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Modeling Hepatitis B-dependent Hepatocellular Carcinoma in Human Embryonic Stem Cells

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Ephie Ng

Committee in charge:

Professor Yang Xu, Chair
Professor Xiang Dong Fu
Professor Cornelius Murre

2012

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University of California, San Diego

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ABSTRACT OF THE THESIS

Modeling Hepatitis B-dependent Hepatocellular Carcinoma in Human Embryonic Stem Cells

by

Ephie Ng

Master of Science in Biology

University of California, San Diego 2012

Professor Yang Xu, Chair

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide; 80% of the cases are caused by chronic hepatitis B (HBV) infections that result in liver cirrhosis. Current disease models are composed primarily of cell lines transfected with HBx, a HBV protein shown to promote tumorigenesis. These models can be improved upon, as the transfected

cell lines are usually cancerous or immortal already. Transfection of primary hepatocyte cell lines is ideal, but studies have demonstrated that the standard plasmid-based method has a very low targeting efficiency of ~3%, and must be replenished due to the limited life span of the cells. Transfection of human embryonic stem cells (hESCs) using a bacterial artificial chromosome (BAC) recombineering system followed by differentiation of positive clones into hepatocytes can be a feasible alternative. Firstly, the BAC recombineering system has been shown to have a vastly improved targeting efficiency of ~20%. Secondly, the immortal lifespan of stem cells ensures that transfection and screening only be performed once. Here, we have created a stem-cell model for HBV-dependent HCC by transfecting HUES 8 embryonic stem cell lines with HBx using a BAC recombineering system. Once differentiated into hepatocytes, they can serve as a cell culture model for HCC as well as an in vivo model via human hepatocyte repopulation in immunodeficient mice and subsequent development into cancerous human liver tissue.

Introduction

Hepatitis B, an infectious and inflammatory liver condition caused by the hepatitis B virus (HBV), affects more than 350 million people worldwide and can result in cirrhosis and eventually, hepatocellular carcinoma (HCC). HBV is the cause of 80% of worldwide HCC cases and is rampant in parts of Asia and Africa, afflicting about ¾ of the local population. In these areas, HBV is contracted during childhood, which increases the risk of chronic hepatitis B (review in Nebbia et al, 2012; review in Kew, 2011). This encourages the development of liver damage and HCC, both of which exhibit lackluster responses to current therapies. Studies have demonstrated that HBx, a protein encoded by the HBV genome, promotes tumorigenesis and contributes to HBV-dependent liver cirrhosis. Though the mechanism for the role of HBx in both diseases has been a popular area of study for many decades, it still has not been completely elucidated.

The HBV genome consists of a partially double-stranded DNA sequence around 3 kb long. Once it enters the host cell, the attached viral DNA polymerase with reverse transcriptase activity completes the short strand of DNA to create a fully double-stranded sequence with joined ends, known as closed circular DNA. This structure is preserved within the lifetime of the afflicted hepatocyte, which can contribute to chronic infection (review in Nebbia et al, 2012). The genome contains four overlapping coding sequences for the proteins C, X, P, and S.

Gene X, also known as HBx, is of particular interest due to its association with both liver cirrhosis and HCC. It is a 17 kDA protein consisting of 154 amino acids. Its function has proven to be incredibly varied and has not been thoroughly investigated; HBx participates in numerous protein-protein interactions and acts as a transactivator of many genes (review in Kew, 2011).

Since HBV is a noncytopathic virus, the immune system is primarily responsible for HBV-related liver damage due to its efforts to clear the disease. Again, the mechanism is not fully understood, since studies have implicated several different immune pathways that lead to HBv-related liver damage (Dunn et al, 2007; Zhang et al, 2010; Iannacone et al, 2005; review in Guidotti, 2001; review in Hsieh et al, 2011; Lee et al, 1998). It is likely that liver cirrhosis as a result of HBV infection is caused by an intricate network of several immune pathways. HBx contributes to this process possibly by sensitizing hepatocytes to apoptotic factors. This in turn can apply selective pressure for neoplastic hepatocytes that develop into cancerous cells (Su et al, 1997).

HBx promotes tumor progression through DNA repair interference and the induction of several cell survival, proliferation, and migratory pathways (review in Ng and Lee, 2011; Fu et al, 2012; Review in Hsieh et al, 2011; Ding et al, 2005; Diao et al, 2001; Tang et al, 2012; Wang et al, 2012; Lee et al, 2012; Wang et al, 2012). Among these are the Ras/Raf/Mapk, JAK1/STAT, c-FOS/c-JUN, and src kinase pathways, all of which are popular pathways in tumorigenesis. HBV lacks a correcting polymerase, which leads to a higher rate of mutation, often resulting in a truncation of the terminal carboxyl region. These deviant forms of HBx are

often more effective at promoting carcinogenesis than its wild-type counterpart (review in Ng and Lee, 2011; review in Kew, 2011).

