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RESEARCH ARTICLE

Modulation of hypoxia-signaling pathways by extracellular linc-RoR

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ABSTRACT

Resistance to adverse environmental conditions, such as hypoxia, contributes to the reduced efficacy of anticancer therapies and tumor progression. Although deregulated expression of many long noncoding RNA (lncRNA) occurs in human cancers, the contribution of such RNA to tumor responses to hypoxia are unknown. RNA expression profiling identified several hypoxia-responsive lncRNAs, including the long intergenic noncoding RNA, regulator of reprogramming (linc-RoR), which is also increased in expression in malignant liver cancer cells. Linc-RoR expression was increased in hypoxic regions within tumor cell xenografts *in vivo*. Tumor cell viability during hypoxia was reduced by small interfering RNA (siRNA) to linc-RoR. Compared with controls, siRNA to linc-RoR decreased phosphorylation of p70S6K1 (RPS6KB1), PDK1 and HIF-1α protein expression and increased expression of the linc-RoR target microRNA-145 (miR-145). Linc-RoR was highly expressed in extracellular RNA released by hepatocellular cancer (HCC) cells during hypoxia. Incubation with extracellular vesicle preparations containing extracellular RNA increased linc-RoR, HIF-1α expression and cell survival in recipient cells. These studies show that linc-RoR is a hypoxia-responsive lncRNA that is functionally linked to hypoxia signaling in HCC through a miR-145–HIF-1α signaling module. Furthermore, this work identifies a mechanistic role for the extracellular transfer of linc-RoR in intercellular signaling to promote cell survival during hypoxic stress.

KEY WORDS: Extracellular vesicle, Exosome, Gene expression, microRNA, Cell stress, Liver cancer, Hypoxia, linc-RoR

INTRODUCTION

Hepatocellular cancer (HCC) is the fifth most common cancer in men and the seventh most common cancer in women worldwide (El-Serag, 2012). HCC is a highly treatment-resistant malignancy and is characterized by the alteration of several nondominant signaling pathways that modulate tumor behavior and contribute to both local spread and a tendency for multifocal tumor development (Whittaker et al., 2010). Resistance to therapy, and tumor progression, is increased by the ability of HCC cells to resist adverse environmental conditions, such as hypoxia. Locoregional therapies, such as transarterial embolization, which result in ischemic necrosis but tumor cell survival within hypoxic regions, might contribute to incomplete eradication or tumor recurrence (Shim et al., 2008; Gadaleta and Ranieri, 2011; Kong et al., 2012). Although the ability of tumors to survive during hypoxia is a crucial determinant of therapeutic response and tumor progression, the cellular mechanisms by which tumor cells respond to, and survive during, hypoxia are poorly understood.

In recent studies, long noncoding RNAs (lncRNAs), which are defined as noncoding RNAs (ncRNA) more than 200 nucleotides in length, have been identified to have crucial regulatory roles in the regulation of gene expression. Several lncRNAs, such as H19, highly upregulated in liver cancer, TUC338, maternally expressed 3, and metastasis-associated lung adenocarcinoma transcript 1 are aberrantly expressed in human HCC (Ariel et al., 1998; Panzitt et al., 2007; Tripathi et al., 2010; Braconi et al., 2011b; Braconi et al., 2011a; Lai et al., 2012). These lncRNAs might contribute to oncogenesis during the development of HCC. Despite the pervasive effects of lncRNA in the regulation of gene expression, the function of lncRNAs in HCC and other cancers is still unclear. In the present study, we sought to examine the functional contribution of lncRNA derived from tumor cells to environmental perturbations, such as hypoxia.

The release of extracellular vesicles, such as exosomes, from cells in response to changes in the tissue environment provides a potential mechanism of intercellular signaling by which tissue responses to hypoxia could be coordinated. These small vesicles are shed from cells and contain membrane and cytoplasmic proteins, lipids and nucleic acids derived from the cytoplasm of the donor cell (Théry et al., 2009; Batagov et al., 2011). The vesicles can contribute to cell-to-cell communication and modulate cellular activities in recipient cells by transfer of their content. We recently reported that extracellular vesicles derived from malignant hepatocytes contain microRNAs that can be transferred to other recipient cells and, moreover, can modulate cell signaling and cell viability in these recipient cells (Kogure et al., 2011). We have also identified lncRNAs, such as TUC339, that can be selectively enriched within these vesicles (Kogure et al., 2013). These observations support the presence of highly selective mechanisms for incorporating ncRNA into extracellular vesicles and support a role for ncRNAs as effectors of intercellular signaling (Valadi et al., 2007; Saunderson et al., 2008; Skog et al., 2008). However, the involvement of extracellular RNA during tumor-cell responses to hypoxia is unknown. Thus, the aims of our study were to identify lncRNA that are expressed in response to hypoxia and can modulate cellular and tissue responses to hypoxia, and to evaluate the potential role of extracellular-RNA-mediated intercellular signaling in these responses. Our findings identify a hypoxia-responsive lncRNA signaling module that can contribute to tissue survival responses in human HCC through a mechanism involving the transfer of extracellular RNA across cells.

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RESULTS

The long noncoding RNA regulator of reprogramming is aberrantly expressed in HCC cells and its expression is regulated by hypoxia

To identify lncRNAs of which the expression is deregulated in malignant hepatocytes, we examined the expression profiles of several known lncRNA in non-malignant hepatocytes and malignant (HepG2) cells using real-time (RT)-PCR-based assays. We identified several lncRNAs with a >2-fold alteration in expression in malignant cells, compared with non-malignant hepatocytes (Fig. 1A). Next, we evaluated the effect of hypoxia on the expression of lncRNA. Compared with control HepG2 cells incubated under normoxia, we identified 20 lncRNAs of which the expression increased by >2-fold during hypoxia (Fig. 1B). These included seven lncRNAs of which the expression was deregulated in malignant cells. Amongst the most substantially altered lncRNA in this group was the long intergenic noncoding RNA, regulator of reprogramming (linc-RoR), and we verified these observations in other tumor cell lines. We first examined the expression of linc-RoR in other malignant hepatocytes and identified that linc-RoR expression was altered by 1.7- to 4.7-fold in several different HCC cell lines (Fig. 2A). Next, we examined linc-RoR in other tumor cells during hypoxia and identified an increase in the levels of linc-RoR in Hep3B and PLC-PRF5 HCC cells, and MzChA-1 gallbladder cancer cells during hypoxia (Fig. 2B). We then examined these responses in PLC-PRF5 tumor cell xenografts *in vivo*. We identified hypoxic areas in tumor tissue by immunostaining for pimonidazole adducts and examined tissue expression of linc-RoR by *in situ* hybridization. Hypoxic areas were mainly located adjacent to areas of necrosis in the middle of the tumors. Illustrative sections are shown in Fig. 3A–D. The expression of linc-RoR was scored in hypoxic or non-hypoxic regions. Consistent with our observations that linc-RoR expression was regulated by hypoxia *in vitro*, we observed an increase in the number of linc-RoR-positive cells in hypoxic areas compared with non-hypoxic areas *in vivo* (Fig. 3E).

Knockdown of linc-RoR decreases cell viability of HCC cells during hypoxia

Having identified linc-RoR as a hypoxia-responsive lncRNA, we examined the functional contribution of this lncRNA to cellular responses to hypoxia. Exposure of HepG2 cells to a hypoxic environment did not alter cell viability for up to 48 hours (Fig. 4A). Small interfering RNA (siRNA) to linc-RoR were designed and their effect on linc-RoR was confirmed by qPCR (Fig. 4B). Cells transfected with siRNA to decrease linc-RoR expression were susceptible to hypoxia, with reduced viability in hypoxia but not during normoxia (Fig. 4C,D). Thus, linc-RoR is a hypoxia-responsive lncRNA that is aberrantly expressed in tumor cells that can modulate susceptibility and cell survival in response to hypoxia.

