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RalA signaling pathway as a therapeutic target in hepatocellular carcinoma (HCC)

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ABSTRACT

Ral (Ras like) leads an important proto-oncogenic signaling pathway down-stream of Ras. In this work, RalA was found to be significantly overactivated in hepatocellular carcinoma (HCC) cells and tissues as compared to non-malignant samples. Other elements of RalA pathway such as RalBP1 and RalGDS were also expressed at higher levels in malignant samples. Inhibition of RalA by gene-specific silencing caused a robust decrease in the viability and invasiveness of HCC cells. Additionally, the use of geranyl–geranyl transferase inhibitor (GGTI, an inhibitor of Ral activation) and Aurora kinase inhibitor II resulted in a significant decrease in the proliferation of HCC cells. Furthermore, RalA activation was found to be at a higher level of activation in HCC stem cells that express CD133. Transgenic mouse model for HCC (FXR-Knockout) also revealed an elevated level of RalA-GTP in the liver tumors as compared to background animals. Finally, subcutaneous mouse model for HCC confirmed effectiveness of inhibition of aurora kinase/RalA pathway in reducing the tumorigenesis of HCC cells in vivo. In conclusion, RalA overactivation is an important determinant of malignant phenotype in differentiated and stem cells of HCC and can be considered as a target for therapeutic intervention.

1. Statement of significance

Hepatocellular carcinoma (HCC) imposes a significant burden on our health system. With lack of effective therapy for this deadly disease, expansion of our knowledge about cell signaling pathways involved is crucially important. In this study, we reveal the RalA signaling pathway as a novel player in biology of HCC and a potential platform development of new therapeutics.

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2. Introduction

As the third leading cause of cancer deaths worldwide and the ninth leading cause of cancer deaths in the United States, liver cancer ranks highly in terms of priority for translational cancer research (Altekruse et al., 2009). Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for about 78% of global HCC cases (Ahmed et al., 2008). However, a series of other major risk factors also contribute significantly to the etiology of HCC including environmental carcinogens such as Aflatoxins, alcoholic/nonalcoholic liver disease, inherited genetic disorders, Wilson's disease, hemochromatosis, tyrosinemia and α-1-antitrypsin deficiency (McGlynn and London, 2005).

In a majority of cases, HCC develops in the background of chronic hepatitis or cirrhosis when continuous inflammation and regeneration of hepatocytes prepares the necessary platform for neoplastic progression. At the molecular level, deregulation of the major signal transduction pathways, including pathways associated with Ras, p53, Wnt/β-catenin, TGFβ and pRB, have been shown to play a role in the development of HCC (Teufel et al., 2007). In another study, increased expression of a cluster of survival genes in tumor tissues was found to be associated with a poor prognosis of HCC patients. These genes include cell cycle regulation, cell proliferation, anti-apoptosis genes as well as histones and hypoxia-inducible factor-1 alpha (HIF-1A) regulators (Lee et al., 2004).

The hallmark of proto-oncogenic activation, *Ras*, is mutated in 30% of HCC cases (Downward, 2003). Therefore investigation about Ras related signaling events in HCC is not only important from a biological point of view, but can also exert direct ramifications on developing novel therapies for this deadly disease.

Ral (Ras-Like) signaling pathway is a Ras down-stream effector (Feig, 2003; Feinstein, 2005) that has not been significantly studied in HCC. Expression levels of RalA was found to be increased in HCC suggesting that it might contribute to the malignant transformation of this disease (Wang et al., 2009). In this work, we introduce the overactivation of RalA signaling pathway in HCC for the first time. Activation of Ral via Ras is regulated by Ral guanine nucleotide dissociation stimulator (RalGDS), one of the several known Ras-regulated guanine nucleotide exchange factors (RasGEFs)(Ferro and Trabalzini, 2010). RalGDS family members, RalGDS, RGL, RGL2/Rlf and RGL3, interact with activated Ras through their Ras-Binding domain (RBD) and can transmit the signal as effectors to other Ras family members, such as Ral (Bodemann and White, 2008). Upon activation of Ral, a number of down-stream effectors such as RalBP1, PLD and CDC42, ZONAB, SEC5 and EXO84 become activated. The activation leads to a series of biological outcomes such as secretory vesicle trafficking through the exocyst, regulation of gene expression and protein translation (Chien and White, 2003).

Aurora kinase A and other yet to be identified kinases phosphorylate Ral while protein phosphatase 2A AB restricts tumor progression mainly by dephosphorylating Ral (Wu et al., 2005). Indeed Aurora kinase A not only promotes RalA activation but also translocation from the plasma membrane and activation of the effector protein RalBP1 (Lim et al., 2009).

In our study RalA was found to be significantly overactive in both HCC cell lines as well as tumor tissues when compared to non-malignant counterparts. Additionally, interrogations of both cell lines and tissues revealed the expression of RalBP1 and RalGDS with more prevalence in cancer samples. One of the most striking differences between cancerous and noncancerous tissues and cell lines in terms of elements involved in Ral signaling pathway was found in the case of Aurora kinases, potential activators for Ral and controllers of mitotic progression. While Aurora kinase was highly activated in HCC, the levels of activation were significantly lower in normal hepatocytes or non-malignant liver tissues. Blockade of Aurora kinase/Ral axis was found in our work to be a potent regimen for inhibiting the growth and invasion of HCC cells in vitro.

The CD133 glycoprotein (prominin1) has been described as a cancer stem cell (CSC) marker in hepatocellular carcinoma (Yin et al., 2007). RalA (along with Ras and phospho-aurora kinase) was found to be more activated in the population of HCC cells enriched for CD133 expression. Finally, while liver tumors from a transgenic mouse model for HCC (*FXR*-knockout) was found to contain high level of RalA activation, in vivo studies using subcutaneous (SC) injection of HCC cells to nude mice revealed the effectiveness of pharmacological blockers of Ral pathway (e.g. geranyl–geranyl transferase inhibitor, GGTI, and aurora-kinase inhibitor II, AKI-II) in inhibiting tumor growth.

