**Cell**

## Supplemental Information

# An HNF4α-miRNA Inflammatory Feedback Circuit regulates Hepatocellular Oncogenesis

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Inventory:

## Hatziapostolou et al, Figure S1

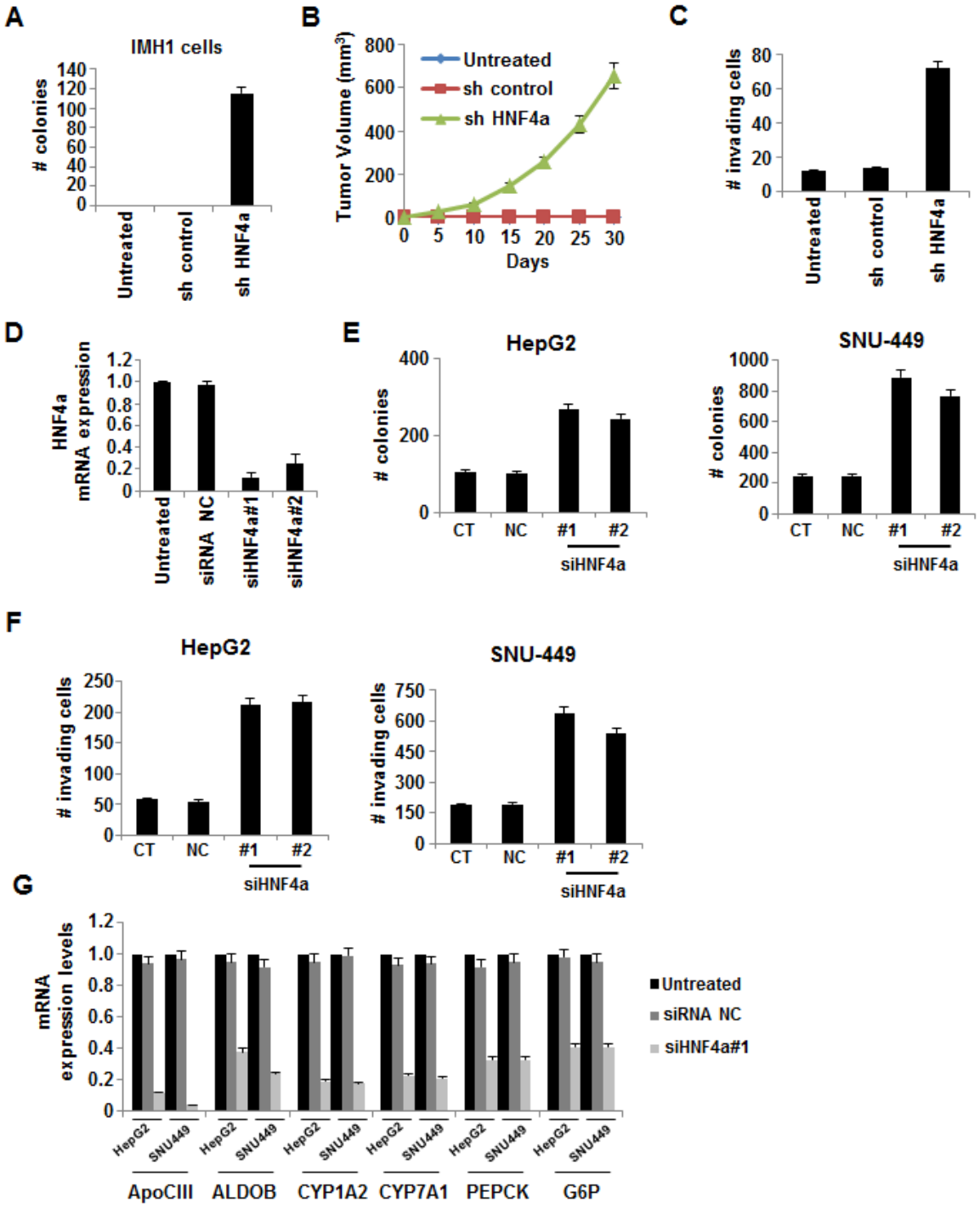



**Figure S1, related to Figure 1. HNF4α suppression induces hepatocellular transformation.** (A) Soft-agar colony assay of non transformed immortalized hepatocytes (IMH1) treated with shRNA empty vector (sh control) or shRNA against HNF4α (sh HNF4α). Colonies 50 μm were counted using a microscope 20 days later. The mean together with SD of a representative experiment done in triplicate is shown.