Linc-RoR modulates expression of HIF-1α

We next examined the effect of linc-RoR on hypoxia-inducible factor 1 α (HIF-1α), a well-established and crucial mediator of cellular responses to hypoxia. First, we assessed expression of HIF-1α in HepG2 cells after transfection with either siRNA to linc-RoR or nontargeting control siRNAs. A reduction in HIF-1α expression was observed with knockdown of linc-RoR compared with control, and this effect was more pronounced under hypoxic conditions (Fig. 5A). Consistent with these changes, the expression of pyruvate dehydrogenase kinase isozyme 1 (PDK1), a HIF-1α responsive protein, was also decreased in HepG2 cells after knockdown of linc-RoR (Fig. 5B). These observations identify the HIF-1α–PDK1 pathway as a downstream target of linc-RoR. We examined the effect of overexpression of HIF-1α on the viability of HepG2 cells following incubation with linc-RoR siRNA and observed a marked, but not complete, effect on the loss of viability (data not shown). HIF-1α has an established and well-characterized

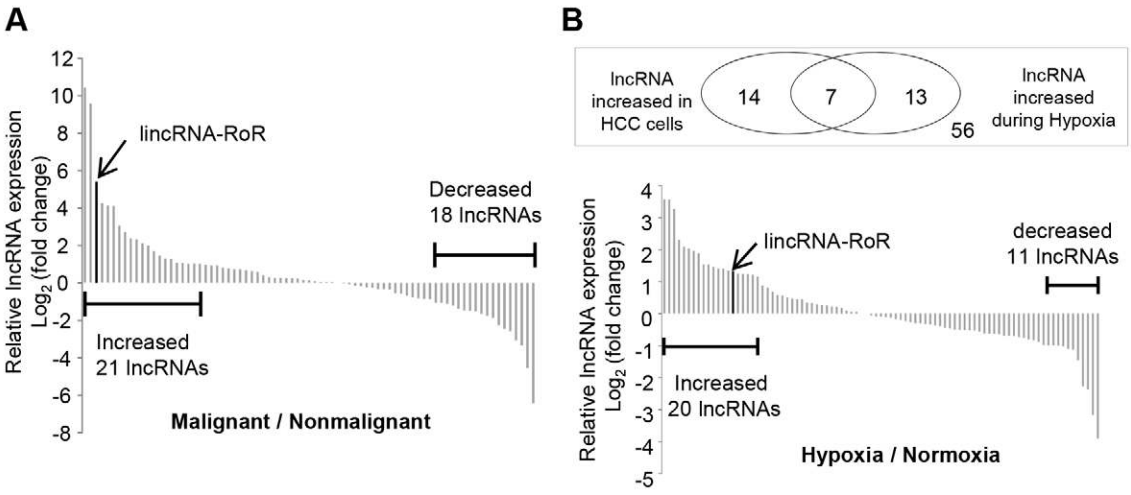

Fig. 1. Expression of lncRNA in HepG2 cells. (A) LncRNA expression was assessed by RT-PCR in malignant HepG2 cells and non-malignant human hepatocytes. Each analysis was performed on three independent samples for each lncRNA. Each bar represents the relative expression for an individual lncRNA. Deregulated lncRNA expression, with a >2-fold change in malignant cells compared to non-malignant cells, was identified for 39 lncRNAs. (B) LncRNA expression was examined in HepG2 HCC cells under conditions of hypoxia or normoxia. 89 lncRNAs were identified in HepG2 cells, of which 20 lncRNA were increased by a >2-fold change under hypoxia conditions. Seven of these lncRNAs, including linc-RoR, were also increased in expression in malignant cells. The Venn diagram depicts the number of individual lncRNAs that were increased in HepG2 cells compared to non-malignant hepatocytes, or in response to hypoxia compared to normoxia.

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

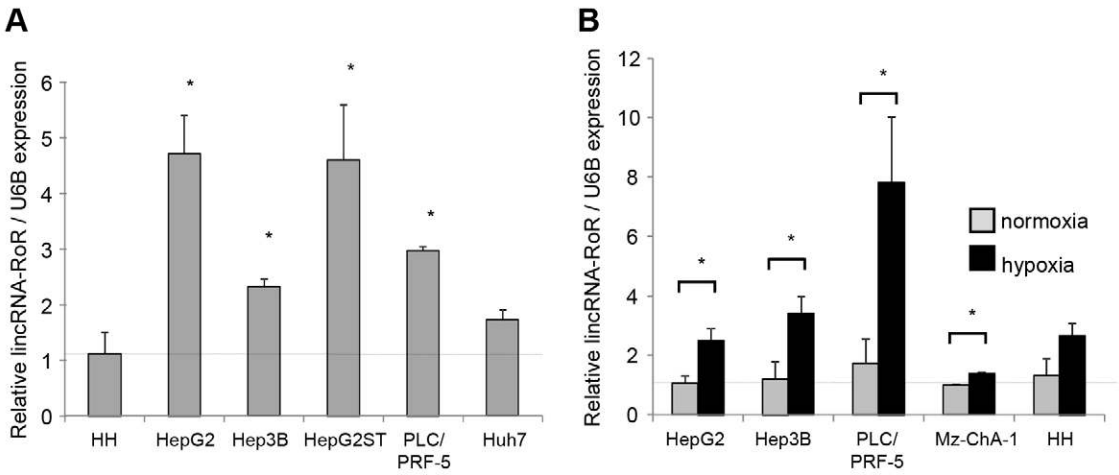

Fig. 2. Expression of linc-RoR expression in human HCC cells during hypoxia. RNA was extracted and RT-PCR performed, as described in the Materials and Methods section. (A) Basal expression level of linc-RoR in non-malignant human hepatocytes (HHs) and HCC cell lines. Expression of linc-RoR was normalized to expression of RNU6B and expressed relative to expression in HHs. (B) Linc-RoR expression was assessed in malignant liver cancer cell lines and HH cells incubated under hypoxia or normoxia for 24 hours. Expression of linc-RoR was normalized to RNU6B expression and expressed relative to the value in normoxia. Ct values of RNU6B were similar across samples and not altered during hypoxia. Bars represent the mean±s.e.m. of three separate studies. *$P<0.05$.

involvement in cell signaling during hypoxia. These findings therefore identify a mechanism by which linc-RoR can modulate cellular responses during hypoxic stress through modulation of HIF-1α and its downstream targets.

