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Gene Editing Solutions for Human and Food Animal Health

Modeling Hepatocellular Carcinoma in the Minipig

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Delivering Better Models to Understand Human Disease

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Abstract

Introduction: Hepatocellular carcinoma (HCC) is the fourth deadliest cancer in the world, with a 5-year survival rate of only 17.7%. Development of safe and effective therapies for HCC patients is desperately needed, since therapeutic options for HCC are limited and insufficient. Rodent models are valuable for identifying potential therapeutic targets and serving as preliminary preclinical models. Additional studies, however, in a disease model more similar to humans in size, anatomy, and metabolism would improve preclinical evaluation of drug dosing, toxicity, and efficacy to inform clinical trial design and improve patient outcomes. Additionally, efficacy studies in a large animal model could aid in the development of novel drug delivery devices, imaging technologies, and surgical approaches for HCC. To address this need, we have developed a platform for modeling HCC by inducing genetic alterations common in human HCC in the liver of the minipig, which has a more comparable size, lifespan, genetics, anatomy, and metabolism to human patients than other animal models. Further, the liver size, function, segmental anatomy, biliary tree, and hepatic vasculature of the pig is particularly similar to human, making swine ideal for evaluating therapies, radiological, and surgical approaches for HCC treatment. **Methods:** We have developed a platform for somatic cell gene delivery to the minipig liver in vivo, targeting either the whole liver or specifically the left liver lobe. Using this technique, we have delivered a combination of expression vectors for oncogenes and targeted nucleases to disrupt tumor suppressor genes commonly altered in human HCC to promote tumorigenesis in the minipig liver. These are being monitored for tumorigenesis using a secreted reporter, detectable through a simple, rapid, luminescence-based blood assay. **Results:** We have developed and optimized methods for efficiently, reproducibly, and stably inducing genetic alterations in the livers of minipigs. We have demonstrated efficient gene-delivery to the left liver lobe and the whole liver using a secreted gene-delivery reporter, as a well as by RT-PCR to detect expression of delivered transgenes. We are monitoring a cohort of minipigs for tumorigenesis using the secreted reporter. **Conclusion:** We have developed an adaptable and efficient hepatic gene-delivery system to model human HCC in the minipig. Since HCC research is progressing rapidly, the flexibility of our platform is critical. The ability to generate models with combinations of genetic alterations tailored to an individual study's needs allows control over the presence of molecular targets for therapeutic testing, the subclass of HCC being modeled, tumor penetrance, and tumor size. Tumorigenesis can be targeted to a single liver lobe or diffuse over the liver. Since the alterations are induced somatically, this can be performed in the context of clinically relevant backgrounds and liver damage. The FDA has emphasized the need for testing new therapies in large animal models, in addition to rodent models, prior to human studies. Our swine HCC models will provide a platform to 1) evaluate the safety and efficacy of molecularly targeted therapies, 2) test drug delivery devices, 3) apply in vivo imaging technology, 4) understand tumor natural history without intervention, and 5) support longitudinal blood and tissue sampling to detect biomarkers. This will lead to earlier HCC detection in patients and safer, more effective HCC treatments entering clinical trials, improving patient outcomes and clinical trial success rates.

Advantages of Swine Preclinical Models of HCC

Limitations of Common HCC Preclinical Models

Rodent Models (general)
- Small size, metabolic, and anatomical differences from human **limits**:
  - Drug dosing evaluation
  - Drug safety/efficacy studies
  - Surgical approaches
  - Imaging technologies
  - Medical device development
  - Repeated tissue sampling for biomarker detection
- RCI's swine model more comparable to human in size, metabolism, liver anatomy, and vasculature

Xenograft Models
- No immune response to tumor
  - Cannot study interaction with microenvironment during tumor progression
- RCI's swine model immunocompetent
  - Tumors develop and progress *in situ*

Chemical Injury Models
- Driver mutations unknown
  - Limited usefulness testing targeted therapies
- RCI's swine model driven by known genetic alterations tailored to a study's requirements
  - Facilitates targeted therapy testing

Genetically Engineered Animal Models
- Driver alterations to germline rather than somatic cells
  - Lack heterogeneity
- Driver alterations to somatic cells models spontaneous cancer development
  - Can generate heterogeneous tumors