3. Results and discussion

RalA induces its signaling pathway once converted to the guanosine triphosphate (GTP)-bound format. Activation of Ral is induced by Ras and Aurora kinases; however, PP2A can negatively regulate RalA activation. Upon RalA activation the signaling cascade continues by involvement of CDC42 and RalBP1 (Feig, 2003) (Figure 1A). In order to study the biological role of Ral signaling in HCC, we have evaluated the levels of active Ral (RalA-GTP) in HCC cell lines in comparison to their levels in primary fetal human hepatocytes (feHH). While RalA- was found to be activated in the majority of HCC cells (SNU449, SNU475, Hep3B2, HepG2, HuH7, Ph5Ch8 and SNU398), feHH cells contained significantly lower RalA-GTP levels (Figure 1B). Figure 1C shows the results of evaluating the band intensity for Ral-GTP and total Ral for the average of HCC cells in comparison to feHH. Once the average intensity for RalA-GTP in all HCC cell lines is considered as 100, the levels of Ral-GTP in feHH cells falls below 20 (relative luminosity). While Ral seems to be overactivated in all HCC cells, the levels of Ras-GTP(active Ras) is elevated in five out of seven HCC cell lines (SNU475, Hep3B2, HuH7, Ph5Ch8 and SNU398) as compared to feHH. Negative regulators of Ral, i.e. PP2A, were found to be equally expressed in malignant and non-malignant cells. Interestingly, Aurora kinases seem to be primarily expressed/activated only in cancerous cell lines. This is strengthened by our observation about lack of expression of RalGDS in feHH as compared to HCC cell lines. These findings point towards an existence of an overactivation pattern for certain components of Ral signaling pathway including RalA, Aurora kinases and RalGDS in HCC. One of the

parameters involving Ral overactivation is the Aurora kinase. Our cell line studies correlate the Ral overactivation observed in HCC cell lines with Aurora kinase phosphorylation. However, within the HCC cells lines, the levels of activated Aurora kinase seemed to be equivalent. Additionally, the lack of Aurora kinase expression in feHH would introduce this enzyme as a potential candidate for further therapeutic evaluations. In the same manner, lack of expression of RalGDS in feHHs would contribute to the lower levels of Ral activation in these cells in response to Ras.

In our next step, tissue interrogations also revealed enhanced levels of RalA-GTP as compared to the non-malignant cases (Figure 1D–E). And, Once again, the levels of phosphorylated and total Aurora kinases were elevated in 60% (6/10) of malignant tissues, while only 30% (3/10) of cases in normal tissues showed detectable levels of P-Aurora kinase. Average P-Aurora kinase band intensity for HCC tissues was close to five folds higher than non-malignant tissues while the same parameter for RalA-GTP was about 35% higher in HCC tissues as compared to non-malignant tissues (Figure 1E). The increased levels of RalBP1 and RalGDS expression was also observed in HCC tissues in resemblance to the HCC cell lines.

If RalA contributes significantly to the biology of HCC cells, its silencing should influence the important characteristics of these cells, such as their viability and invasiveness. In order to test this, we infected HuH7 HCC cells with a lentivirus expressing anti-RalA shRNA (referred to as anti-RalA virus) or a negative control virus (void of any anti-RalA element) both at 10 colony forming unit (cfu)/cell and measured the cell viability up to day 12 post-infection (Figure 2A). Such treatment was successful in reducing the RalA levels in a meaningful manner by day 6 post-infection (Figure 2B, protein concentrations are normalized for each time-point independent of other time-points). Under such conditions, a statistically significant decrease was observed in cell proliferation as of the seventh day forward; by day 12 post-infection, almost all cells treated with anti-RalA lentivirus were eliminated (proliferation is graphed as a percentage of control at each day). A similar reduction was also observed in the invasiveness of HCC cells at day 2 after treating these cells with anti-RalA virus and introducing them to the invasiveness assay. In Figure 2C, blue staining of the nucleus by DAPI visualizes the fraction of cells that have invaded through a layer of matrigel after being exposed to anti-RalA or negative control lentiviruses. As is seen in this figure, a reduction of more than 50% is observed in cells which were exposed to anti-RalA lentivirus. Also, it is important to note that the reduction in invasion is happening at a time point when no statistically significant change in cell viability was observed. Therefore, Ral activation might directly influence the machinery in charge of invasiveness and the underlying signaling mechanism. Such observation is also in harmony with our previous studies in other tumor models (Bodempudi et al., 2009).

On the basis of our observations about increased levels of activation for RalA and Aurora kinase, our next task was to investigate if pharmacological inhibition of these two proteins would alter the viability of HCC cells. The activity of Aurora kinases can be blocked by using Aurora kinase Inhibitor II (AKI-II) (Heron et al., 2006). This compound is a cell-permeable anilinoquinazoline that acts as a potent, selective, and ATP-competitive inhibitor of Aurora kinases (Heron et al., 2006). Once tested on SNU398 HCC cells at 10 μM concentration, a cytostatic effect was seen on these cells at 24 h post-exposure; the number of cells compared to the control (DMSO) was reduced significantly but the majority of cells stayed alive (Figure 2F, left panel). However, upon prolongation of the cells' exposure time to the inhibitor from 1 day to 2–3 days (Figure 2D), or a modest increase in the dosage of AKI-II to 20 μM (Figure 2E), a significant decrease was observed in the proliferation rate of HCC cells up to 3 days post-treatment. In the next step we used GGTI-2133 (Chiba et al., 2009) in order to inhibit RalA activation. Although there are no specific Ral inhibitors, we have shown before that inhibition of geranyl-geranylation by GGTIs can result in reduction of Ral activity (Bodempudi et al., 2009). Once again, upon exposure to GGTI-2133, a significant reduction was observed in the proliferation capabilities of HCC cells (Figure 2F, right panel). The middle panel in Figure 2F confirms the effects of AKI-II and GGTI-2133 on RalA-GTP levels as well as the effects of AKI-II on phospho-Histone (a known target for Aurora kinases) as the proof for effectiveness for these inhibitors at concentrations used.

