Role of cancer stem cells and claudins in the pathogenesis of hepatocellular carcinoma and metastatic liver cancer

Outline booklet of the Ph.D. thesis

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1. INTRODUCTION

Primary liver cancer (PLC) is the sixth most common cancer worldwide, with more than 850,000 new cases annually. Hepatocellular carcinoma (HCC), the most common type of PLC in adults, is a complex, genetically and phenotypically heterogeneous disease with poor clinical outcome. The development of HCC is a slow, multistep process during which genetic and epigenetic changes in cellular proto-oncogenes and tumor suppressor genes progressively alter the hepatocellular phenotype. The origin of heterogeneity in HCC is not clearly understood; however, it is recognized that the same genomic changes in cells at different stages of differentiation can affect both malignant potential and tumor phenotype. Metastatic liver cancers are far more frequent than PLCs, representing 95% of all hepatic malignancies. Liver metastases confer a poor prognosis, as metastatic lesions disrupt the function of the liver, leading to hepatic failure. Colorectal and pancreatic cancers are frequent sources of metastatic liver cancers, yet the complex pathogenesis of liver metastases of colorectal cancer (CRLM) and pancreatic cancer (PLM) is poorly understood. Differentiating HCC from CRLM and PLM, especially when the tumors are poorly differentiated, can be challenging for pathologists. Diagnosis often requires additional immunohistochemical work-up besides routine histopathology.

The cancer stem cell (CSC) model of tumor heterogeneity proposes that tumors are organized in a cellular hierarchy. At the top, CSCs have the capacity to self-renew, and to differentiate into the heterogeneous cancer cell types that reside at the bottom of the hierarchy. Cancer stem cells have been identified in leukemia and a variety of solid malignancies, including HCC, using cell surface markers and functional assays. Cancer stem cells are not only affiliated with tumor initiation and invasive growth, but are likely to be responsible for metastasis formation and therapeutic resistance as well.

Tight juctions, the apicalmost part of intercellular junctions, form a circumferential belt at the boundary between the apical and basolateral plasma membrane domains and create a paracellular barrier in epithelial and endothelial cells. The backbones of tight junction strands are constituted by claudin proteins. Claudins comprise a large gene family, to date, 26 members in humans and 27 in mice have been identified. Dysregulation of claudin expression has been demonstrated in various cancer types. In particular, claudin-1, -3, -4, and -7 are among the most frequently altered members of the claudin family. However, relatively little is known about the role of claudins in carcinogenesis and progression to metastasis.

2. OBJECTIVES

- To assess the ability of mouse hepatic lineage cells at distinct differentiation stages (i.e. bipotential hepatic progenitor cells, lineage-committed hepatoblasts and adult hepatocytes) to become cancer stem cells.
- To explore the influence of cell-of-origin on the phenotype of mouse liver tumors derived from distinct mouse hepatic lineage cells.
- To identify common and cell-of-origin-specific gene expression signatures in mouse liver tumors derived from distinct mouse hepatic lineage cells.
- To investigate the role of c-Myc in the acquisition of cancer stem cell properties in mouse adult hepatocytes.
- To characterize the mRNA and protein expression of claudin-1, -2, -3, -4, and -7 in HCC, and liver metastases of colorectal adenocarcinoma and pancreatic adenocarcinoma.

3. METHODS

3.1 Isolation and transduction of mouse hepatic lineage cells

Hepatic progenitor cells (HPCs), hepatoblasts (HBs) and adult hepatocytes (AHs) were isolated from C57BL/6NCr mice. Genetically labeled AHs were isolated from B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J mice. HPCs were activated with a 0.1% 3,5-diethoxy-carbonyl-1,4-dihydrocollidine diet, hepatic non-parenchymal cells were isolated using a modified two-step collagenase perfusion technique, and then EpCAM^{+} HPCs were purified using fluorescence-activated cell sorting (FACS). E-cadherin^{+} HBs were isolated using a magnetic-activated cell sorting system from embryonic day 16.5 fetal livers. I obtained fully differentiated AHs from 3-month-old male mice using a two-step collagenase perfusion method followed by Percoll purification. Primary cells were co-transduced with oncogenic H-Ras-luciferase/enhanced green fluorescent protein (EGFP) and SV40LT-mCherry lentiviral vectors and cultured for 3 weeks. To test the cancer stem cell properties of the resulting cell populations both *in vitro* and *in vivo*, EGFP^{+}/mCherry^{+} HPCs, HBs, and AHs were sorted using the same gating parameters to ensure comparable viral load and transgene expression. I performed all animal studies in accordance with protocols approved by the Animal Care and Use Committee of the National Institutes of Health (USA).