(B) Tumor volume (mean ± SD) in mice injected with untreated or treated with shcontrol or shHNF4α IMH1 cells. Tumor volume was monitored 30 days post injection. (C) Invasion assay of non transformed immortalized hepatocytes (IMH1) treated with shRNA empty vector (sh control) or shRNA against HNF4α (sh HNF4α). (D) HNF4α expression levels in xenograft tumors. HNF4α mRNA expression assessed by real-time RT-PCR analysis in tumors (55 days) derived from injected IMH1 cells that were untreated or treated for 48h with 1nM siRNA negative control (siRNA NC) or two different siRNAs against HNF4α (siHNF4α#1, siHNF4α#2). (E) Soft agar colony assay and (F) invasion assays in HepG2 and SNU-449 hepatocellular cancer cell lines untreated (CT) or treated for 48h with siRNA negative control (NC) and two siRNAs against HNF4α. The experiments have been performed in triplicate and the data show mean ± SD. (G) Expression levels of HNF4α direct downstream targets in liver cells. ApoCIII, ALDOB, CYP1A2, CYP7A1 and GP6 mRNA levels were assessed by real-time RT-PCR analysis in HepG2 and SNU449 cells untreated or treated with 1nM siRNA negative control (siRNA NC) or siRNA against HNF4α (siHNF4α#1).

## Hatziapostolou et al, Figure S2

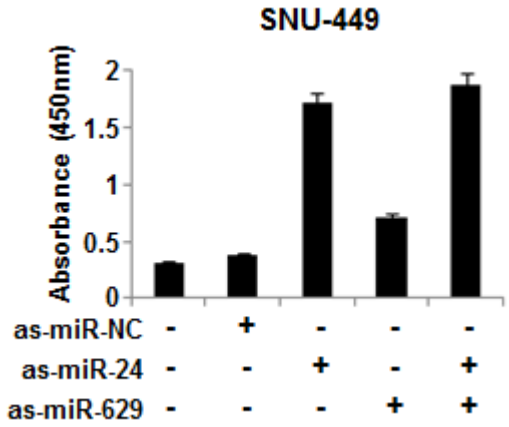



**Figure S2, related to Figure 2. Suppression of miR-24 and miR-629 induces apoptosis in HCC xenograft tumors.** The levels of cleaved caspase-3 activity were measured by ELISA assay in the same tumors, described in Figure 2G. The data suggest that inhibition of miR-24 and/or miR-629 by antisense-microRNAs leads to decreased tumor growth due to induction of apoptosis.