Therefore, targeting RalA pathway at the level of RalA with gene therapy (anti-RalA lentivirus) resulted in a significant reduction in proliferation and invasiveness of HCC cells. Targeting RalA with drug therapy (GGTI 2133 and AKI-II) resulted in a significant reduction in proliferation. These observations are not only important from the biological point of view, as they reveal the outcome of blocking Ral signaling pathway on the malignant phenotype of HCC cells, but are also significant in terms of divulging elements of Ral signaling as potential therapeutic targets in HCC. Additionally, observations by other groups about the outcome of inhibition of Aurora kinase-B in HCC (Jeng et al., 2004) and its predictive value in aggressive recurrence of HCC after hepatectomy (Tanaka et al., 2008) is better understood in light of placement of Aurora kinase as a signaling element upstream Ral pathway. The possibility of a combination therapy for enhanced inhibition of multiple elements in Ras and Ral pathway along with inhibition of Aurora kinases is another logical extension of these data that can be pursued in future studies.

We were also interested to evaluate the activation levels of Ral in the cancer stem cell (CSC) fraction of HCC cells. On a theoretical basis, the architecture of each tumor shelters a fraction of "multi-potential" cells which are capable of dividing into a variety of tumor cells, repopulating the tumor mass after any kind of treatment (Monteiro and Fodde, 2010). Such cells are found to be more resistant to chemotherapy and apoptosis and, therefore, are capable of repopulating tumors following treatment. To this day, the presence of CSCs has been shown in many malignancies including those of leukemia, breast cancer, glioblastoma, pancreatic cancer and colon cancer (Clevers, 2011; Francipane et al., 2013). It has been claimed that expression of CD133, a glycosylated surface antigen, can be used as a marker for selection of CSCs. Such cells exhibit a greater colony-forming efficiency, higher proliferation capabilities and enhanced in vivo

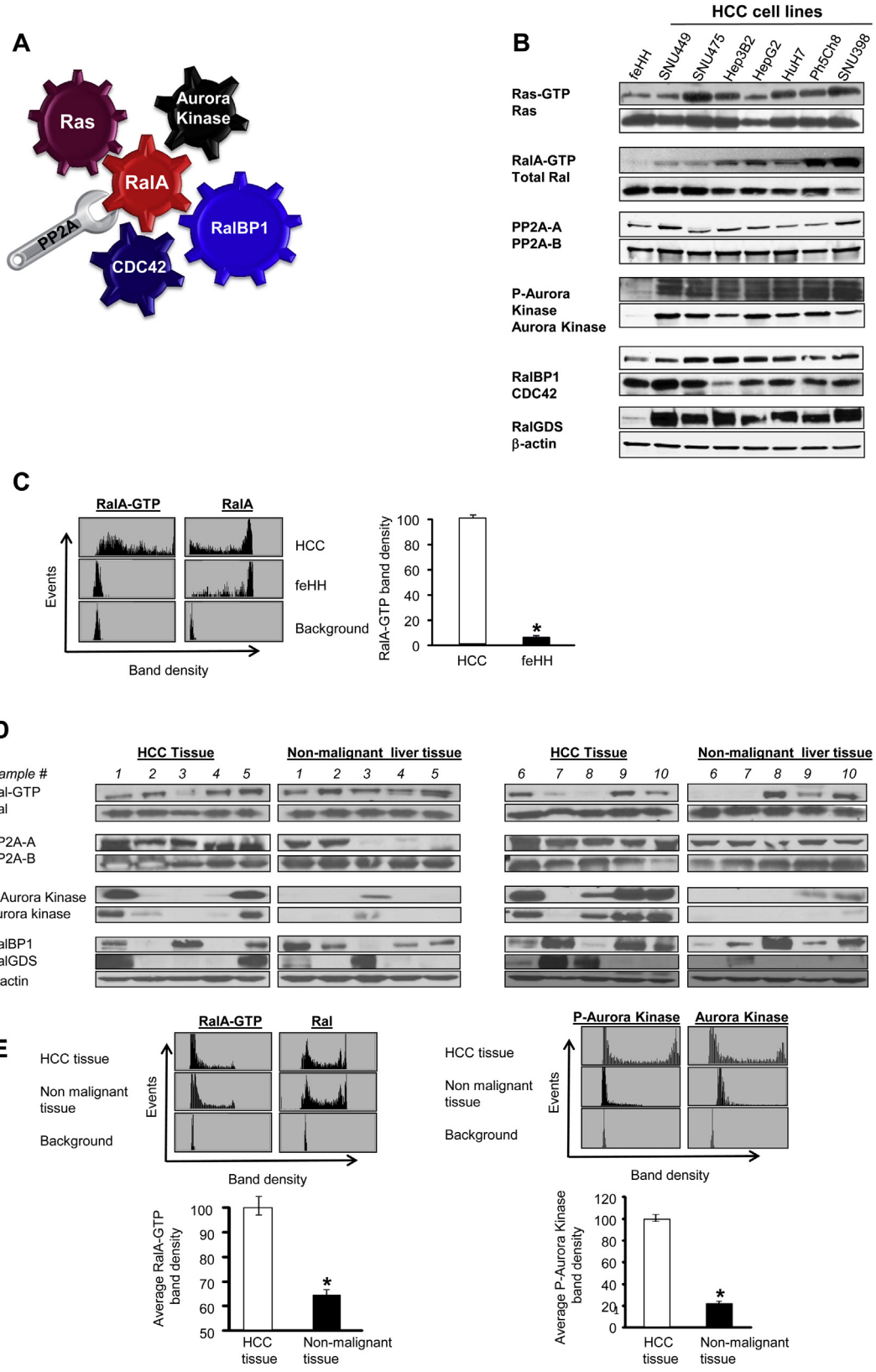

Figure 1 – Activation levels of elements of Ral signaling pathway in HCC cell lines and tumoral tissues. A: Ral pathway is activated by interaction with active form of Ras. Aurora kinase A also phosphorylates and activates Ral. Once Ral is activated, the signal is passed on to a number of different downstream elements such as RalBP1 and CDC42. B: A series of HCC cell lines including SNU449, SNU475, Hep3B2, HepG2, HuH7, Ph5Ch8 and SNU398 along with non-malignant fetal human hepatocytes (feHH) were tested. It should be noted that HepG2 is referred to as a Hepatoblastoma cell line, a form of liver cancer in children. The activated form of Ral and Ras (both in GTP associated forms) was evaluated by affinity precipitation assay. Signaling molecules PP2A, Aurora kinase, RalBP1, CDC42 and RalGDS were studied using specific antibodies