3.2 Transplantation mouse models

I used male NOD/SCID mice (6-9 weeks of age) for cell transplantation experiments. For limiting dilution assay, 10^1, 10^2, or 10^3 H-Ras-$EGFP^+$/SV40LT-$mCherry^+$ HPCs, HBs, and AHs were injected subcutaneously into both flanks (4 mice/group). Orthotopic growth was assessed after injecting 1.5×10^5 H-Ras-$EGFP^+$/SV40LT-$mCherry^+$ HPCs, HBs, and AHs into the left liver lobe (5 mice/group). Primary tumor growth and metastases were detected using *in vivo* and *ex vivo* bioluminescence imaging, respectively. I sorted H-Ras-$EGFP^+$/SV40LT-$mCherry^+$ cells from primary liver tumors to establish tumor cell lines (4/group). To assess the effect of c-Myc knockdown on tumor growth, 10^2 H-Ras-$EGFP^+$/SV40LT-$mCherry^+$ AHs expressing c-Myc short hairpin RNA (shRNA) or scrambled shRNA were injected subcutaneously (5 mice/group). For immunohistochemistry, western blot, microarray analysis, and quantitative reverse transcription polymerase chain reaction (qRT-PCR), I generated mouse liver tumors by intrasplenic injection of 10^5 H-Ras-$EGFP^+$/SV40LT-$mCherry^+$ HPCs, HBs, and AHs to ensure tumor formation in the liver from engraftment of a single cell.

3.3 Flow cytometry and sphere formation assay

I analyzed nuclear ploidy, frequency of side population (SP) cells, and expression of hepatic lineage and CSC markers by flow cytometry. For sphere formation assay, 500 cells/well were seeded in ultra-low attachment 96-well plates in serum-free growth medium

containing 1% methylcellulose. Tumor spheroids were dissociated and cells were replated once a week for 6 weeks as described above.

3.4 Tissue specimens

For claudin studies, I used formalin-fixed, paraffin-embedded (FFPE) surgical resection specimens from 20 HCCs, 20 CRLMs, and 15 PLMs with paired surrounding non-tumorous liver tissues from the archives of the 2nd Department of Pathology, Semmelweis University, Budapest, with the permission of the Regional Ethical Committee of the Semmelweis University (#137/2008). Median age and female to male ratio were as follows: 65 years, 7:13 (HCC); 65 years, 7:13 (CRLM) and 58 years, 9:6 (PLM). Five normal liver samples were used as control. Semiquantitative histological evaluation and immunohistochemical analysis of hepatocyte, progenitor/biliary cell, and mesenchymal markers were performed on FFPE mouse liver tumors (14 HPC-, 28 HB-, and 28 AH-derived). I used frozen tumors for western blotting (2 AH-derived), qRT-PCR (6 samples each), and microarray analysis (10 HPC-, 20 HB-, and 20 AH-derived).

3.5 Immunostaining, morphometry and western blotting

Purity of primary mouse HBs was assessed by immunofluorescence staining for E-cadherin, albumin, alpha-fetoprotein (AFP), and cytokeratin 18 (CK18). Mouse liver tumor sections were stained manually with anti-H-Ras, anti-SV40LT, anti-hepatocyte nuclear factor 4 alpha (HNF4A), anti-CK19, anti-laminin, anti-vimentin, and anti-A6 antibodies. Immunoreactions for claudin-1, -2, -3, -4, and -7 in human

samples were carried out using the Ventana ES automatic immunostainer. Immunoreactions were visualized with 3,3′-diaminobenzidine. I measured the percentage of claudin-positive area using Leica QWin V3 software. The mean percentage of tumor areas occupied by HCC-, cholangiocarcinoma (CCA)-, and epithelial-mesenchymal transition (EMT)-like phenotypes was evaluated semiquantitatively on hematoxylin-eosin-stained mouse liver tumor sections. I analyzed the expression of H-Ras, SV40LT, and c-Myc protein in mouse cells via western blotting. Immunoreactive bands were visualized using chemiluminescence.