## Hatziapostolou et al, Figure S3

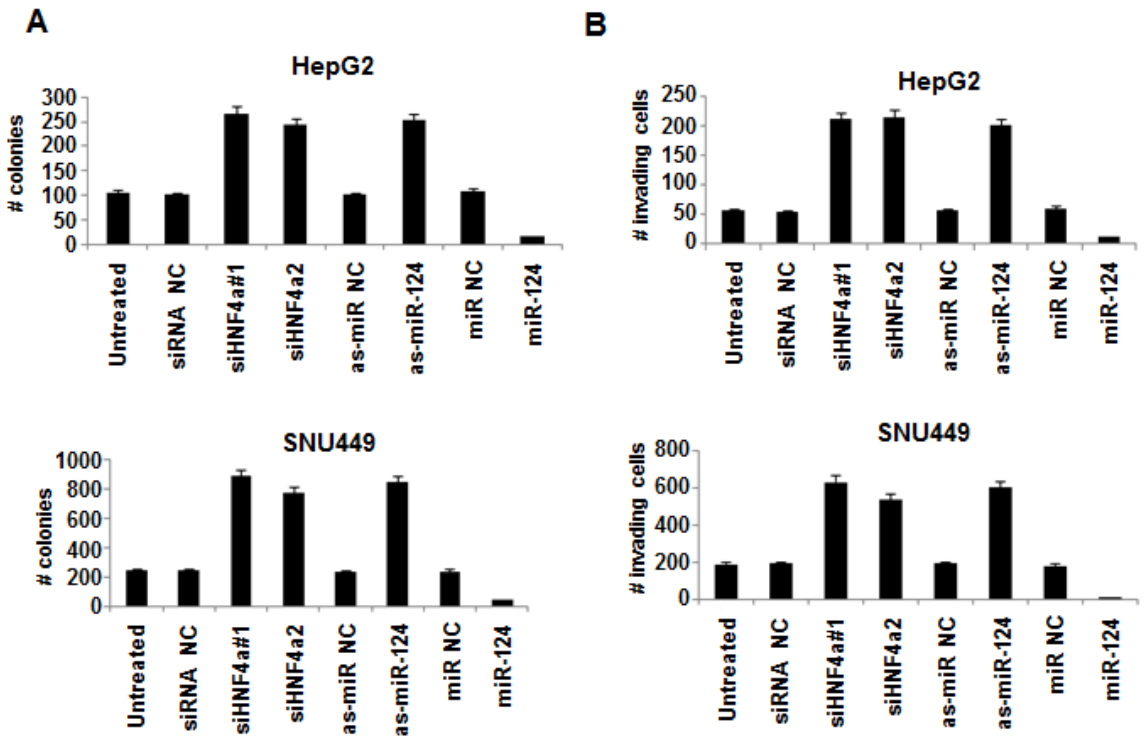



**Figure S3, related to Figure 4. Inhibition of miR-124 expression has similar effects with HNF4α inhibition in the colony formation and invasive ability of hepatocellular cancer cells.** (A) Soft agar colony assay and (B) invasion assays in HepG2 and SNU-449 hepatocellular cancer cell lines untreated or treated for 24h with siRNA negative control (siRNA NC) and two siRNAs against HNF4α or antisense-microRNA negative control (as-miR-NC), antisense-microRNA-124 (as-miR-124), microRNA negative control (miR NC) and microRNA-124 (miR-124). The experiments have been performed in triplicate and the data show mean ± SD.