Linc-RoR modulates expression of miR-145 in HCC cells

The mechanisms by which lncRNA can modulate the expression of target genes are very diverse. We next sought to identify mechanisms by which linc-RoR knockdown could decrease HIF-1α expression. A marked reduction in HIF-1α mRNA was observed in cells transfected with siRNA to linc-RoR, compared with controls, during hypoxia (Fig. 6A). Analysis of the 3′-UTR of HIF-1α mRNA did not identify any regions with a greater than 7-nucleotide-sequence identity to linc-RoR. Because of the lack of regions with sequence complementarity to linc-RoR, it is unlikely that linc-RoR interacts directly with HIF-1α mRNA to modulate transcript levels. Linc-RoR has been shown to function as an endogenous microRNA-145 (miR-145) sponge and to limit increases in miR-145 expression in self-renewing human embryonic stem cells (Wang et al., 2013). Furthermore, miR-145 has been reported to downregulate expression of HIF-1α by targeting p70S6K1 (RPS6KB1), a kinase that phosphorylates the S6 ribosomal protein to induce protein synthesis at the ribosome (Xu et al., 2012). Indeed, we observed that siRNA to linc-RoR increased miR-145 expression in HCC cells during hypoxia (Fig. 6B) and normoxia (Fig. 6C). Changes in the relative levels of miR-145 expression were more pronounced during hypoxia than normoxia with a mean (±s.e.m.) relative expression of

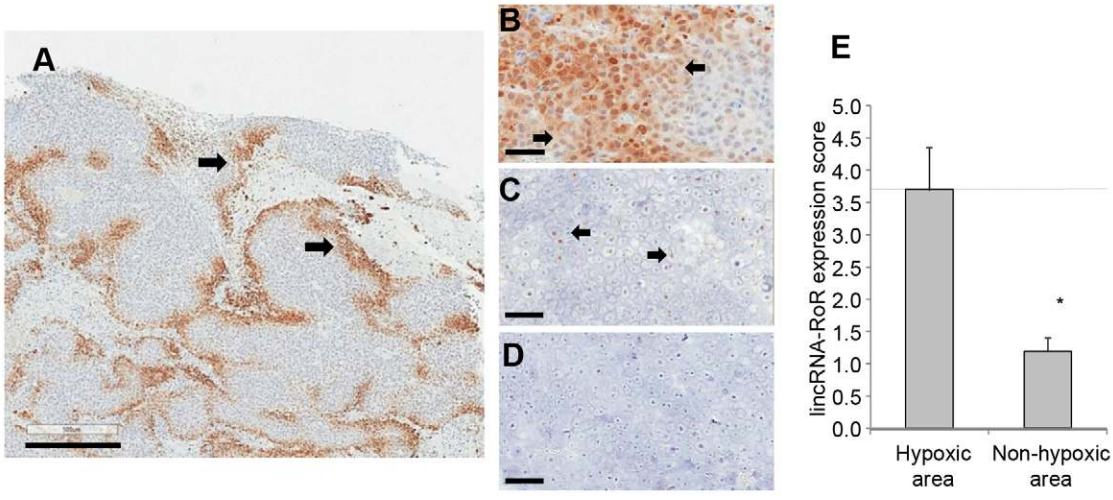

Fig. 3. Linc-RoR is increased in hypoxic areas *in vivo*. HCC tumor cell xenografts were established in athymic mice to examine the expression of linc-RoR *in vivo*. Intratumoral hypoxic areas were identified by immunohistochemistry for Hypoxyprobe-1 (A). Scale bar: 500 μm. Immunohistochemistry for Hypoxyprobe-1 (B), *in situ* hybridization for linc-RoR (C) or *in situ* hybridization for negative control probe (D) in representative high-power fields from adjacent sections. Scale bars: 50 μm. The arrows show Hypoxyprobe-1- or linc-RoR-positive cells. (E) The number of linc-RoR-positive cells was quantified in hypoxic or nonhypoxic areas of tumor tissues. Data represents mean±s.e.m. of the number of positive cells with detectable linc-RoR in ten high-power fields. *$P<0.05$.

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

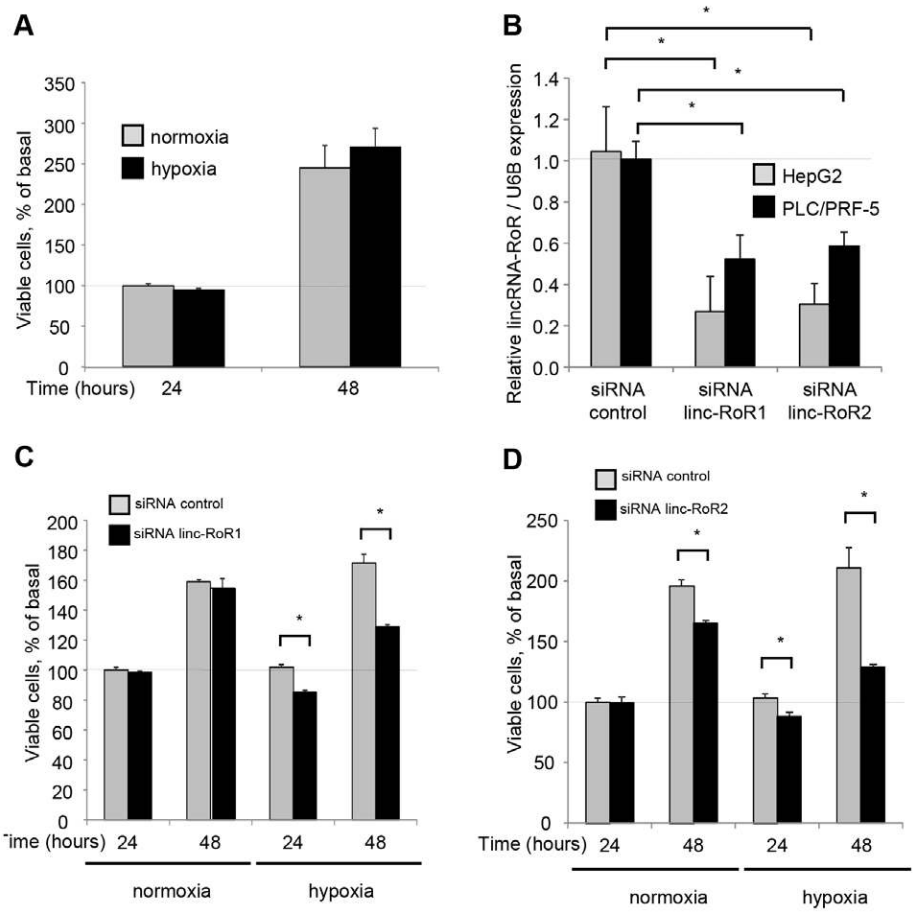

Fig. 4. Knockdown of linc-RoR decreases tumor cell viability during hypoxia. (A) HepG2 cells were seeded (1×10^4/well) into 96-well collagen-coated plates and cultured under conditions of normoxia or hypoxia, and cell viability was assessed after 24 or 48 hours. (B) HepG2 cells or PLC-PRF-5 cells were transfected with two different siRNAs to linc-RoR (1 or 2) or nontargeting control siRNA. After 48 hours, RNA was isolated and qPCR for linc-RoR was performed. (C,D) HepG2 cells were transfected with siRNA 1 or 2 to linc-RoR (C and D, respectively) or nontargeting control. After 24 hours, cells were plated (1×10^4/well) in 96-well plates and cultured under normoxia or hypoxia conditions. Cell viability was assessed after 24 or 48 hours and is expressed relative to controls.

1.43±0.01 compared with 1.26±0.04 (P=0.03). HIF-1α mRNA expression was reduced during normoxia and hypoxia to a similar extent, with a relative expression of 0.44±0.05 in normoxia and 0.52±0.01 in hypoxia (P=0.12). We speculate that the expression of target genes might be sensitive to small changes in regulatory RNA genes, such as noncoding RNA, and that the lack of a difference in the expression of HIF-1α between normoxia and hypoxia reflects a maximal effect of experimental knockdown of linc-RoR. Furthermore, we noted a reduction in the expression of the p70S6K1 protein, as well as a decrease in constitutive phosphorylation of the active site of p70S6K1 after linc-RoR knockdown (Fig. 6D,E). Thus, linc-RoR could alter the expression of HIF-1α mRNA by sequestering and modulating the expression of miR-145. Furthermore, concomitant overexpression of HIF-1α and knockdown of linc-RoR had a considerable, but not complete, effect on the recovery of cell viability, suggesting that these effects are mediated, in part, through HIF-1α (Fig. 6F).