tumorigenesis (Hagiwara et al., 2010; Kohga et al.). CD133 has also been studied as a marker for CSC in HCC (Yin et al., 2007). We confirmed this matter by performing a western blot for expression of CD133 in our tissue samples (Figure 3A). As expected, a significantly higher level of CD133 expression was observed in tumoral tissues as compared to non-malignant tissues (Figures 3A and 1D are generated from same lysates therefore the β-actin control in Figure 1D is also valid for Figure 3A and has not been duplicated). Once investigated for HCC cell lines, a pattern was observed where the levels of CD133 expression matched with the levels of RalA activation in HCC and feHH cells. To be precise, cells with higher levels of RalA activation (presented by upward arrow) showed elevated levels of CD133 expression (Figure 3B). On such basis we divided the HCC cell lines into two groups of High CD133/High RalA activation (Figure 3B left panel) and LowD133/Low RalA activation (Figure 3B, right panel). Further, we embarked on studying the expression/activation levels of Ras, RalA and phospho-Aurora kinase in CSC subpopulation of HCC cells. In order to achieve this, the population of HuH7 CD133 + cells was enriched to ~35% once these cells were processed with Miltenyi Biotec's magnetic activated cell sorting (MACS) with affinity for CD133 (Figure 3C, upper panel). The fraction of CD133 + cells in the flow-through population were reduced to 7%. Under such conditions, the expression/activation level of RalA was found to be higher in the population of cells enriched for CD133. The same situation was also observed for total and GTP-bound levels of Ras and total and activated (phosphorylated) levels of Aurora kinase (Figure 3C, lower panels). Correlation of CD133 expression with high RalA activity as well as increased activation of elements of Ras and RalA signaling pathway in CD133 + cells reveals a novel characteristic of these cells, which potentially serves as one of the mechanisms for enhanced resistance of these cells to therapy. Such overactivation patterns might render HCC stem cells more sensitive to the pharmacological inhibition of Ras and Ral pathways providing a novel way to preferentially target these cells. Our unpublished data in other tumor models are also in harmony with this concept.

Finally, in order to study the in vivo effects of inhibition of Ral pathway on HCC cells we incorporated the use of SC mouse model using athymic nude hairless mice *(Hsd:Athymic Nude-Foxn1^{nu})*. This mouse model bears an autosomal recessive mutation on *nu* locus on chromosome 11, thymic aplasia and is phenotypically hairless. T-cell immunity is deficient; however, B-cell function is normal. Animals ($n = 14$) were injected with 1.2×10^6 Huh7 cells (in 1:1 mixture of growth media: matrigel for a total of volume of 200 μl). The cells were pre-treated with AKI-II [20 μM, $n = 5$] and GGTI-2133 [380 nM, $n = 4$] or DMSO ($n = 5$) overnight and then injected via SC in the right flank (AKI-II or GGTI-2133) or left flank (DMSO). Tumors were allowed to grow for three weeks. Figure 4A shows animals treated with AKI-II and three of the largest tumors extracted from the control (DMSO) treated side. No tumors were observed in the right (AKI-II treated) side. Figure 4B shows tumor volumes (mm^3) for each subject in this study at 20 days post SC injection. A strong inhibition was observed for AKI-II with no tumors in any of the subjects while 3 out of 4 subjects treated with GGTI-2133 also remained tumor free.

Collectively, these data suggest an important role for the inhibition of Ral pathway in reducing the tumorigenesis of HCC in vivo. One reason for the stronger effects of AKI-II can be explained by the impact of this inhibitor on phosphorylation of histones (Figure 2F, middle panel). Aurora kinases have been shown to affect phosphorylation of histone 3, which is implicated in the proper functioning of both mitosis and meiosis. Such effects along with inhibition of Ral activation can impose synergistic inhibitory effects on the growth of HCC cells.

In order to strengthen the correlation between HCC and RalA activation in vivo we also studied the levels of active RalA in Farnesoid X Receptor (FXR)-knockout spontaneous mouse model for HCC (Guo et al., 2006). Deficiency in FXR (the primary bile acid sensing nuclear receptor) results in development of HCC with mechanism that are not well understood. Recent data revealed a possible role for sustained activation of Wnt/β-catenin pathway in FXR-KO mice (Wolfe et al., 2011). Figure 4C shows livers extracted from 6 months old (upper panel) versus 18 months old (lower panel) animals (three FXR-KO mice versus three normal background mice). While in early stages (6 months) there are no tumors visible, a number of tumors are developed at the age 18 months.

Once liver tumors from three independent FXR-KO subjects were compared with liver from three wild-type subjects for activation level of RalA (Figure 4D), a significantly higher level of RalA-GTP was observed in FXR-KO subjects. This data not only further confirms RalA overactivation as a contributor to the developmental process underlying HCC progression but also portrays FXR-KO mouse as a suitable model for future studies with the purpose of inhibiting Ral signaling for treatment of HCC.

3.1. *Conclusion and future directions*

The results of this study highlight the importance of Ral signaling pathway in the biology of HCC at cell line and tissue levels. The impact of inhibition of RalA is imposed across all translational related characteristics of HCC cells such as viability, invasiveness and in vivo tumorigenesis.

However, the above fact is better explained once the element of CSCs is also entered to this equation. Although the role of CSCs is still under intense investigation in different cancer models, revelation of their signaling characteristics

against these targets. C: Evaluation of band density (left) and comparing the averages (right) for RalA-GTP in HCC cell lines versus feHH shows a significantly higher level of activation in cancer cells. D: A series ($n = 10$) of tissues with confirmed HCC pathology reports, as well as non-malignant liver tissues ($n = 10$), were analyzed for expression of Ral signaling pathway elements such as Ral-GTP, PP2A, Aurora kinase, RalBP1 and RalGDS. Enhanced levels of Ral-GTP, phospho-Aurora kinase, RalBP1 and RalGDS were observed for HCC tissues. E: Evaluation of band density (upper panels) showed a ~35% increase in RalA-GTP levels in HCC tissue as compared to non-malignant tissues. In the case of phospho-Aurora kinase, a ~80% increase was observed in HCC cells (lower panels).