## Hatziapostolou et al, Figure S4

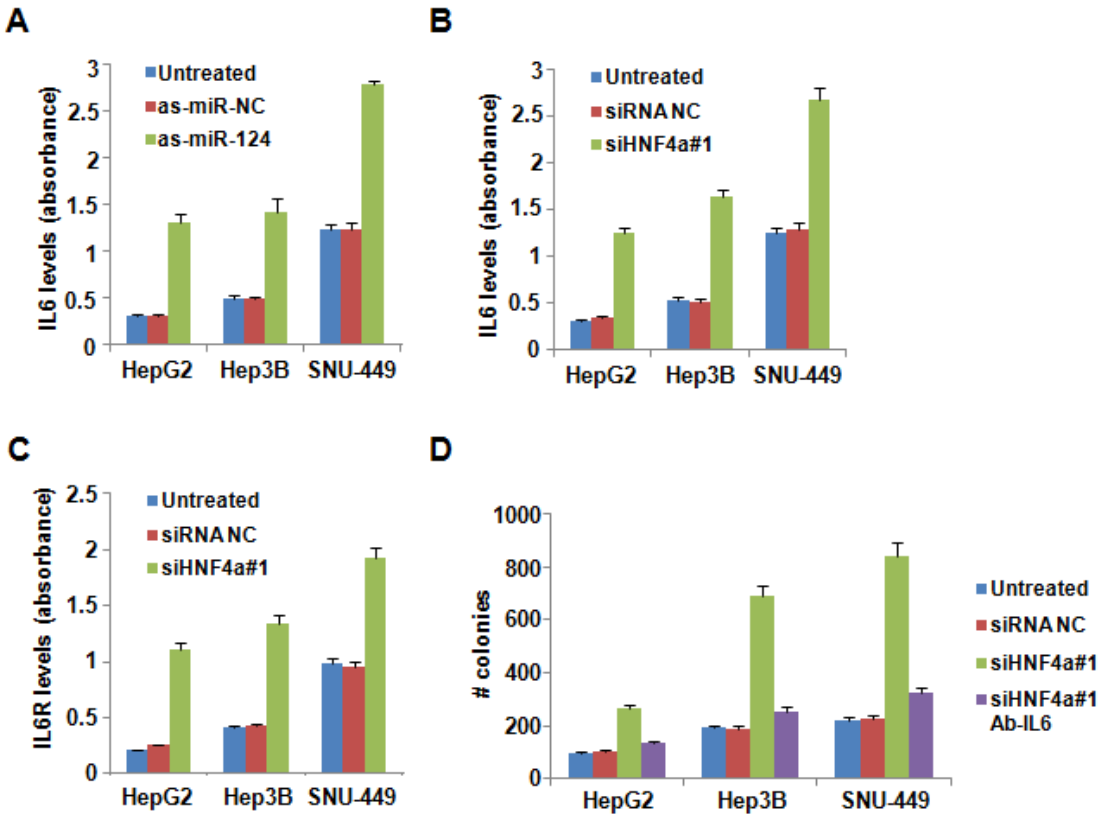



**Figure S4, related to Figure 4. HNF4a circuit regulates the production of IL6 and IL6R in liver cancer cells.** (A) Soluble IL6 levels were measured in untreated, as-miR-NC or as-miR-124 treated HepG2, Hep3B and SNU-449 cells, 24h post transfection by ELISA assay. (B) Soluble IL6 and (C) IL6R levels were measured in untreated, 1nM of siRNA NC or 1nM of siRNA against HNF4a (siHNF4a#1) in HepG2, Hep3B and SNU-449 cells, 24h post transfection by ELISA assay. The data are presented as mean ± SD of three independent experiments and show that inhibition of HNF4a expression increases the levels of soluble IL6 and IL6R in liver cancer cells, suggesting that perturbation of the HNF4a circuit drives liver cancer cells to an activated inflammatory state. (D) Soft-agar colony assay in HepG2, Hep3B and SNU-449 cells treated for 24h with 1nM siRNA NC or siHNF4a#1 or siHNF4a#1 followed by Ab-IL6 for 24h. The data are presented as mean ± SD of three independent experiments.

