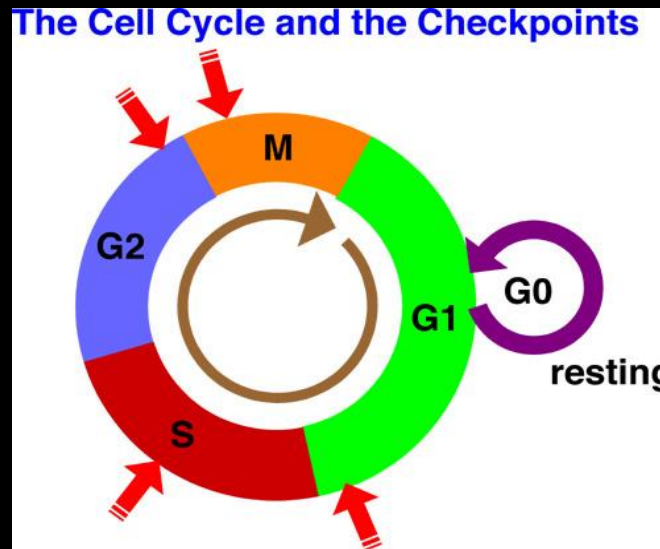
Cell proliferation

G2/M phase arrest

Cyclin B1
(Yu et.al., 2002)

Cyclin E (sherr et.al.,1999)

Rps27a- early growth response
gene G1 phase cell division

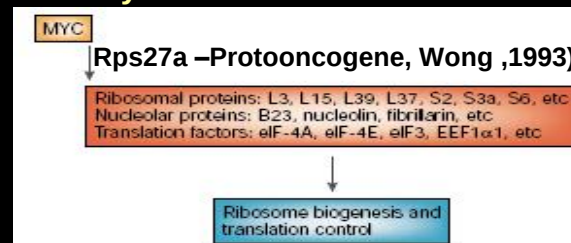
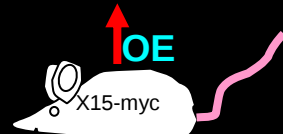


“S”PHASE ↑
DNA replication

(Rps27a & CEP) ↑

Induction of
Cyclin D-CDK4
Cyclin D-CDK2

OE



DeBenedetti et al., 1990

In yeast, UB13 deletion-Slow growth of phenotype (1.6 hr-6.8hr) (Finley et.al., 1989)

Many small & Large Rib.Sub unit Protein ↑ variety of p.Tumor- Ex RPL36A. OE-Over Expression

Summary

- Rps27a, a ubiquitin precursor protein fused to the 80 amino acid carboxyl extension protein (CEP)
- The flow cytometry analysis revealed that Rps27a overexpressing cells accelerated cells to enter G1 phase to S phase with enhanced cell proliferation
- Immunohistochemical staining of Rps27a expression was moderately reduced from 3 months to 18 months old Transgenic liver tissues.

Overall, The sensitivity of over expressed Rps27a in HCC might be a general biomarker for tumor proliferation.

THANKS