3.6 Gene expression studies

I isolated total RNA from human FFPE tissue samples using High Pure RNA Paraffin Kit. I used TRIzol reagent in combination with RNeasy Mini Kit to isolate total RNA from mouse tumors and freshly isolated mouse HPCs, HBs, and AHs (7 samples each). qRT-PCR was perfomed to measure relative mRNA expression of claudin-1, -2, -3, -4, -7 (human tissue samples), AFP, and albumin (primary HPCs, HBs, and AHs) and to validate microarray results. The mRNA expression levels of target genes were normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase mRNA expression. For transcriptomic analysis of HPC-, HB-, and AH-derived tumors, linear amplification of 400 ng RNA isolated from the tumors and their normal counterparts (4 samples each) was performed using Illumina TotalPrep RNA

Amplification Kit. Biotinylated complementary RNA (750 ng/sample) was then hybridized onto MouseRef-8 v2.0 Expression BeadChips.

3.7 Statistical analysis

Lilliefors and Pearson normality tests were used to assess the normality of data. Significant differences in the number of spheres among normal and transformed hepatic lineage cells were evaluated by Poisson generalized linear model. I calculated frequencies of tumor-initiating cells and probability of tumor initiation by hepatic stem cells (HSCs) using Poisson and binomial distribution, respectively. Significant differences in the proportion of different phenotypes in mouse liver tumors were calculated by one-way analysis of variance and Tukey post hoc test. Microarray data was analyzed using bioequivalence test, bootstrap t-test, hierarchical clustering, and gene set enrichment analysis. I used Mann-Whitney U test to analyze the expression of genes selected for validation by qRT-PCR from microarray analyses. Effect of c-Myc knockdown was evaluated by Poisson generalized linear model and Student's t-test. I conducted Kruskal-Wallis and post hoc tests to compare the protein and mRNA expression of individual claudins in the different groups.

4. RESULTS

4.1 Contribution of distinct murine hepatic lineage cells to the evolution of liver CSCs and heterogeneity of HCC

4.1.1 H-Ras/SV40LT reprogram mouse hepatic lineage cells into cancer stem cells

Freshly isolated primary cells displayed high purity. Over 99% of HBs exhibited positive staining for E-cadherin, AFP, albumin, and CK18 by immunocytochemistry. Only HBs expressed AFP, whereas AHs expressed the highest levels of albumin, as measured by qRT-PCR. High percentages of H-Ras-EGFP$^+$/SV40LT-mCherry$^+$ HPCs, HBs, and AHs were detected by flow cytometry (89%, 89%, and 96%, respectively) 10 days after lentiviral transduction. Western blot analysis confirmed similar levels of H-Ras and SV40LT protein expressions in sorted EGFP$^+$/mCherry$^+$ HPCs, HBs, and AHs. All three types of hepatic lineage cells were effectively transformed by H-Ras/SV40LT and acquired CSC properties as defined by an increase and/or acquisition of SP fraction, CD133 expression, and ability to grow as self-renewing spheres. Interestingly, limiting dilution assay revealed that the ratio of tumor-initiating cells was significantly higher in H-Ras$^+$/SV40LT$^+$ HPCs (1/7 cells, 95% confidence interval [CI]: 1/3 – 1/17) as compared to H-Ras$^+$/SV40LT$^+$ HBs (1/26 cells, 95% CI: 1/11 – 1/62, $P = 0.04$), and H-Ras$^+$/SV40LT$^+$ AHs (1/42 cells, 95% CI: 1/19 – 1/91, $P = 0.003$). H-Ras$^+$/SV40LT$^+$ HPCs, HBs, and AHs initiated aggressive tumors that gave rise to multiple metastatic foci throughout

liver, lungs and brain both in subcutaneous and orthotopic transplantation experiments. Irrespective of tumor cell-of-origin, cell lines established from liver tumors expressed hepatic progenitor/biliary cell (CK19, EpCAM, A6) and CSC-associated markers (CD133, CD44, CD29, CD49f, CD90, Sca-1), had comparable size of SP fraction, and possessed high self-renewal capacity through 6 serial passages.