## Hatziapostolou et al, Figure S5

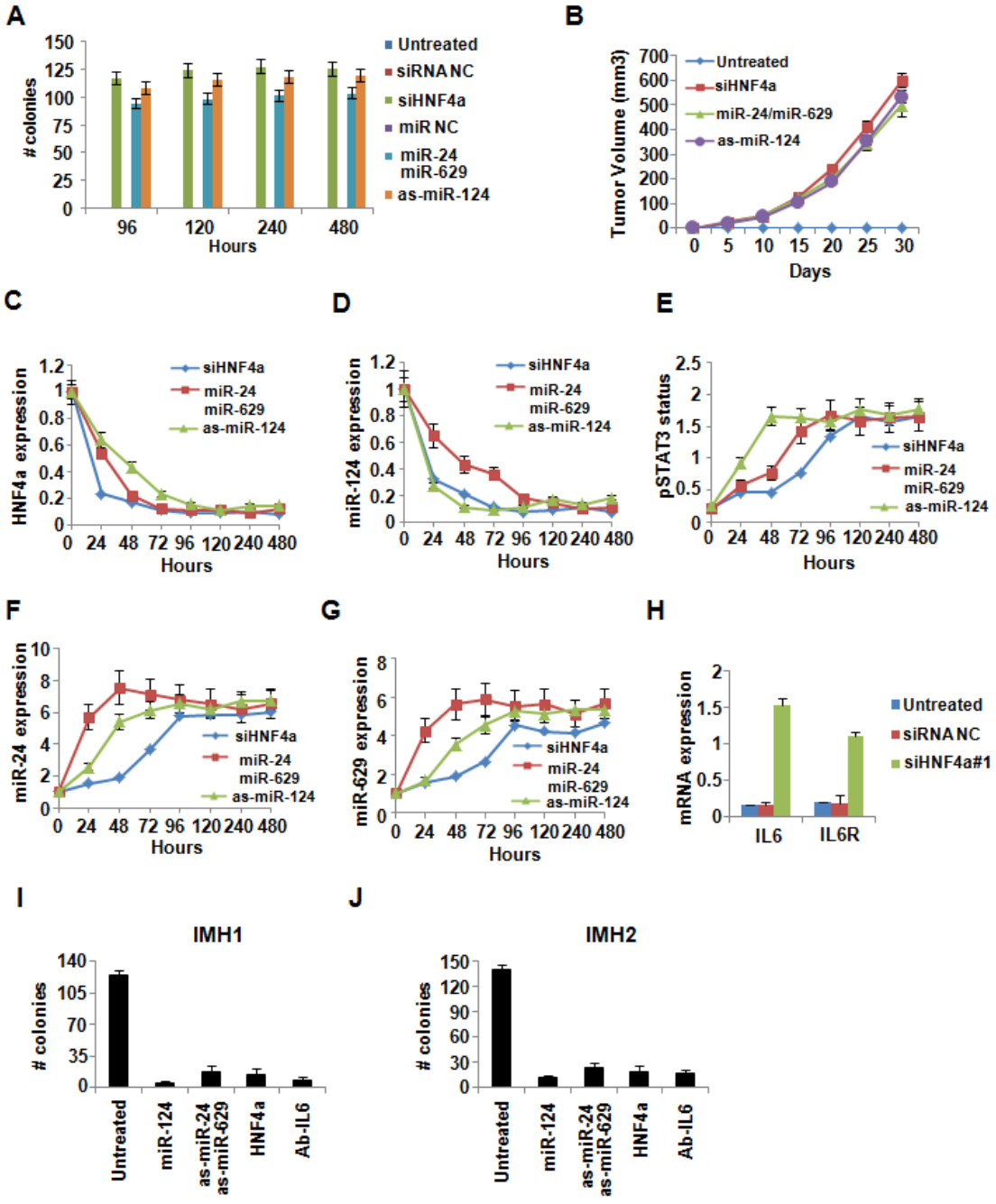
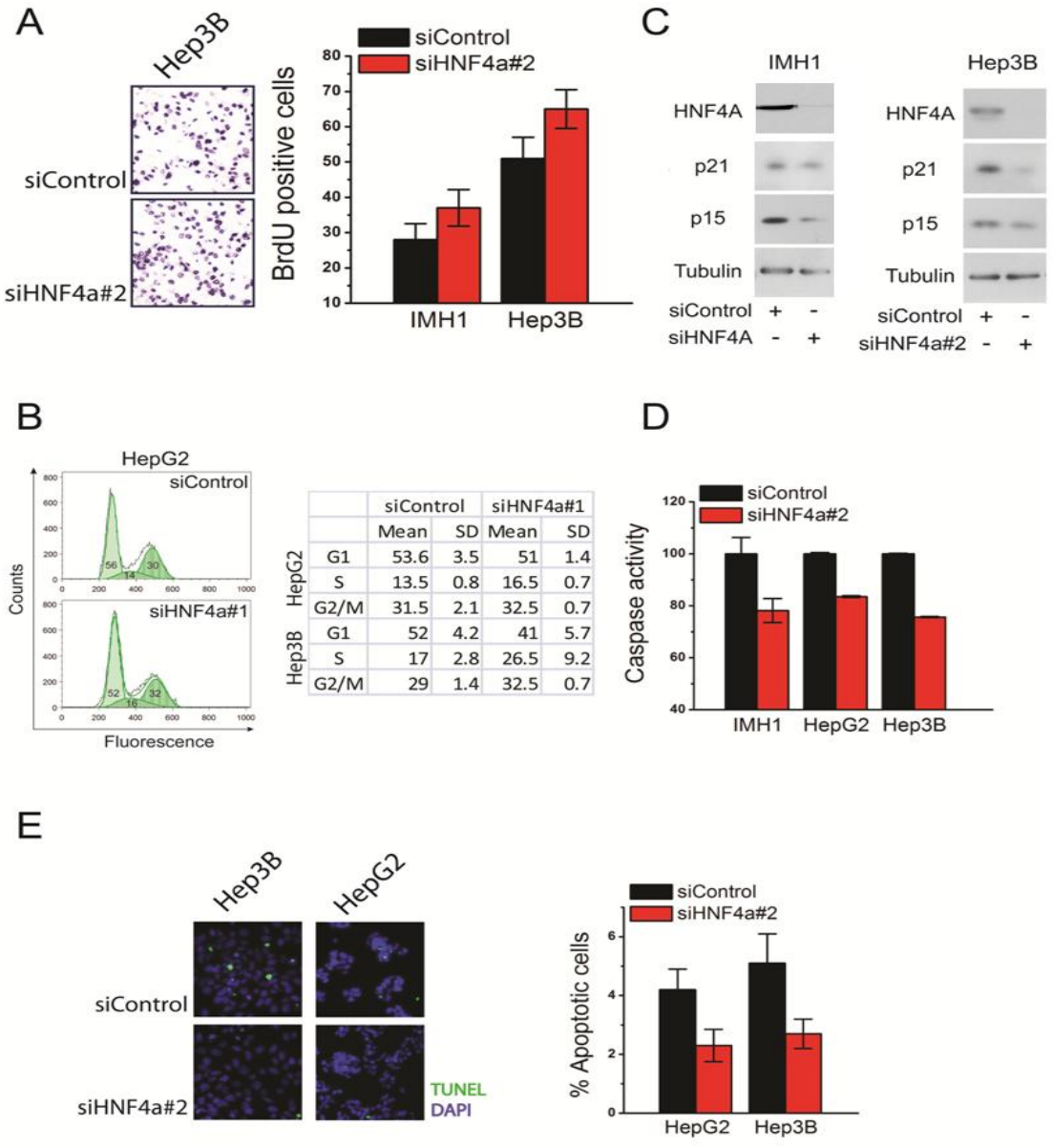



