With over 660,000 new cases and 630,000 resultant deaths estimated in 2009, primary liver cancer, or hepatocellular carcinoma (HCC), is the fifth most prevalent malignancy and the third leading cause of cancer-related deaths worldwide. Although 80% of cases occur in developing regions where hepatitis B infection is endemic, the incidence of HCC in the United States is rising at epidemic proportions as a result of the rampant spread of hepatitis C four decades ago. The incidence of hepatitis C cirrhosis is projected to peak in 2015. However, a sharp decline in HCC is not anticipated for several decades due to the current epidemic of obesity which will in part replace hepatitis C as the etiology of HCC. Obese patients with metabolic syndrome can develop non-alcoholic steatohepatitis (NASH) with progression to cirrhosis and resultant HCC. The lethality of HCC is demonstrated by its equal annual incidence and mortality, and the dismal 8-month median survival without treatment. However, when HCC is detected at an early stage, curative treatments such as surgical resection, liver transplantation, and ablative therapies can be implemented, achieving 5-year survival rates of up to 75%, highlighting the importance of early detection.

Challenge #1: Accurate Radiographic Diagnosis
The diagnosis of early stage HCC is heavily reliant on quality multiphase, contrast-enhanced computed tomography (CT) and magnetic resonance (MR) imaging, the current gold standards. While characteristic arterial enhancement with portal venous washout of a liver lesion on CT or MR is diagnostic of HCC, indeterminate lesions are frequently detected. This is especially true in cases where the tumor is small (< 1cm), the tumor exhibits atypical contrast enhancement characteristics, and when concomitant regenerative and dysplastic nodules from cirrhosis are present. As the sensitivity of CT and MR continues to improve, and surveillance imaging for HCC is adopted, more such indeterminate lesions will be detected. This uncertainty translates into costly repeat imaging or unnecessary biopsy with its inherent risks of bleeding or tumor seeding, all resulting in delay of treatment and potential complications. Furthermore, eligibility for liver resection or transplantation based on suboptimal scans either results in early recurrences and poor outcomes, or missed treatment opportunities. Studies comparing tumor extent observed on preoperative imaging to pathologic evaluation of the liver explant demonstrate troubling rates of discordance. The false positive rate for CT or MR in the setting of cirrhosis is estimated to be as high as 15%. As a result, roughly 7% of donor livers in the U.S. are transplanted into patients who do not have cancer. These are unacceptable figures, especially given the shortage of donor organs. Imaging that definitively captures small volume, atypical disease, and distinguishes from benign processes would be invaluable in improving our ability to stage and risk-stratify these patients for potentially curative therapies.

Challenge #2: More Effective Treatment
Treatment of HCC remains a formidable challenge. While surgical resection, liver transplantation, and radiofrequency ablation are the mainstay of potentially curative therapy, and can provide 5-year-survival figures of up to 75%, only 10-15% of patients present with disease that is amenable to these modalities. Trans-arterial chemoembolization (TACE), a catheter-based therapy, can be employed as an effective bridging therapy or for symptom control, but is largely palliative. Numerous systemic chemotherapy regimens have been explored for HCC treatment with marginal results. Based on a large multicenter trial, sorafenib, a multi-kinase inhibitor, has emerged as the current standard of care.
The long-term goal of this research is to develop a multifunctional HCC-specific magnetic nanovector that would enable cancer-specific targeting and effective delivery of a sufficient dose of siRNA to target cells to induce gene silencing, while providing the capability of carrier monitoring through MRI and bioluminescence imaging.

Research Objective
To overcome the above mentioned challenges and improve the diagnosis and treatment of HCC, a collaborative effort has been forged with Professor Miqin Zhang’s group in the UW Department of Materials Sciences and Engineering. The long-term goal of this research is to develop a multifunctional HCC-specific magnetic nanovector that would enable cancer-specific targeting and effective delivery of a sufficient dose of siRNA to target cells to induce gene silencing, while providing the capability of carrier monitoring through MRI and bioluminescence imaging. This biocompatible and biodegradable nanovector will target ligands highly expressed by most HCC cells (e.g., glypican-3) and deliver siRNA that inhibits components within the Wnt/β-Catenin pathway that are implicated in the modulation of HCC tumorigenesis, cell proliferation, survival, and cell fate. Our research aims are illustrated in Figure 1.

Nanotechnology
A magnetic nanoparticle (NP) platform, consisting of an iron oxide core coated with a cationic copolymer of chitosan-grafted-polyethylene glycol (PEG), is utilized. The superparamagnetic core enables real-time monitoring by MR imaging. The nanopolymer substratum consists of a chitosan polymer backbone grafted with a low molecular weight, heterobifunctional polyethylene glycol (PEG). This platform is not only biocompatible and biodegradable, rendering it safe for human use, but the anti-fouling properties of PEG prevents agglomeration and uptake by macrophages, prolonging blood circulation time and bioavailability. The PEG grafting not only improves the aqueous solubility of chitosan, adding to its biostability, but also provides terminal functional groups for covalent conjugation of targeting and signaling components. The hydrodynamic size (50nm) is large enough (> 5nm) to circumvent prompt renal clearance, while small enough (< 200nm) to evade sequestration and elimination by the reticuloendothelial system. The negative zeta potential minimizes non-specific binding and uptake by surrounding tissues, allowing for deeper tissue penetration toward the target. Moreover, the higher binding capacity of a NP with more functional amine groups is carefully balanced against the steric hindrance created by its bulk. Near-infrared fluorescent (NIRF) fluorophores make up the second signaling component of the NP, which are detectable by laser confocal microscopy and the Xenogen IVIS fluorescence imaging system.