Linc-RoR modulates miR-145 and HIF-1α expression and downstream signaling *in vivo*

We next examined the effect of linc-RoR on the growth of tumor-cell xenografts *in vivo*. Tumor cells were subcutaneously injected

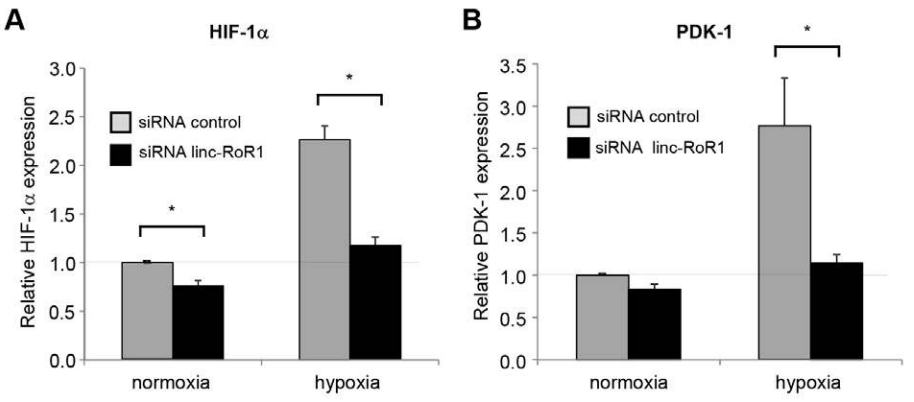

Fig. 5. Knockdown of linc-RoR decreases HIF-1α and PDK1 expression. HepG2 cells were transfected using siRNA 1 to linc-RoR or 'ontargeting' control. After 48 hours, cells were plated (1×10^4/well) on 96-well amine-coated plates and incubated under normoxia or hypoxia conditions. After 48 hours of incubation, quantitative immunocytochemistry for (A) HIF-1α and (B) PDK1 were performed using a HIF-1α + PDK1 Hypoxia Human In-Cell ELISA kit (Abcam) and imaged using a LI-COR Odyssey system. Expression of HIF-1α and PDK1 were normalized to the corresponding Janus Green fluorescence for each well.

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

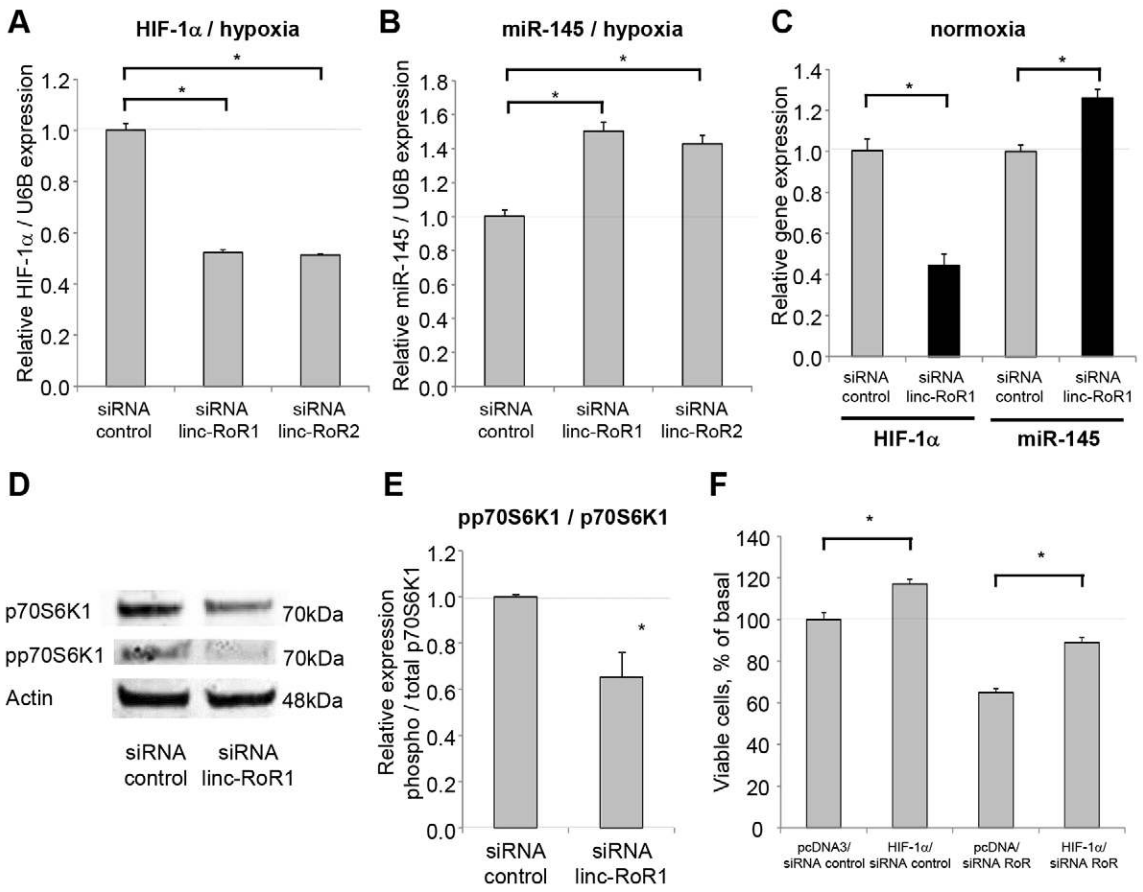

Fig. 6. Linc-RoR modulates expression of miR-145 and downstream signaling. (A–C) HepG2 cells were transfected with two different siRNAs to linc-RoR (1 or 2) or nontargeting control as indicated, and cultured under hypoxia (A,B) or normoxia (C). After 48 hours, RNA was isolated and RT-PCR for (A,C) HIF-1α or (B,C) miR-145 was performed. (D,E) HepG2 cells were transfected with siRNA 1 to linc-RoR, or nontargeting control. After 48 hours, cells were lysed and immunoblot analysis was performed using specific antibodies against p70S6K1 and phospho-p70S6K1 (pp70S6K1). A representative immunoblot (D) and quantitative densitometric data (E) of the ratio of phosphorylated to total p70S6K1, from three independent experiments, are shown. (F) HepG2 cells were transfected with either HA-HIF-1α-pcDNA3 or pcDNA3 vector and, 24 hours later, cells were transfected with siRNA no. 1 to linc-RoR, or nontargeting control. After 24 hours, cells were seeded (1×10^4/well) into 96-well plates and cultured under hypoxia conditions; cell viability was assessed after 72 hours.

following *ex vivo* transfection with siRNA to linc-RoR or control siRNA (Fig. 7A). Intriguingly, tumor growth was reduced (Fig. 7B) and a sustained reduction in the expression of linc-RoR was observed after linc-RoR knockdown (Fig. 7C). These results indicate an important contribution of linc-RoR to tumor growth *in vivo*. In tumoral tissues, miR-145 expression was increased whereas HIF-1α mRNA expression was decreased (Fig. 7D,E). A decrease in constitutive active-site phosphorylation of p70S6K1 was also detected (Fig. 7F). These results *in vivo* are consistent with those observed *in vitro* and confirm a role for linc-RoR as a modulator of cellular responses to hypoxia.