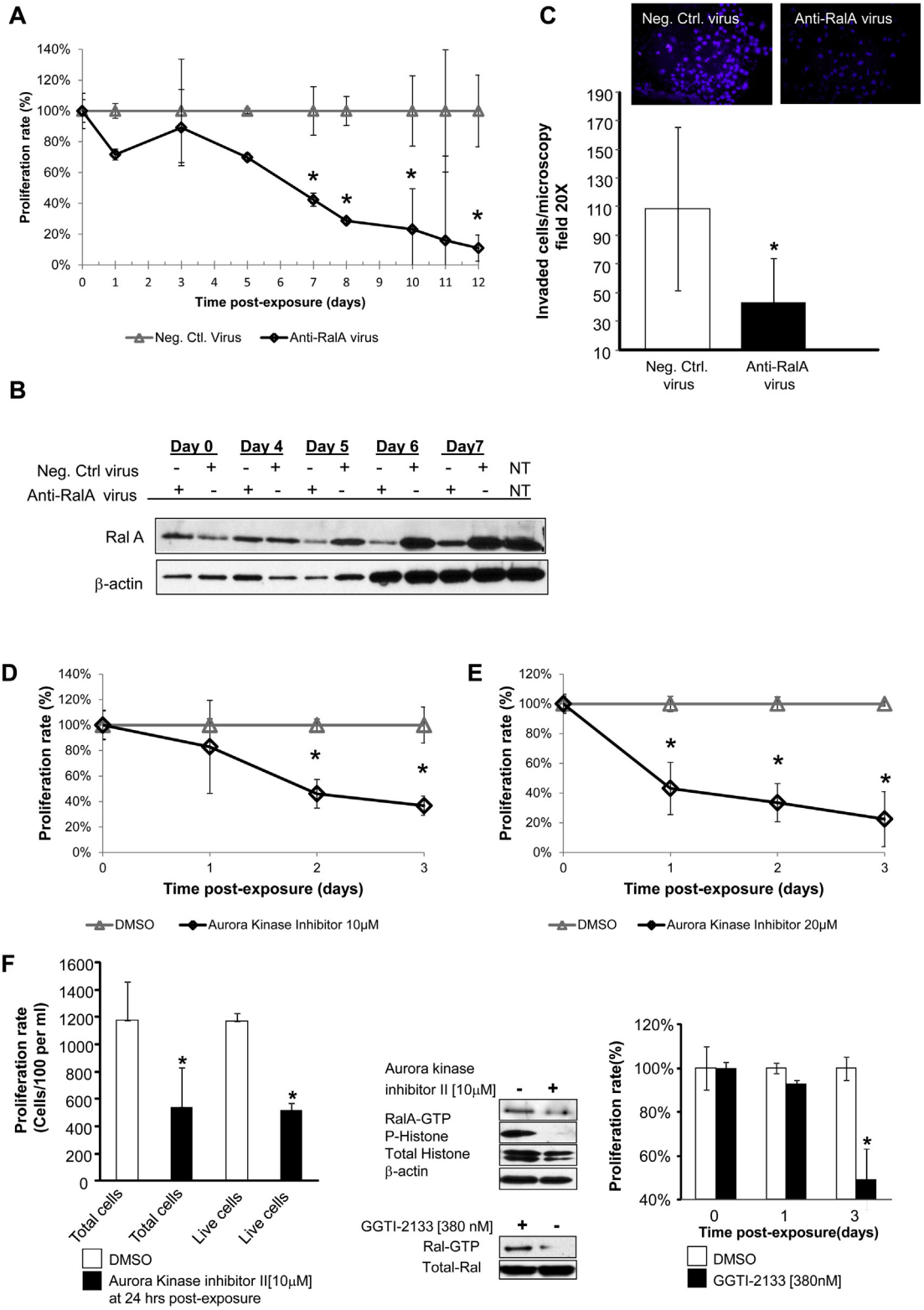

Figure 2 – The outcome of inhibition of RalA signaling pathway on proliferation and invasiveness of HCC cells. A: In order to evaluate the effects of inhibiting RalA on the proliferation rate of HCC cells, HuH7 cells were exposed to a lentivirus expressing anti-RalA shRNA or a negative control lentivirus (containing no Anti-RalA sequences) at the MOI ~ 10 cfu/cell. At each time point, the proliferation rate of anti-RalA virus-treated cells were reported as a fraction of control virus-treated cells resulting in loss of proliferation and close to a complete elimination of these cells at day 12 post-infection. B: Expression of RalA was investigated by Western blotting for this protein. Samples are normalized for total protein concentration within each time point (and not between time points). Effective silencing is initiated by day 6 and continues at day 6 and 7. No treatment (NT) controls were harvested at the last time point. C: Once cells were exposed to the Anti-RalA lentivirus, their capabilities to invade through a layer of matrigel in invasiveness assay (as a representation of their metastatic capabilities) was also reduced to about 40% of the cells