**Figure S5, related to Figure 4. The HNF4α feedback loop circuit is pertubated during hepatocellular transformation in vitro and in vivo**. (A) Soft-agar colony assay of IMH cells transiently transfected with the respective miRNAs or siRNAs for 24h. These cells were maintained in culture 96-480h post-transfection and then were plated in soft agar and colony number was assessed 20 days later. (B) Tumor volume (mean ±

SD) in mice injected with IMH cells untreated or treated with siHNF4α, miR-24 and miR-629, as-miR-124 for 24h and then maintained in culture for 120h before being injected in nude mice. (C-G) Assessment of HNF4α, miR-124, miR-24, miR-629 levels by real-time RT-PCR analysis and of STAT3 phosphorylation levels by ELISA in IMH1 cells transiently transfected with 1nM siHNF4α or miR-24 and miR-629 or as-miR-124. (H) Assessment of soluble IL6 and IL6R by ELISA assay in IMH1 cells, 96h after transfection with 1nM siRNA NC or siHNF4a#1. (I) Assessment of colony formation in soft agar of IMH1 cells and (J) IMH2 cells untreated or treated with 100nM miR-124 or as-miR-24 and as-miR-629 or HNF4α for 48h. These cells were propagated for 20 days (480h) post treatments and then were plated in soft agar and their colony formation ability was tested 20 days later.

## Hatziapostolou et al, Figure S6




| | | siControl | | siHNF4a#1 | |
|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD |
| HepG2 | G1 | 53.6 | 3.5 | 51 | 1.4 |
| | S | 13.5 | 0.8 | 16.5 | 0.7 |
| | G2/M | 31.5 | 2.1 | 32.5 | 0.7 |
| Hep3B | G1 | 52 | 4.2 | 41 | 5.7 |
| | S | 17 | 2.8 | 26.5 | 9.2 |
| | G2/M | 29 | 1.4 | 32.5 | 0.7 |