Extracellular transfer of lncRNA can modulate responses to hypoxia

We have recently reported a mechanism by which HCC cells can modulate cellular responses in neighboring cells through extracellular-vesicle-mediated transfer of noncoding RNA. To explore the potential of linc-RoR to act as a signaling mediator during hypoxia, and to evaluate the contribution of this intercellular signaling paradigm on cellular responses to hypoxia, we first evaluated the effect of extracellular RNA (exRNA), released from cells, on cell viability during hypoxia. exRNA was isolated from preparations of extracellular vesicles (EVs) obtained by ultracentrifugation, as described in the Materials and Methods section. In studies using density gradient centrifugation of EV preparations, we found ~16–20% of total RNA was present in fractions that did not contain quantifiable vesicles as determined by NanoSight analysis. Cellular uptake of RNA within vesicles, with subsequent effects on intercellular processes, has been demonstrated in HCC and several other cell types but similar data for cellular uptake, and functional effects, of extracellular non-vesicular RNA is lacking. Nevertheless, the possibility remains that non-vesicular RNA released within the extracellular milieu could be involved in the observed effects. Incubation of exRNA, obtained from cells during hypoxia, enhanced the survival of tumor cells under hypoxia (Fig. 8A). It is possible that the effects of extracellular linc-RoR could have been influenced by the siRNA in the target cells and diminished the effects on the recovery of cell viability that were observed. Expression profiling of lncRNAs within tumor-cell-derived exRNA identified linc-RoR as amongst the most highly enriched lncRNA within these preparations (Fig. 8B). Moreover, the content of linc-RoR was also increased in exRNA obtained from tumor cells undergoing hypoxia (Fig. 8C). For these studies, we used the same total amount of exRNA for each experiment because the amount of exRNA that was obtained from cells under

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

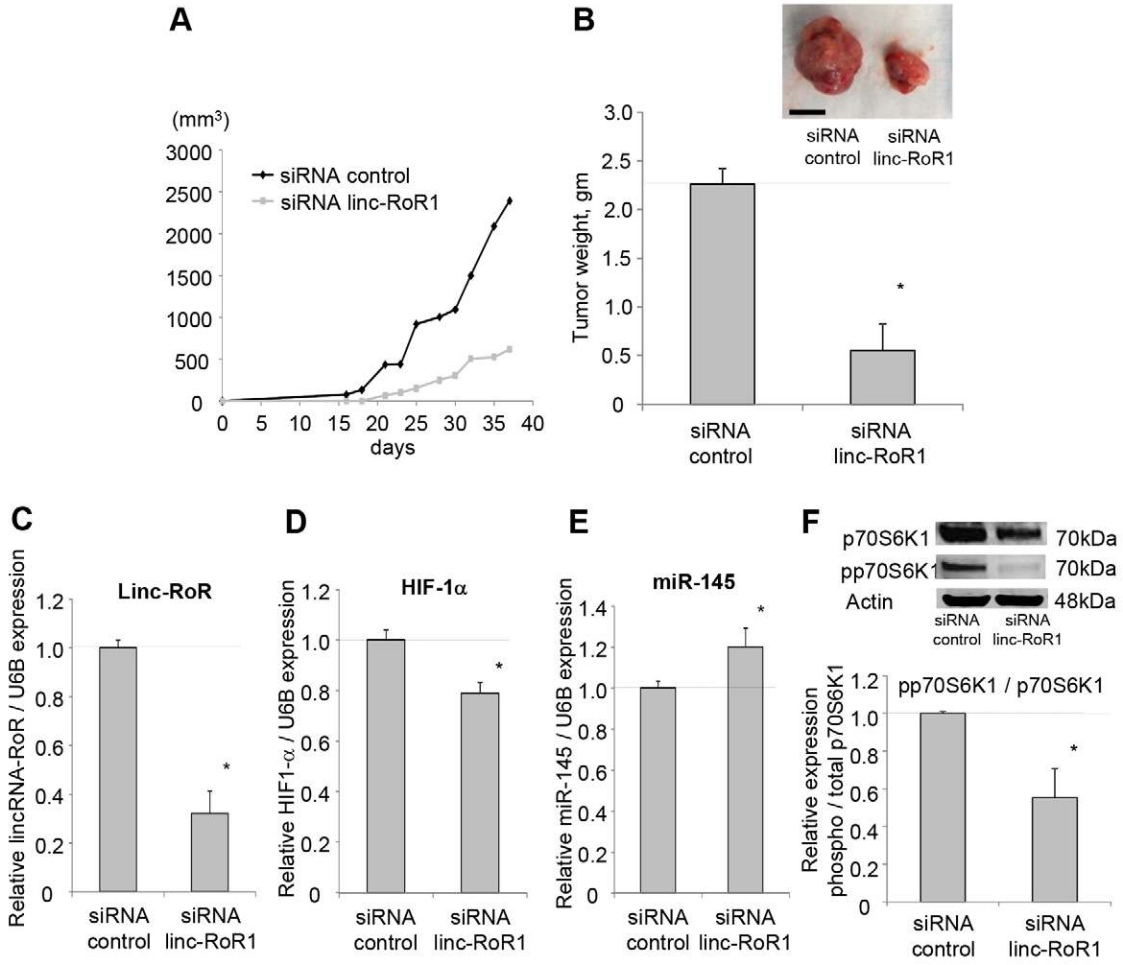

Fig. 7. Effect of linc-RoR knockdown in tumor xenografts *in vivo*. Xenograft tumors were established following *ex vivo* transfection of PLC-PRF-5 cells with siRNA 1 to linc-RoR or control siRNA as described in the Materials and Methods section. (A) Tumor volume was estimated at the indicated time-points. Data represent the average of estimated tumor volume from three separate xenografts. (B) Tumors were excised at 6 weeks after implantation. The bars represent average and standard deviation of xenograft tumor weight. Scale bar: 10 mm. (C–E) RNA was isolated from xenograft tumors and PCR was performed for (C) linc-RoR, (D) HIF-1α mRNA or (E) miR-145. (F) Immunoblot analysis from tumor lysates was performed using specific antibodies against p70S6K1 or phospho-p70S6K1. A representative immunoblot and quantitative densitometric data showing the ratio of phosphorylated to total p70S6K1 from three separate tumors is shown.

normoxia or hypoxia condition varied with cell type and duration. For HepG2 cells, an average of 303.0 ng/10^6 cells was obtained during hypoxia, whereas 290.1 ng/10^6 cells was obtained during normoxia. For normalization, we used median normalization across all samples for the data shown in Fig. 8B. We analyzed several potential normalization controls in both donor cell lines and extracellular RNA from these cells, and chose RNU6B on the basis of consistency across experiments and low variance (Table 1). HIF-1α protein expression was increased in a concentration-dependent manner in recipient cells incubated with EV isolated from HepG2 cells. A greater increase in HIF-1α was observed with EVs obtained from cells during hypoxia compared to cells during normoxia (Fig. 8D). Cells incubated with EVs obtained from cells cultured under conditions of normoxia demonstrated a concentration-dependent increase in HIF-1α mRNA and a decrease in the expression of miR-145 (Fig. 8E). To verify that the increased linc-RoR within exRNA could sequester miR-145, we quantified miR-145 transcript levels within exRNA following linc-RoR knockdown. Because the concentrations of this miRNA were beyond the sensitivity of detection using RT-PCR, and a suitable endogenous invariant control was lacking, we performed absolute quantification using digital PCR (Fig. 8F). An increase in extracellular miR-145 content was observed in exRNA from cells in which linc-RoR had been knocked down. In addition, we performed loss-of-function studies in which exRNA obtained from cells transfected with siRNA to linc-RoR (or controls), was shown to reduce linc-RoR, as well as HIF-1α, in recipient cells (Fig. 8G). Taken together, these studies indicate that intercellular responses to hypoxia can be modulated through the transfer of extracellular linc-RoR across cells.