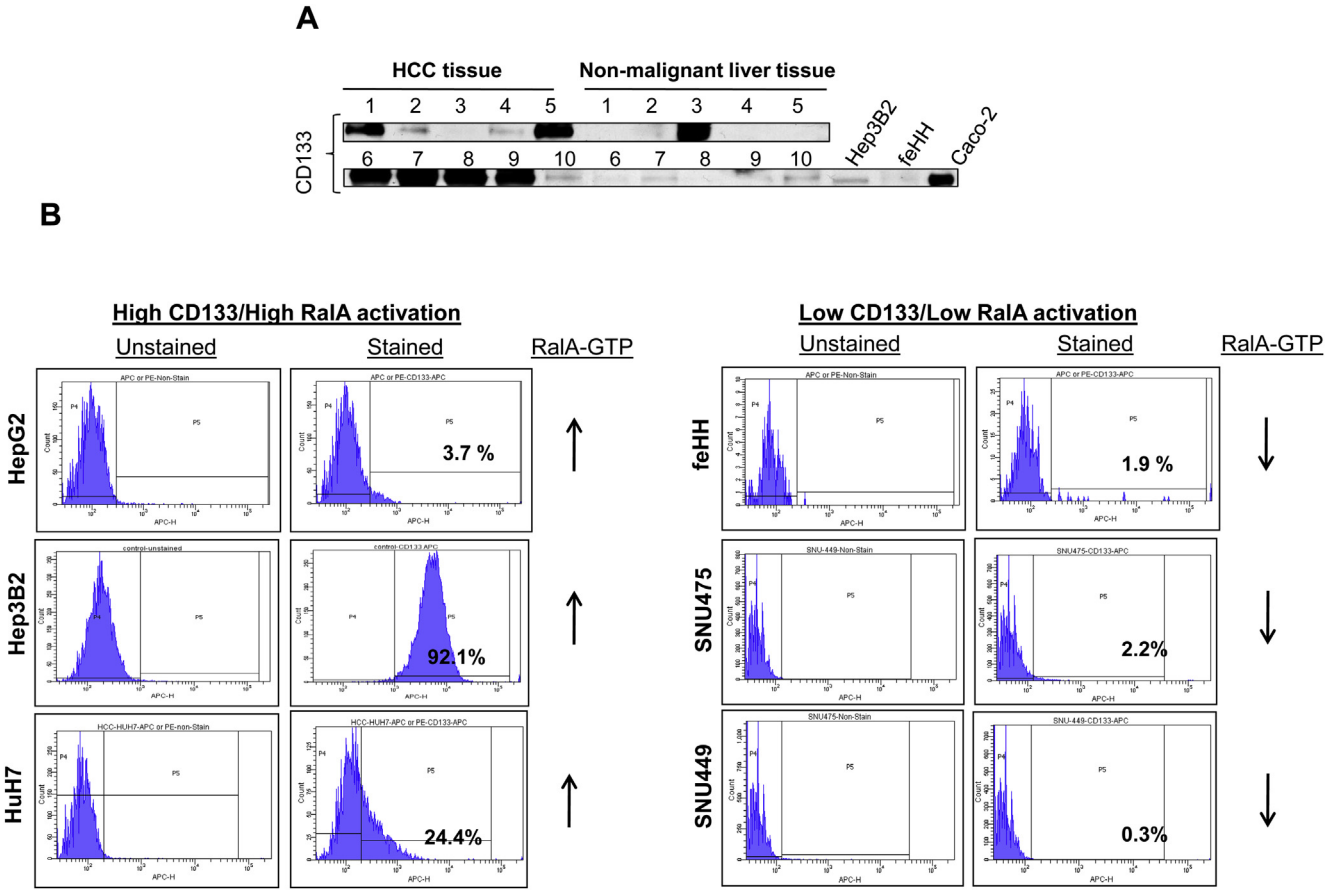

Figure 3 – Ral, Ras and Aurora kinase are overactivated in HCC stem cell subpopulation. A: CD133 is dominantly expressed in HCC tissues as compared to non-malignant tissues. The samples are the same as used for Figure 2 and β-actin control was presented in Figure 2A. Caco (colorectal cancer cell line) and Hep3B were used as positive controls for CD133 expression. FeHH cells did not express a major level of CD133 expression. B: In HCC cell lines, CD133 expression follows the pattern observed for overactivation of RalA in a majority of cases. Two groups can be separated on the basis of the levels of CD133/RalA activation. HepG2, Hep3B2 and HuH7 belong to high CD133/high RalA activation group and feHH, SNU475 and SNU449 form low CD133/low RalA activation group. Cells with higher expression of CD133 also contained higher levels of Ral-GTP. Arrows to the right represent the comparative levels of activated RalA in HCC cell lines (↑ = High Ral-GTP, ↓ = Low Ral-GTP).C: CSCs are generally considered as a subpopulation of cells with enhanced pluripotential capabilities expressing certain markers, such as CD133 (upper panel). The fraction of CD133 + HuH7 cells was enriched (35%) or depleted (7%) using magnetic assisted cell sorting (MACS)(middle panel). The CD133-enriched fraction contained higher levels of activated Ras (Ras-GTP), activated Ral (Ral-GTP) and activated Aurora kinase (phospho-Aurora kinase)(lower panel). It should be noted that while the basal level of expression of Ras was the same in these two populations, Ral and Aurora kinase were expressed at higher levels in CD133-enriched fraction.

would allow us to develop strategies which can efficiently eradicate these cells. These strategies include novel agents targeted to the signaling characteristics of CSCs, such as Ras or Ral signaling pathway. A range of possibilities from oncolytic viruses (Pan et al., 2009) to small molecule inhibitors show great potential for this purpose. Our team has recently developed an oncolytic virus model capable of targeting cells with overactivated Ras signaling pathway named Signal-Smart 1 or SS1 virus (Esfandyari et al., 2009; Farassati et al., 2008, 2001; Pan et al., 2009). SS1 has the potential to target CD133 + cells with enhanced specificity due to their Ras signaling overactivation.

If we submit into the idea of "tumor as an organ" with a series of cells performing different biological roles (rather than a coalition of highly resembling cells), the axis of Aurora Kinase/Ral pathway seems to carry a fundamental role not only in

treated with the control virus. Statistical significance was determined by Student's *t*-test at $\alpha = 0.05$. D–E: Exposure of HuH7 cells to Aurora kinase inhibitor II (AKI-II) at 10 and 20 μM reduces the proliferation rate of these cells in a significant manner as compared to DMSO treated cells ($n = 3$). Proliferation was measured by the WST-1 assay. F: AKI-II applied at 10 μM for 24 h seems to exert a cytostatic effect on HCC cells while exposure to GGTI-2133 (a blocker of geranyl-geranylation of Ral) at 380 nM reduces cell viability at 72 h post-treatment. The middle panel confirms the effectiveness of AKI-II in reducing RalA activation as well as another target of this inhibitor, phospho-histone (upper panel) and the effects of GGTI-2133 in reducing Ral-GTP levels (lower panel). Statistical significance was determined by Student's *t*-test at $\alpha = 0.05$.