HCC Targeting
Glypican-3 (GPC3) was selected as a novel molecular target for HCC based on qualities crucial to successful targeting and promising for future applications. GPC3 is a heparan sulfate proteoglycan essential in regulating embryonal cell growth, as evidenced by its mutation causing the Simpson-Golabi-Behmel overgrowth syndrome. While its expression is absent in normal adult tissues, GPC3 is significantly over-expressed in up to 80% of human HCCs. Attached to the cell membrane via a glycosyl-phosphatidylinositol anchor, GPC3 is readily accessible for antibody-mediated targeting and binding. Moreover, GPC3 has been shown to promote HCC growth by stimulating the canonical Wnt signaling pathway, exhibiting potential as an important therapeutic target. These auspicious attributes make GPC3 an ideal biomarker for HCC targeting. We have successfully demonstrated specific targeting of GPC3 expressing HCC cells using biotin conjugated GPC3 antibody with subsequent detection of the NP via fluorescence microscopy and MR. GPC3 antibody production has been conducted by Dr. Elizabeth Wayner’s group at the Fred Hutchinson Cancer Research Center.
Therapeutics

Various therapeutic payloads can be delivered to tumor cells using the nanocarrier construct. One such payload is siRNA for RNAi. Ribonucleic acid interference (RNAi) is an endogenous mechanism by which cells regulate gene expression using small RNA molecules. Short interfering RNA (siRNA), a non-coding 21 base-pair RNA duplex, binds complementary messenger RNA to direct gene silencing via endonuclease degradation within the RNA-induced silencing complex. siRNA-based RNAi is a rapidly developing gene therapy frontier with immense potential.

**Aim 1:** Anti-glypican-3 (GPC3) immunoglobulin (IgG) and its antigen-binding fragment (F(\(\text{ab'}\)\(_2\))) are generated and subsequently conjugated to magnetic chitosan-polyethylene glycol (PEG) iron-oxide nanoparticles (NP). The binding of nanoparticles to GPC3 expressing hepatocellular carcinoma (HCC) cells is tested *in vitro*.

**Aim 2:** Gene silencing using an established green fluorescent protein (GFP) short-interfering ribonucleic acid (siRNA)-nanoparticle system is tested in HCC cells, and then siRNA targeting \(\beta\)-Catenin is optimized for the NP *in vitro*.

**Aim 3:** The GPC3-specific \(\beta\)-Catenin silencing NP is tested *in vivo*.

*Figure 1. Research Aims*
application in cancer therapy. However, effective delivery of the siRNA to the target cancer cell is a major hurdle to in vivo applications. The anionic, hydrophilic siRNA exhibits limited internalization by cell diffusion, and ineffective intracellular trafficking in cancer cells hinders potency. Poor site specificity, low gene silencing efficacy, and lack of non-invasive delivery monitoring are additional challenges.

The pathogenesis and progression of HCC is a complex multi-step process involving several signal transduction pathways (e.g. Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, and Wnt/β-Catenin). Aberrant activation of the canonical Wnt/β-Catenin signaling pathway, resulting in cytoplasmic stabilization and nuclear accumulation of β-Catenin with consequent induction of downstream regulators of cell proliferation (e.g. c-Myc, glutamine synthetase, and cyclin D1), is a frequently observed, important event implicated in HCC tumorigenesis, making this an attractive molecular treatment target for HCC. Over-expression of the Wnt3 glycoprotein ligand and its activation of the Wnt/β-Catenin signal transduction cascade through interaction with the Frizzled7 receptor have been previously demonstrated. Hence, siRNA-mediated inhibition of these components and β-Catenin has been chosen for further study.

Our NP platform is designed to address the limitations of siRNA delivery. The siRNA are covalently attached to the NPs to prevent degradation by extracellular or intracellular enzymes, enabling proper intracellular trafficking and thus improving the efficacy in gene silencing. The targeting of GPC3 adds to improve the efficacy of tumor-specific delivery of the siRNA.

Future Clinical Applications
The NP construct has the unique advantages of dual modality imaging; targeted MR imaging, which can be used for pretreatment staging/planning; and targeted NIRF imaging which can be used in the operating theater to ensure adequate surgical margins during tumor resection.

RELATED PUBLICATIONS

OTHER CO-INVESTIGATORS
From UW Department of Materials Science and Engineering: Miqin Zhang, PhD, Professor / Omid Veiseh, PhD, Conroy Sun, PhD, Hyejung Mok, PhD, Post-doctorates / Forrest M. Kievit, Zachary Stephen, Chen Fang, Matthew Leung, Graduate students