DISCUSSION

The mechanisms by which tumor cells respond and adapt to hypoxic stress are important in cancer pathobiology, and, in particular for tumors, such as HCC, for which therapeutic strategies involving arterial embolization are frequently used. Linc-RoR was identified as a hypoxia-responsive lncRNA that is aberrantly expressed in malignant hepatocytes *in vitro* and *in*

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

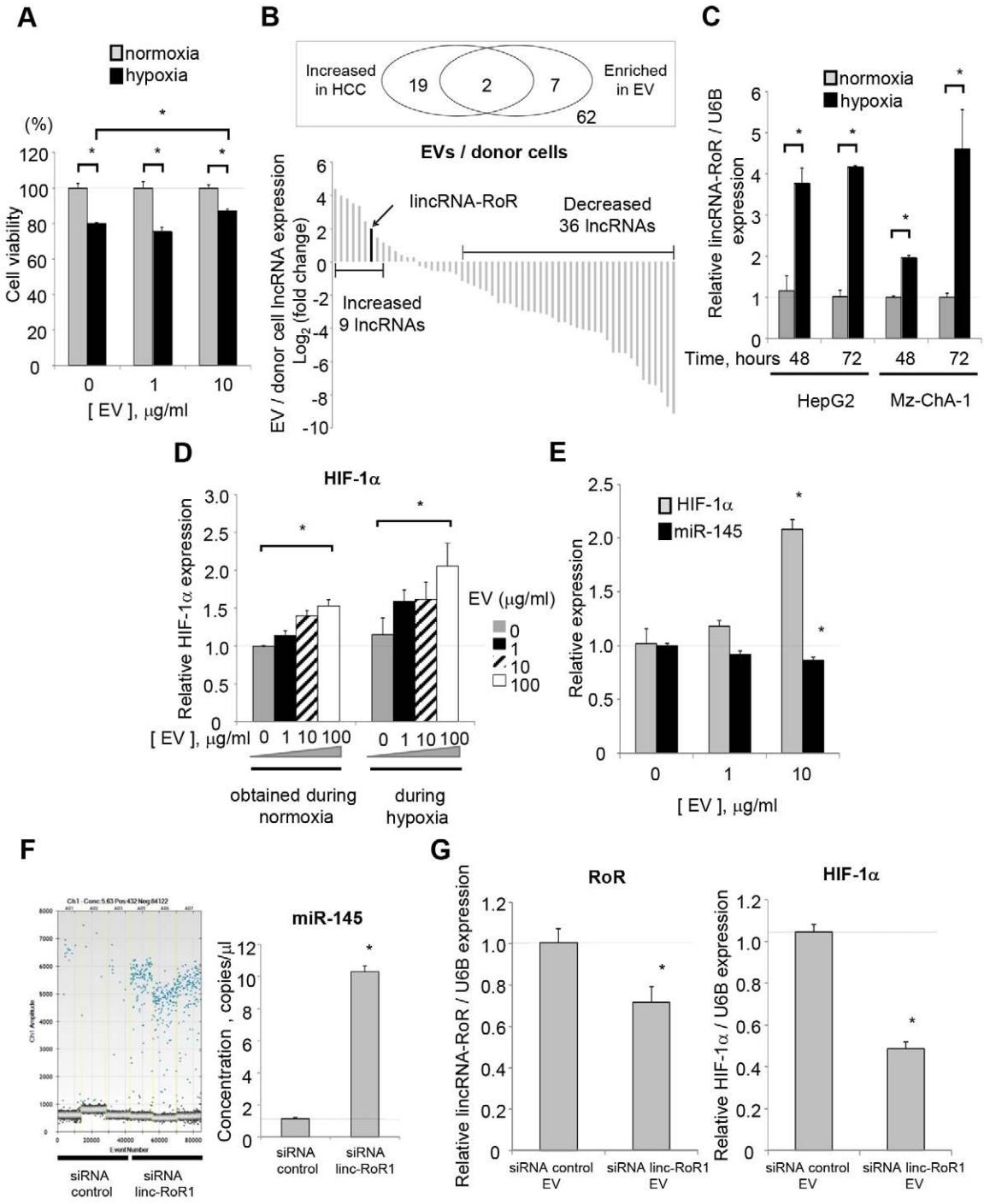

Fig. 8. Extracellular linc-RoR during tumor cell responses to hypoxia. (A) HepG2 cells were transfected with siRNA 1 to linc-RoR. After 48 hours, cells were plated (1×10^4/well) into 96-well plates in vesicle-depleted medium and incubated with varying concentrations of EVs under conditions of normoxia or hypoxia. Cell viability was assessed after 48 hours. (B) The expression of lncRNA, within EV preparations, released by HepG2 tumor cells from three independent replicates was assessed using LncProfiler™ qPCR Array Kit. Each bar represents the relative expression of extracellular RNA and donor-cell RNA for an individual lncRNA. Nine lncRNAs, including linc-RoR, were predominantly expressed in extracellular-RNA isolations compared to their donor cells. (C) Tumor cells were incubated under hypoxia or normoxia conditions, and extracellular RNA released by these cells was obtained after 48 or 72 hours. qRT-PCR for linc-RoR was then performed. (D) HepG2 cells were plated (1×10^4/well) on 96-well amine-coated plates in vesicle-depleted medium and incubated with varying concentrations of EVs that had been derived from HepG2 cells under normoxia or hypoxia conditions. Recipient cells were then cultured under hypoxia conditions for 48 hours. Quantitative immunocytochemistry for HIF-1α was performed in recipient cells using an in-cell ELISA assay. (E) EVs were isolated from HepG2 cells under normoxia and added to recipient HepG2 cells. After 48 hours incubation under normoxia with those EVs, recipient-cell RNA was isolated and qRT-PCR for HIF-1α or miR-145 was performed. (F,G) HepG2 cells were transfected with siRNA 1 to linc-RoR, or nontargeting control, and cultured under normoxia. (F) After 72 hours, extracellular RNA from HepG2 cells was isolated and droplet digital PCR for miR-145 was performed. The number of positive droplets and concentration of miR-145 from three independent experiments are shown. (G) After 72 hours, EVs were collected from each group, and 10 μg/ml of EV was added to recipient HepG2 cells. After 48 hours incubation under normoxia, recipient-cell RNA was isolated and RT-PCR for linc-RoR or HIF-1α was performed.

Journal of Cell Science (2014) 127, 1585–1594 doi:10.1242/jcs.141069

Table 1. Expression of selected genes in donor cells and exRNA derived from these cells

	exRNA		Cells	
	Average Ct	Variance	Average Ct	Variance
18S rRNA	27.33	2.74	17.66	8.61
RNU43 (snoRNA)	34.62	50.06	25.88	8.54
GAPDH	36.88	1.25	20.79	5.87
RNU6B	33.37	1.41	23.15	4.27

qPCR was used to determine the level of gene expression. The average Ct value and variance across four independent samples are shown for each.

vivo. Linc-RoR is enriched within the extracellular RNA that is released by tumor cells during hypoxia and can modulate cellular signaling and cell survival in recipient cells. This lncRNA is a large intergenic noncoding RNA (lincRNAs) that is associated with epigenetic regulators (Boyer et al., 2006; Rinn et al., 2007; Nagano et al., 2008; Zhao et al., 2008) and involved in pluripotency and lineage commitment (Lee et al., 2006). A functional role of linc-RoR in maintaining pluripotency and modulation of cell reprogramming in induced human pluripotent stem cells (iPSCs) has been recognized (Loewer et al., 2010). Our observations support a much broader role for this lncRNA as a stress-responsive lncRNA, and furthermore, identify a mechanistic pathway by which linc-RoR can coordinate responses between cells and their local environment. In support of such a role, linc-RoR has been shown to promote survival of iPSCs and embryonic stem cells by preventing the activation of cellular stress pathways, such as the p53 response (Loewer et al., 2010). Further studies to examine the roles of this lncRNA in tumor genesis and behavior are therefore warranted as they might provide unique targets for therapeutic intervention.