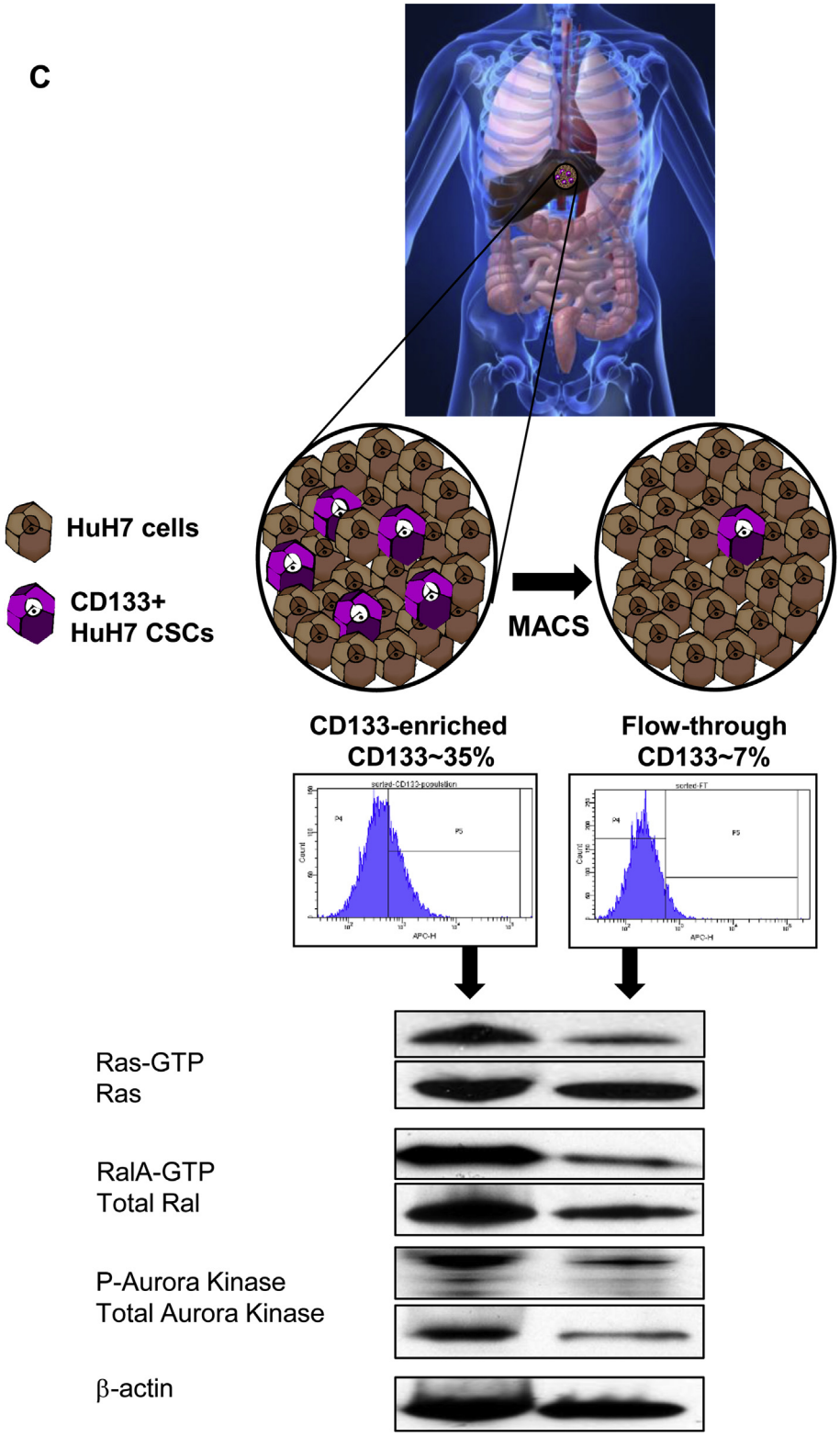

Figure 3 — (*continued*)

differentiated HCC cells but also in the subpopulation of HCC stem cells. Further studies are also needed with the goal of revealing the molecular details of the impact of Ral signaling on the invasiveness of HCC cells, such as the relationship between Ral and epithelial–mesenchymal transition (EMT). For a cancer scientist, the development of specific inhibitors against this pathway and its signaling partners can hold many promises including the availability of better tools to study the biology of HCC, as well as potential uses for such agents in the treatment of HCC.