Advantages of Recombinetics' Model Over Common HCC Preclinical Models

Minipigs and human livers are similar in:
- Size
- Function
- Segmental anatomy
- Biliary tree
- Hepatic vasculature
- More prone to fibrosis than mice
- Ability to develop ALD from ethanol feeding alone

At 6 months of age, swine hepatic portal vein closely resembles human in terms of:
- Caliber
- Wall thickness
- Lumen
- Wall area
- Collagen, elastin

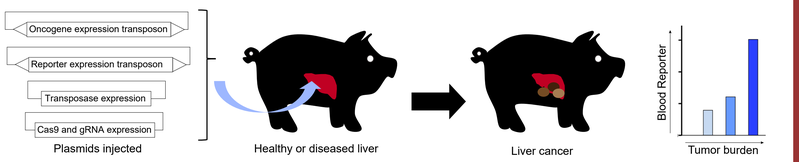
Efficient tumor model production:
- Tumors will likely develop within weeks or months
- No cloning or breeding required; inject into wild-type pigs
- Minimally invasive approach

Flexibility in driver alterations and background:
- Can generate models with any combination of genes for targeted therapy testing
- Model various molecular subtypes
- Can be layered onto clinically-relevant backgrounds:
  - NASH (genetic and/or diet)
  - ASH: Alcoholic steatohepatitis (diet and alcohol)
  - HBV and HCV Viral: can express viral genes from transposons
  - Fibrosis: CCl4 or other another damaging agents

- A swine model closely resembling human HCC genetically and structurally, with similar intratumoral heterogeneity, vasculature, and immune function would allow the development and evaluation of new therapies before or in parallel to clinical trials, improving success rates
- HCC minipig models should lead to new, safer and more effective treatments for HCC

Approach for Modeling HCC in Swine

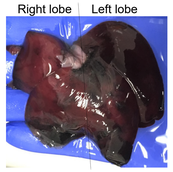
Somatic Mutation Induction Platform for Modeling HCC in the Minipig



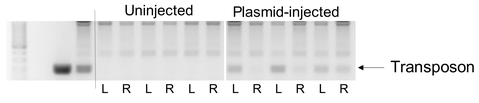
- We developed a hydrodynamic injection approach for to transfect the minipig liver
- Tumors can be induced by stable oncogene delivery on transposons and knockout of tumor suppressor genes by CRISPR/Cas9
- A secreted reporter can also be delivered on a stably integrated transposon.

Targeted Gene Delivery to the Minipig Liver

Preferential Delivery to the Minipig Left Liver Lobe

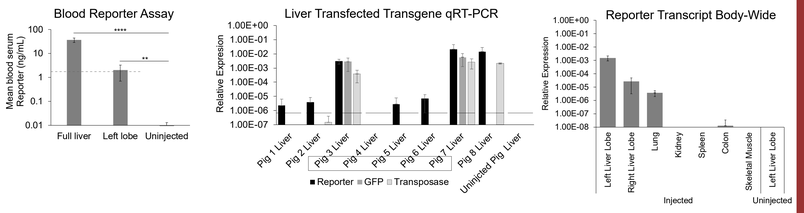


PCR Detection of Injected Transposon in Minipig Liver

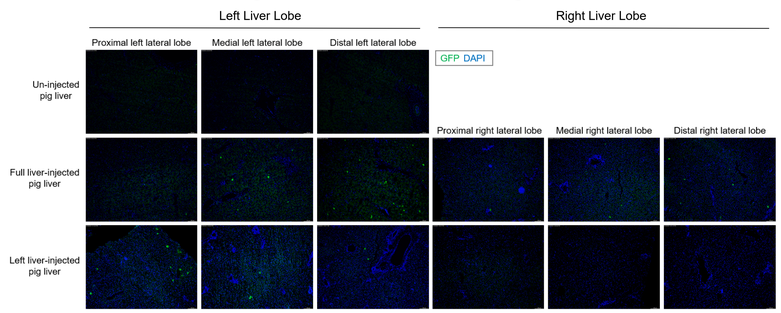


L, left liver lobe sample/ R, right liver lobe sample.