A novel observation of these studies is the recognition that lncRNA, like mRNA and miRNA, can function as modulators of cellular responses. Diverse mechanisms have been reported by which lncRNA can epigenetically modulate gene expression, such as chromatin remodeling and modulation of transcription and translation. In the case of linc-RoR, a potential mechanism of action involves lncRNA–miRNA interactions to modulate miRNA-dependent effects. Linc-RoR functions as a miRNA sponge to limit miRNAs, such as miR-145, that can modulate the expression of key effectors of the hypoxia response, such as HIF-1α expression. Knockdown of linc-RoR significantly decreased HIF-1α expression, as well as expression of PDK1, especially under hypoxia stress. Levels of HIF-1α are generally increased in aggressive tumors (Keith et al., 2012) and could be useful as a molecular predictor of poor prognosis and treatment response in HCC (Xiang et al., 2011; Nath and Szabo, 2012; Zheng et al., 2013). HIF-1α contributes to the acute hypoxic response and can promote the expression of several hypoxia-inducible genes that are associated with diverse cellular processes, such as angiogenesis, cell growth, differentiation, survival and apoptosis (Nath and Szabo, 2012). Amongst the many genes that can be regulated by HIF-1α are vascular endothelial growth factor and transforming growth factor β, both of which have been implicated in the progression of HCC. PDK1, a HIF-1α target gene, is a hypoxia-responsive protein that regulates mitochondrial function during hypoxia by reducing pyruvate entry into the tricarboxylic acid cycle (Papandreou et al., 2006; Majmundar et al., 2010). Further studies to examine the contribution of the linc-RoR–miR-145–HIF-1α axis in modulating cell metabolism, during cell stress, are warranted.

The potential for liver cancer cells themselves to exert paracrine, or even autocrine, effects, mediated by functional lncRNA or other noncoding RNA within extracellular vesicles, provides a unique mechanism by which cells undergoing stress can signal to other cells within the local hepatic microenvironment. Our studies have focused on a single lncRNA mediator of intercellular responses to hypoxia; however, extracellular vesicles can contain several different proteins or genes that could, in combination, modulate diverse cellular effects. Thus, other contents of extracellular vesicles could potentially contribute to the observed differences in the magnitude of changes in HIF-1α expression in response to EV, when compared with changes in response to siRNA to linc-RoR. As previously noted, the possibility remains that non-vesicular RNA released within the extracellular milieu could contribute to the observed effects. An understanding of crucial mediators, and the processes by which these are mobilized for cell-to-cell communication, is necessary in order to understand tissue homeostasis and responses, and their contribution to tumor behavior. Such responses might also be crucial determinants of tissue adaptation and survival in normal physiological processes. In contrast to the large amount of information regarding intracellular events, signaling pathways, intermediates and responses to hypoxia, the mechanisms and contribution of intercellular signaling to normal, or transformed epithelia, undergoing cellular stress are poorly understood. In addition to the implications for liver, or other, cancers, the mechanisms described herein will therefore have broader relevance to other pathophysiological conditions affecting the liver.

MATERIALS AND METHODS

Cell lines, culture and reagents

Human HCC cell lines HepG2, Hep3B, PLC-PRF5 and Huh-7 were obtained from the American Type Culture Collection (Manassas, VA), and HepG2.ST was derived from HepG2 cells by spontaneous transformation (Kogure et al., 2013). Cells were cultured in Dulbecco's modified Eagle's high-glucose medium (HyClone, Logan, UT), containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY), at 37℃ under 5% CO_2. Non-malignant human hepatocytes (HH) were obtained from Sciencell (Carlsbad, CA) and cultured as recommended by the supplier. Human cholangiocarcinoma (CCA) cell line Mz-ChA-1 was cultured in Connaught Medical Research Laboratories medium 1066 (Life Technologies) with 10% FBS, 1% L-glutamine and 1% antibiotic-antimycotic mix. For studies of enforced HIF-1α expression, cells were passaged in 6-well plates and transfected with 8 µg HA-HIF-1α-pcDNA3, a pcDNA3 vector encoding haemagluttinin-tagged HIF-1α (Addgene, Cambridge, MA) or pcDNA3 $3.1^{(+)}$ vector (Life Technologies, Grand Island, NY) using Lipofectamine 2000 for 24 hours before further experiments. For all studies related to extracellular vesicles, vesicle-depleted medium was prepared by centrifuging cell-culture medium at 100,000 ***g*** overnight to spin down any pre-existing vesicle content. Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO), and Sorafenib was obtained from Selleck (Houston, TX). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and diluted with culture medium to the desired concentration with a final DMSO concentration of 0.1%. DMSO 0.1% (v/v) was used as a solvent control. For hypoxia studies, cells were cultured in 10-cm dishes or 96-well culture plates at 37℃ in a hypoxia chamber (Billups-Rothenburg, Del Mar, CA); left open in a humidified incubator in 21% O_2, 5% CO_2, balance N_2 (for normoxia studies); or gassed with a pre-analyzed gas mixture containing 5% CO_2 and 95% N_2 and then sealed (for hypoxia studies).

Cell viability and cell growth assays

For cell viability assays, cells were seeded (1×10^4/well) in collagen-coated 24-well plates. At each time point, trypan-blue staining was performed and the number of viable cells was expressed relative to cell

counts at baseline. For cell proliferation assays, HepG2 cells were seeded (1×10^4/well) into 96-well collagen-coated plates in appropriate media. At selected time-points, proliferation was assessed using MTS solution (Promega, Madison, WI) and a FLUOstar Omega microplate reader (BMG Labtech, Cary, NC). Background correction was performed by subtracting background fluorescence from wells without cells.

Tumor xenografts

All animals received humane care and studies were performed under an institutionally approved animal care protocol. Male athymic nu/nu mice, at 8 weeks of age, were obtained from Jackson Laboratory (Bar Harbor, ME) and fed food and water *ad libitum*. Fluorescent light was controlled to provide alternate light and dark cycles of 12 hours each. Mice were anesthetized under isoflurane (1–2% by inhalation to effect during induction) before subcutaneous injections to either flank with PLC-PRF-5 cells (1×10^6 viable cells) transfected *ex vivo* with siRNA to nontargeting control or linc-RoR ($n=3$ mice each) and suspended in 0.5 ml BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA). Tumor cell xenograft growth was monitored by serial measurements in mm^3 and the volume was estimated using the following equation: tumor volume$=\pi/6\times$[minor axis]$^2\times$major axis. After 6 weeks, mice were euthanized and tumors excised for histologic examination or RNA and protein extraction.

In vivo detection of hypoxia

At 6 weeks after tumor cell implantation, mice received a single intraperitoneal injection of 60 mg/kg of body weight of pimonidazole hydrochloride (Hypoxyprobe, Burlington, MA). After 4 hours, mice were euthanized and tumors excised. Tissue sections were prepared and immunohistochemistry, using Hypoxyprobe-1 monoclonal antibody (Hypoxyprobe, Burlington, MA), was performed to detect pimonidazole adducts, as a marker of hypoxia, and to localize the presence, and extent, of hypoxia *in vivo*.

In situ hybridization

In situ hybridization for linc-RoR was performed using RNAscope® 2.0 formalin-fixed paraffin-embedded assay (Advanced Cell Diagnostics, Hayward, CA). A linc-RoR expression score was calculated as the product of semi-quantitative assessment of the number of positive cells and staining intensity from ten separate high-power fields. The percentage of cells positive for dot clusters was scored accordingly: 0 (none), 1 (1–5%), 2 (6–10%) or 3 (>10%). Staining intensity was scored as 0 (none or less than 1 dot/cell), 1 (1–3 dots/cell), 2 (4–10 dots/cell) and 3 (>10 dots/cell).