**Figure S6, related to Figure 4**: **Knockdown of HNF4α induces cell growth and renders cells resistant to apoptosis.** (A) Cell proliferation as determined by the BrdU assay. Cells were transfected with siHNF4α or with siRNA NC and 48 h later cell proliferation was determined using the BrdU assay. Data are mean percentages of BrdU positive cells ± SEM. (B) Cell cycle analysis. The DNA contents of cells harvested 48 h after HNF4α knockdown were determined. (Left) Representative plots of FACS analysis in HepG2 cells transfected with siControl (upper panel) and si HNF4α (lower panel). (Right) The percentages of cells in the G1, S, and G2/M phases of the cell cycle were measured and the mean percentage of cells in a given phase ± the SEM is derived from three independent experiments. (C) Lysates of cells were harvested 48 h after the transfection and analyzed by Western blotting for the detection of cell cycle regulation proteins. Tubulin was used as the loading control. (D) Cells were transfected with siHNF4α or with siControl and 48 h later caspase3/7 activity was determined. Data are mean percentages of caspase3/7 activity ± SEM. (E) Apoptosis was determined by the TUNEL assay. 48 h after transfection of the cells with the indicated siRNAs tunel staining was performed and the samples were examined by fluorescence microscopy. Data are expressed as the mean percentages of apoptotic cells ± SEM.

## Hatziapostolou et al, Figure S7

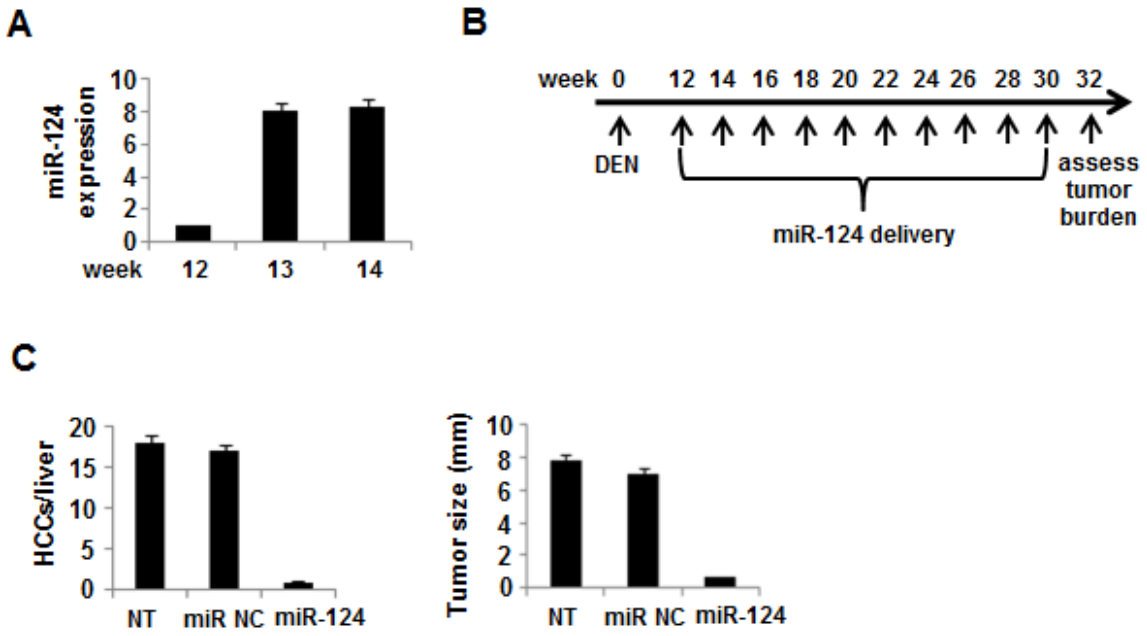



**Figure S7, related to Figure 6. Preventive effects of miR-124 administration in hepatocellular oncogenesis** (A) Assessment of miR-124 levels (mean ± SD) in liver tissues derived from DEN-treated mice (weeks 12, 13, 14) after systemic delivery of miR-124 on the first day of week 12. (B) Timeline of miR-124 prevention delivery experiment. (C) Number of tumors/liver and tumor size ($mm^3$) in non-treated (NT), miR-NC and miR-124 treated mice (week 32).