Delivered Transgenes Expressed in the Minipig Liver



Delivered Transgene Protein Product in Targeted Liver Lobes



- Left lobe of liver injected with ink by left lobe-targeting approach is darkly stained as expected
- Transposon detected in left liver lobe genomic DNA samples fom transposon-injected minipig
- Secreted gene delivery reporter detected in blood of injected minipigs
- Delivered transgene transcript expression detected in injected minipig livers
- Protein expression from delivered transgene detected in targeted liver lobes
- Preferential gene delivery to the left liver lobe, with highest transcript and protein expression detected in the left liver lobe from minipig injected with left lobe-targeting conditions

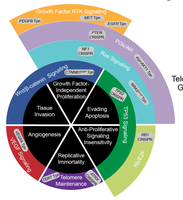
Gene-Editing *in Vivo*

Mutations Detected at gRNA Target Locus in Transfected Minipig Livers

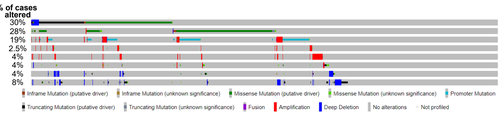
- Targeted gene modification detected in three of four transfected minipig livers examined by NGS

Common Human HCC Pathway Alterations

Genes in Key Signaling Pathways Are Frequently Altered in Human HCC



Pathway	Gene	% of cases altered
TP53 Signaling	*TP53*	30%
Wnt/β-catenin	*CTNNB1*	28%
Telomere Maintenance	*TERT*	19%
Growth Factor RTK	*MET*	2.5%
VEGF Signaling	*VEGFA*	4%
Ras Signaling	*NF1*	4%
PI3K/Akt	*PTEN*	4%
Rb/E2F	*RB1*	8%

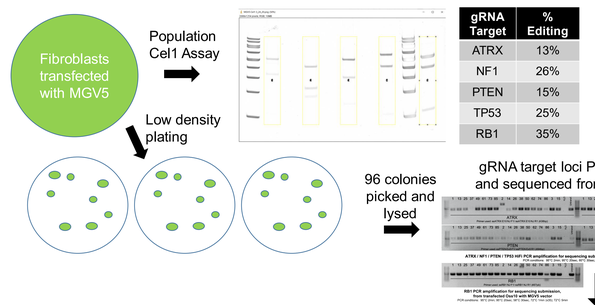


Left: Properties required for tumorigenesis in black wedges. Ring segments show pathways commonly altered in HCC to confer those effects. Gray boxes show constructs we propose to deliver. Right: Each bar represents one sequenced HCC case on The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov.ezp3.lib.umn.edu/).

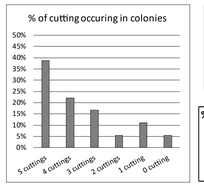
- Minipig HCC tumors will be driven by alterations to pathways commonly altered in human HCC

Gene-Editing Tools Validated *in Vitro*

Editing up to Five Loci in the Same Cell with Multi-gRNA Vector

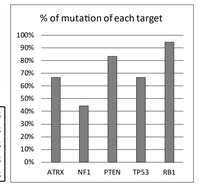


gRNA Target	% Editing
ATRX	13%
NF1	26%
PTEN	15%
TP53	25%
RB1	35%



% of colonies with:			
5 cuttings	39% <=>	7 / 18
4 cuttings	22% <=>	4 / 18
3 cuttings	17% <=>	3 / 18
2 cuttings	6% <=>	1 / 18
1 cutting	11% <=>	2 / 18
0 cutting	6% <=>	1 / 18

% mutations in:		
ATRX	67% <=>	12 / 18
NF1	44% <=>	8 / 18
PTEN	83% <=>	15 / 18
TP53	67% <=>	12 / 18
RB1	94% <=>	17 / 18



MGV, multi-gRNA vector expressing gRNAs targeting swine *ATRX*, *NF1*, *PTEN*, *TP53*, and *RB1*

- Multi-gRNA vector alllows concurrent targeted mutation of up to five tumor suppressor genes
- We are deploying the multi-gRNA vector in minipigs to induce HCC tumors

Next Steps

- Monitor cohort of transfected minipigs for tumorigeneisis by monthly reporter assay
- Apply this method to model various molecular subclasses of human HCC
- Generate models in context of clinically relevant backgrounds such as cirrhosis, fatty liver disease, nonalcoholic steatohepatitis (NASH), and other types of liver damage