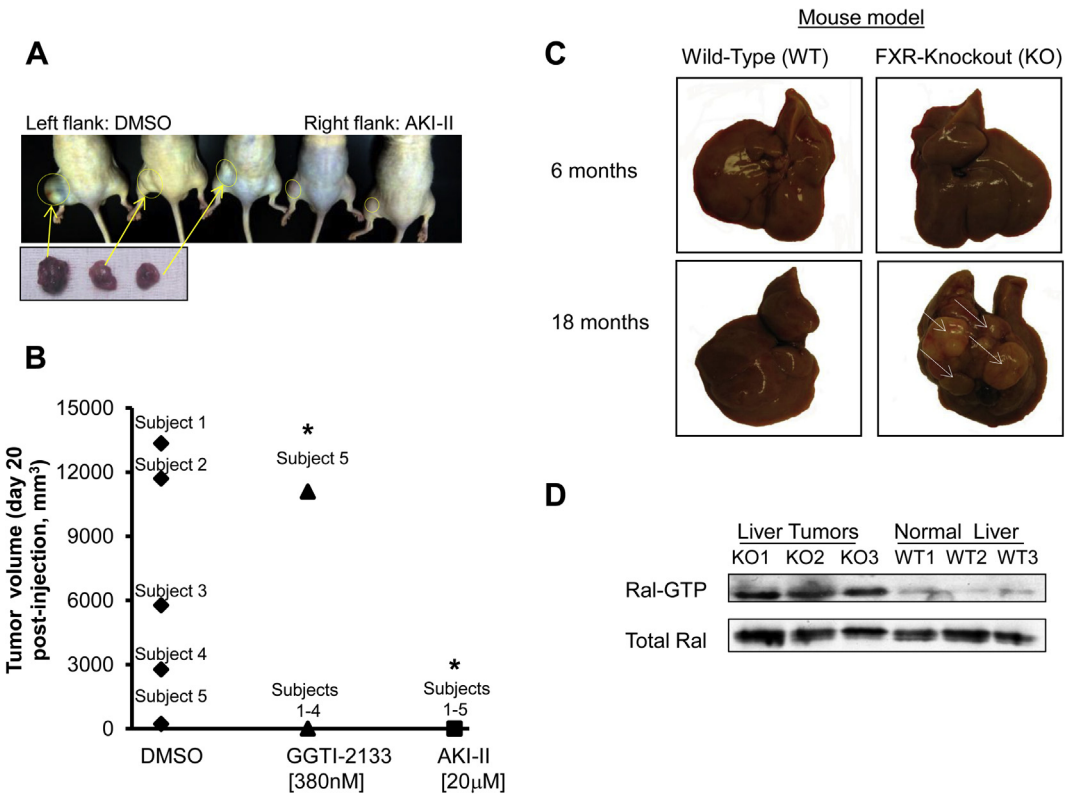

Figure 4 – The role of RalA signaling in in-vivo tumorigenesis of HCC cells. A: Once exposed to the AKI-II [20 μM], the growth of subcutaneously (SC) injected HuH7 cells were significantly reduced in nude hairless mouse model. The upper panel shows the appearance of tumors at 20 days post-injection. Left flank was injected with 1.2 million HuH7 cells treated for 24 h with DMSO while the same number of cells treated with AKI-II was injected to the right flank. The three largest extracted tumors are shown in the bottom panel. B: The graph shows the subcutaneous tumor sizes for each subject. Cell were pretreated with DMSO, GGTI-2133 [380 nM] and AKI-II [20 μM] overnight and injected to the nude mice (*Hsd:Athymic Nude-Foxn1^{nu}*). SC tumors were measured at 20 days post injection. C: Livers were extracted from 6 months old (upper panel) versus 18 months old (lower panel) animals. In early stages (6 months), there were no tumors visible in FXR-KO mice. However a number of tumors are developed at the age 18 months. Arrows point to the tumoral areas. D: Liver tumors from three independent FXR-KO subjects were compared with liver tissue from three wild-type subjects for the activation level of RalA. A significantly higher level of RalA-GTP was observed in FXR-KO subjects.

4. Material and methods

4.1. *Cells, viruses and chemicals*

All HCC cells were purchased from American Tissue Culture Collection (ATCC). HuH7 cells were obtained from Japanese Collection of Research Bioresources (JCRB) distributed by Health Sciences Resource Research Bank (HSRRB). Primary Fetal human hepatocytes (feHH) were purchased from ScienCell, CA. Anti-RalA and negative control lentiviruses were obtained from Santa Cruz, CA. GGTI-2133 and Aurora Kinase inhibitor-II were obtained from Calbiochem, NJ. Primary antibodies were purchased from Cell Signaling, MA, excluding anti-RalA and anti-Ras antibodies which were included in the Ral and Ras activation assay kits from Millipore, CA. All experiments have been repeated for 2–3 series.

4.2. *Affinity pull-down assays for Ras and Ral*

Different cells were grown in 10 cm tissue culture dishes and lysed at 75–80% confluency; Magnesium Lysis Buffer (MLB) was used for Ras assay and Ral lysis buffer (RLB) was used for Ral assay. Ras and Ral activation assays were performed according to the manufacturer's (Milipore, CA) instructions.

4.3. *SDS-PAGE and western blot analysis*

For Western blotting purposes, different cells were lysed with a single detergent lysis buffer (Cell Signaling, MA) and normalized for the amount of total protein. They were then subjected to SDS-PAGE using a Criterion system and then subjected to electroblotting, primary and secondary antibody exposure followed by exposure to Lumigel detection solution and autoradiography. Band densitometry was performed using Adobe Photoshop software.

4.4. *Cell invasion assay*

A commercial kit was used (BD Biosciences, CA) in order to evaluate cell invasiveness. 50,000 cells were introduced into Matrigel-coated inserts fitting 24 well plates. As the cells penetrated the layer of Matrigel, the fraction of invaded cells was

detected by staining nuclei with DAPI (4′-6-Diamidino-2-phenylindole).

4.5. Cell proliferation assay

A formazan-based kit from Millipore (CA) was used for the proliferation assay. Briefly, 10^4 cells/well were seeded in a 96-well microplate in a volume of 100 μL/well. At different times, 10 μL WST-1/ECS solution was added to each well. The plates were incubated for 4 h in standard culture conditions and absorbance was measured at 480 nm.

4.6. Isolation of CD133 + enriched population

The HuH7 HCC cell line was dissociated with cell dissociation solution (SIGMA, MO). The HCC cells positive for CD133 were selected using the CD133 microBeads kit from Miltenyi Biotech, CA. The protocol was modified, labeling 10^7 cells with 400 μl of CD133 microbeads. In addition the background fluorescence was reduced using 400 μl of FcR blocking.

4.7. FACS analysis for immunophenotyping

HCC and feHH cells were treated with cell dissociation solution (Sigma, MO, USA) and then washed using cold 1X phosphate buffered saline (PBS). 250,000 cells were stained for CD133, according to the manufacturer's protocols (Miltenyi Biotech, CA). The samples were analyzed by FACS using an LSRII instrument (BD Biosciences).

4.8. FXR-knockout mouse model for HCC

The FXR-knockout (KO) mice were generated and back-crossed into the C57BL/6 genetic background, as described previously (Guo et al., 2006). C57BL/6 mice were used as wild-type (WT) control. The genotype was confirmed by a PCR-based genotyping method. Mice were allowed free access to water and food and were exposed to a 12 h light/12 h dark cycle. At 18 months of age, livers were removed from male mice and snap-frozen in liquid nitrogen. This research has been conducted in accordance to a protocol approved by Kansas University Medical Center's institutional animal care and use committee.

4.9. Subcutaneous (SC) nude mouse model for HCC

The animals for this study, nude hairless mice *(Hsd:Athymic Nude-Foxn1^{nu} 5–7 weeks of age* < Harlan, IN) were injected subcutaneously with 1.2 million HuH7 cells, suspended in 1:1 mixture of growth media/Matrigel (Matrigel from BD Biosciences, CA) with a total volume of 200 μl. The tumor progression was followed up by measuring the dimension of tumors with a digital caliper. The following formula was used for calculating SC tumor volumes:

$$\text{Tumor volume}\left[\text{mm}^3\right] = (\text{length}[\text{mm}]) \times (\text{width}[\text{mm}])^2 \times 0.52.$$

5. Statistical analysis

Results are reported as means ± Standard Deviation (SD). Student's *t*-test was used to analyze statistical differences between groups. α–level was set at 0.05.

Conflict of interest

Authors have disclosed no conflict of interest in correlation with this work.

Author's contribution

Performed the experiments: ME, EBD, MT, ALW, WP, AR, AAK, MS, KL, KW, FF, Analyzed the data: FF, SW, TE, MO, Wrote the manuscript: FF, Contributed necessary agents/material: BA.

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