To confirm that primary AHs and not the occasional contaminating hepatic stem cells (HSCs) were targeted by H-Ras and SV40LT, I used three experimental approaches. Based on the tumor yield (2-3 liver tumors/mouse) after intrasplenic injection of a low number of H-Ras/SV40LT-transduced primary AHs (10^3 cells) and the estimated frequency of HSCs in primary AH culture ($\leq$ 2 HSCs per 10^6 AHs), the probability of tumor initiation by transduced HSCs is negligible ($\leq 2.1 \times 10^{-6}$). Next, intrasplenic injection of genetically labeled (tdTomato), H-Ras/SV40LT-transduced AHs yielded tumors that displayed overlapping luciferin and tdTomato signals, indicating that the tumors originated from AHs. Lastly, I found a significant increase in nuclear ploidy in AH tumor-derived cell lines, a characteristic of normal AHs. In contrast, HPC tumor-derived cells were predominantly diploid, similarly to normal HPCs.

4.1.2 H-Ras/SV40LT induce liver cancer of multilineage differentiation

Liver tumors initiated by H-Ras$^+$/SV40LT$^+$ HPCs, HBs, and AHs were moderately to poorly differentiated with varying contribution of

EMT-, CCA-, and HCC-like phenotypes, resembling subtypes of human PLC. Hepatic progenitor cell-derived tumors displayed predominantly EMT-like phenotype characterized by spindle-shaped cancer cells. HB-derived tumors displayed mostly CCA-like phenotype composed of columnar or cuboidal cancer cells arranged in tubular structures. AH-derived tumors showed a dominant HCC-like phenotype of polygonal, hepatocyte-like tumor cells arranged in solid pattern. All tumor cells expressed HNF4A, CK19, and A6. Furthermore, EMT- and HCC-like tumor cells showed intense staining for laminin and vimentin.

4.1.3 Transcriptomic analysis of HPC-, HB-, and AH-derived tumors

Bioequivalence test revealed that tumor groups displayed higher degree of similarity to each other than to their cell-of-origin. Notably, HPC-derived tumors showed the highest (71%) and AH-derived tumors the lowest (53%) level of similarity to their normal counterparts. In line with this, AH-derived tumors showed the largest number of differentially expressed genes compared to their cell-of-origin by bootstrap t-test, 2826 versus 574 and 906 genes in HB- and HPC-derived tumors, respectively, suggesting that reprogramming of AHs into tumor-initiating cells required more substantial genomic changes as compared to HBs or HPCs. A significant proportion of the 590 genes with common dysregulation among the three tumor groups was associated with EMT, which was also confirmed by qRT-PCR. Hierarchical clustering of HPC-, HB-, and AH-derived tumors based on the 590

common genes separated tumors according to their cell-of-origin, suggesting that distinct hepatic lineage cells dysregulate cell-type-specific transcriptional programs in response to the same oncogenic stimuli. Network analysis identified a higher number of transcription factors in AH-derived (e.g., *E2f1*, *Klf6*, *Myc*) compared to HB- (e.g., *Sp1*, *Foxo1*) and HPC-derived tumors (e.g., *Cebpb*, *Esrrb*). Significantly, *Myc* was highly upregulated (21-fold) in AH-derived tumors but expressed at a lower level in HB- and HPC-derived tumors compared to their normal counterparts. Gene set enrichment analysis using a list of 229 E-box containing c-Myc target genes confirmed a significant enrichment in AH- ($P < 0.0001$) but not in HPC- or HB-derived tumors.

4.1.4 *Myc* is required for H-Ras/SV40LT-mediated oncogenic reprogramming of adult hepatocytes

Stable knockdown of c-Myc in H-Ras^{+}/SV40LT^{+} AHs significantly reduced the number of CD133^{+} cells (1.5% versus 21.4% in control cells), decreased the frequency of SP cells (0.07% versus 0.46% in control cells), and diminished sphere forming capacity and sphere size. Furthermore, subcutaneous growth of c-Myc shRNA-expressing AHs in NOD/SCID mice was significantly reduced compared to control cells transduced with scrambled shRNA.