Transfection of siRNA

siRNA against linc-RoR: siRNA 1, 5′-GGAGAGGAAGCCTGAGAGT-3′; siRNA-2, 5′-GGTTAAAGACACAGGGGAA-3′ or nontargeting (NT) control siRNA (siGENOME Non-Targeting siRNA) were purchased from Dharmacon (Lafayette, CO). Cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Life Technologies) for 48 hours before further experiments.

Isolation of RNA

Total RNA was extracted from cells or tumor tissue following homogenization using Trizol (Life Technologies) or from extracellular vesicles using ExoQuick-TC (System Biosciences, Mountain View, CA). For the latter, HCC cells (1×10^6) were plated in 11 ml of EV-depleted medium on collagen-coated 10-cm dishes. After 3–4 days, the medium was collected and sequentially centrifuged at 3000 ***g*** for 15 minutes to remove cells and cell debris. The supernatant was transferred to a sterile vessel and combined with 2 ml ExoQuick-TC. After an overnight precipitation at 4℃, total RNA was extracted using SeraMir™ exosome RNA amplification kit (System Biosciences). RNA concentration was measured using NanoDrop ND-2000 (Nano-Drop Technologies, Wilmington, DE).

Real-time PCR analysis

RNA was treated with RNase-free DNase-I (Qiagen, Valencia, CA). A total of 1 µg of RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using a Mx3000p System (Stratagene, La Jolla, CA) or LightCycler® 96 (Roche, Indianapolis, IN) to detect RNU6B, linc-RoR or HIF1α with SYBR green I (SYBR® Advantage® qPCR Premix, Clontech, Mountain View, CA). The following PCR primers were used: linc-RoR forward, 5′-AGGAAGCCTGAGAGTTGGC-3′; reverse, 5′-CTCAGTGGGGAAGA-CTCCAG-3′; HIF-1α, forward, 5′-GGCAGCAACGACACAGAAAC-3′; reverse, 5′-TGATTGAGTGCAGGGTCAGC-3′; RNU6B, forward, 5′-CTCGCTTCGGCAGCACA-3′, reverse, 5′-AACGCTTCACGAAT-TTGCGT-3′. Expression of mature miRNA-145 was assessed using TaqMan human MicroRNA Assay Kit (Applied Biosystem, Foster City, CA) and normalized to that of RNU6B. Expression profiling of lncRNA was performed using the LncProfiler™ qPCR array kit (System Biosciences, Mountain View, CA). RNA from EVs or donor cells ($n=3$ per cell line) were treated with DNase I and 2 µg of DNase-treated-RNA was reverse transcribed. Real-time PCR was performed (2X Maxima® SYBR Green with Rox; Fermentas, Glen Burnie, MD) and the cycle number at which the reaction crossed a threshold (CT) was determined for each gene. Raw CT values were normalized to the median across the array. For each lncRNA, the relative amount in malignant relative to non-malignant cells was determined using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT=\Delta CT_{HCC\ cell}-\Delta CT_{non\text{-}malignant\ hepatocytes}$, and the content in extracellular vesicles relative to donor cells was described using the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT=\Delta CT_{vesicles}-\Delta CT_{donor\ cell}$.

Droplet digital PCR

cDNA was transcribed from RNA treated with RNase-free DNase-I (Qiagen) and RNase inhibitor (10 U/µl) using the iScript cDNA synthesis kit (Bio-Rad) in a 10 µl reaction volume. Droplet digital (dd) PCR reactions were performed using 10 µl ddPCR 2×Master Mix (Bio-Rad, Hercules, CA), 1 µl 20×Primer and TaqMan Probe Mix (Applied Biosystems), 5 µl nuclease-free water and 4 µl reverse-transcribed product per reaction using a QX100 Droplet Digital PCR system (Bio-Rad). Data were analyzed using QuantaSoft software with automatic threshold setting.

HIF1α and PDK1 ELISA assay

HepG2 cells were seeded (1×10^4/well) into 96-well amine-coated plates in appropriate medium. At selected time-points, HIF-1α and PDK1 expression were assessed using Hypoxic Response Human In-Cell ELISA Panel (Abcam, Cambridge, MA) and analyzed using an Odyssey imager (LI-COR, Lincoln, NE). Expression values were obtained by subtracting background values from wells without cells and normalized to the corresponding Janus Green fluorescence values for each well.

Western blotting

Cells were serum-starved for 24 hours and then transfected as described above. After 48 hours, total protein was extracted from cultured cells using cOmplete Lysis-M, EDTA-free and cOmplete Mini, EDTA-free, protease inhibitor cocktail tablet (Roche, Indianapolis, IN). For analysis of xenograft tumors, the tissue was homogenized, and lysates were obtained using cell lysis buffer (Cell Signaling, Danvers, MA). Equivalent amounts of protein sample were mixed with NuPAGE® LDS Sample Buffer (4×) (Life Technologies) and separated on NuPAGE® 4–12% Bis-Tris gel (Life Technologies) and then transferred onto pure nitrocellulose membranes. The membranes were blocked with blocking buffer (LI-COR, Lincoln, NE) for 30 minutes and then incubated overnight at 4℃ with the appropriate primary antibody against the following proteins: rabbit polyclonal against anti-p70S6 kinase α (p70S6K1) (1:500, Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal against phospho-p70S6 Kinase (1:500, Cell Signaling), goat polyclonal against actin (1:5000, Santa Cruz Biotechnology). The membrane was washed three times for 10 minutes with TBST (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% Tween-20) and then incubated with Alexa Fluor® 680 goat anti-rabbit IgG (H&L) (1:5000, Life Technologies) for p70S6K1 and phospho-p70S6K1, and Alexa Fluor® 680 rabbit anti-goat IgG (H&L) (1:5000, Life Technologies) for actin for 20 minutes. Visualization and

quantification of protein expression was performed using the Odyssey imaging system (LI-COR, Lincoln, NE), and expressed relative to that of β-actin.

Isolation of extracellular RNA

Cells (1×10^6) were plated in 11 ml of vesicle-depleted medium on collagen-coated 10-cm dishes. After 3–4 days, the medium was collected and sequential centrifugation was performed. The medium was first centrifuged at 300 ***g*** for 10 minutes, then at 2000 ***g*** for 20 minutes in 4℃ to remove cells and cell debris. The supernatant was then centrifuged at 10,000 ***g*** for 70 minutes at 4℃. The supernatant was further ultracentrifuged at 100,000 ***g*** for 70 minutes at 4℃ to pellet vesicles, which were then washed by resuspending in PBS and ultracentrifuged at 100,000 ***g*** for 70 minutes in 4℃. The final pellet comprised an EV preparation that contained a homogenous population of extracellular vesicles, and was used for isolation of extracellular RNA (exRNA) or other studies, or was re-suspended in 50–100 µl of PBS and stored at −80℃. The size of vesicles was quantified by nanoparticle-tracking analysis using NanoSight LM10 (NanoSight, Amesbury, UK), and the morphology was identified by transmission electron microscopy. Protein content was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL).

Statistical analysis

Data were expressed as the mean±s.e.m. from at least four independent studies, with each containing three replicates unless indicated otherwise. Comparisons between groups were performed using the two-tailed Student's *t*-test, and results were considered to be statistically significant when $P<0.05$.

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Competing interests

The authors declare no competing interests.

Author contributions

K.T. and T.P. designed the project, organized data and wrote the manuscript. K.T. performed the experiments and analyzed data. I.Y. and H.H. performed additional experiments, and reviewed the data and manuscript.

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