4.1.5 Distinct claudin expression profiles of HCC, CRLM, and PLM

Claudin-1 immunohistochemistry resulted in moderate apical membrane staining in HCC and circumferential membrane staining in

CRLM and PLM. In normal and non-tumorous livers, intense apical staining appeared in bile duct cells, whereas hepatocytes exhibited weak apical positivity. Morphometric analysis revealed the highest claudin-1 expression level in CRLMs. Claudin-2 staining showed cytoplasmic, granular positivity in both tumorous and normal cells. By morphometry, significantly lower expression was observed in all three tumor groups compared to surrounding non-tumorous liver tissue. Significantly increased claudin-3 membrane staining was detected in CRLMs compared to HCCs and PLMs. Non-tumorous hepatocytes displayed weak, scattered membrane staining, while bile duct cells were strongly positive for claudin-3. Tumor cells of all CRLM and PLM samples, and normal bile duct cells exhibited a strong membranous staining pattern for claudin-4, whereas HCC cells and normal hepatocytes were negative. Claudin-7 immunostaining was significantly increased in CRLMs in comparison with the other groups. Weak membrane staining was present in normal hepatocytes, and normal bile ducts were uniformly claudin-7-positive.

Claudin-2, -3, -4, and -7 showed overall good concordance between their mRNA and protein expression patterns indicating that they are regulated largely at the level of transcription. However, claudin-1 mRNA expression was significantly downregulated in CRLM when compared with HCC, surrounding non-tumorous, and normal livers.

5. CONCLUSIONS

1. I am the first to demonstrate that any cell within the murine hepatic lineage can be a target of oncogenic reprogramming and acquire cancer stem cell traits; however, hepatic progenitor cells are more susceptible to oncogenic reprogramming than more differentiated cells.

2. Oncogenic transformation of distinct murine hepatic lineage cells may give rise to liver cancer of multilineage differentiation resembling human primary liver cancers; nevertheless, tumors display different predominant phenotypes (hepatocellular carcinoma-, cholangiocarcinoma-, and epithelial-mesenchymal transition-like) according to their cell-of-origin.

3. Activation of common pathways and diverse, hepatic-lineage-stage-dependent transcriptional programs contribute to oncogenic transformation of distinct murine hepatic lineage cells.

4. Upregulation of c-Myc in adult hepatocytes is required for acquisition of cancer stem cell phenotype.

5. Hepatocellular carcinoma and liver metastases of colorectal adenocarcinoma and pancreatic adenocarcinoma display distinct claudin expression profiles.

6. LIST OF PUBLICATIONS

Cumulative impact factor (IF): 85.654

Publications related to the doctoral thesis (IF: 16.329):

Holczbauer Á, Factor VM, Andersen JB, Marquardt JU, Kleiner D, Raggi C, Kitade M, Seo D, Akita H, Durkin M, Thorgeirsson SS. (2013) Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. Gastroenterology, 145: 221-231. **IF: 13.926**

Holczbauer Á*, Gyöngyösi B*, Lotz G, Szijártó A, Kupcsulik P, Schaff Z, Kiss A. (2013) Distinct claudin expression profiles of hepatocellular carcinoma and metastatic colorectal and pancreatic carcinomas. J Histochem Cytochem, 61: 294-305. **IF: 2.403** (*These authors contributed equally)

Publications unrelated to the doctoral thesis (IF: 69.325):

Vermulst M, Denney AS, Lang MJ, Hung CW, Moore S, Moseley MA, Thompson JW, Madden V, Gauer J, Wolfe KJ, Summers DW, Schleit J, Sutphin GL, Haroon S, **Holczbauer A**, Caine J, Jorgenson J, Cyr D, Kaeberlein M, Strathern JN, Duncan MC, Erie DA. (2015) Transcription errors induce proteotoxic stress and shorten cellular lifespan. Nat Commun, 6: 8065. **IF: 11.329**

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