This project expands the scope of current HBV-dependent HCC models, and can be utilized both as a cell-culture and in vivo model. As a cell culture model, it has the advantage of being non-cancerous before transfection of HBx. Most cell models currently used utilize cancerous cell lines transfected with HBx, limiting the study of the protein's scope of cancerous effects. Transfection of primary hepatocytes using the standard plasmid recombination system is incredibly inefficient, with a targeting efficiency of ~3% (Diao et al, 2000). In addition, the resulting cells are not immortal and therefore necessitate repeated transfections to replenish the model. Transfection of stem cells and subsequent differentiation into hepatocytes is one method to sidestep the issue of replenishing the disease model. However, transfection of stem cells using the plasmid recombination system yields a low targeting efficiency of around 1.5% as well (Ruby and Zheng, 2009; Zwaka and Thomson, 2003). Thus, we use a BAC recombination system, which has been previously shown to achieve a targeting efficiency of about 20%. (Song et al, 2010).

As for mouse models, they cannot be infected with HBV. Researchers have circumvented this by creating transgenic mice expressing HBx that develop liver cancers. Nevertheless, mice and humans have many physiological differences that create a need for more human models. This can be attained by implanting the final differentiated hepatocytes expressing HBx into

immunodeficient mice in order to develop human liver cancer models in an in vivo system (review in Koike, 2002).

Here, we have created an albumin BAC construct containing the HBx coding sequence and transfected it into the HUES 8 cell line via homologous recombination. Rather than utilizing plasmids, we used BAC, large DNA constructs around 150 kb in size. This allows for longer homologous arms, and therefore, a higher and more practical targeting efficiency. This stem cell model of HCC can prove to be a valuable tool for the further study of complex HBx mechanisms, as it is more efficiently constructed and applicable to human systems than many disease models used today.

Materials and Methods

Using human albumin BAC clone RP11.788G14 (Invitrogen) as the PCR template, around 500 bp of homologous arms were inserted into a pBlueScript vector. The HBx gene was cloned from the Hepatitis B genome into the modified vector behind the human serum albumin ATG promoter. An internal ribosome entry site (IRES)-puro-polyA construct, as well as a CMV early enhancer/chicken β acting promoter (CAG)-Neo-IRES-puro sequence subcloned from PL452CNP plasmid, was added. The targeting construct was transferred via electroporation using a Biorad Gene Pulser II (1.75 kV, 200 μF) and homologous recombination into the albumin BAC in SW102 *E. coli* strain. One of the homologous arms of the BAC was shortened and selected via homologous recombination with an ampicillin construct flanked with 50 bp of the target sequence. The finished construct was linearized with the homing endonuclease PI-Sce (New England Biolabs Inc).

The targeting vector was then inserted into the HUES 8 cell line via electroporation using a Biorad Gene Pulser II (2.5 kV, 200 μF). 20 million cells and 500 μg of DNA were used. Transfected cells were cultured in a 37° incubator in 5% CO_2 on a feeder layer with Rock Inhibitor Y-27632 (StemGent) and bFGF (Invitrogen). Clones were selected 24-48 hours after transfection using 100 mg/ml puromycin for two weeks. Positive colonies were picked and cultured individually. They were passaged by washing with PBS, trypsinized for 5 minutes, resuspended in culture media, and plated.

A Southern blot was performed to identify clones with a homologous recombination event. CAG-Cre plasmid was then transfected into the positive clones to remove the loxp-flanked CAG-Neo-IRES-puro sequence. Cre excision was confirmed by performing PCR on resulting clones.

Former lab manager Kelly Stewart assisted with the DNA construct, and postdoctoral fellow Xuefeng Lu with the stem cell portion of this project.

Results

HUES 8 cells were utilized due to their ability to survive and expand when passaged as single clones. In addition, they remain genomically stable and do not differentiate throughout the process (Cowan et al, 2004). Finally, it has a propensity for differentiation into endodermal tissue such as liver (Osafune et al, 2007).These traits ensure that subsequent colonies are monoclonal, free of extraneous mutations, and remain pluripotent.

Insertion of the HBx coding sequence was accomplished by BAC-based recombineering. Due to the significantly larger sizes of the homologous arms, the targeting efficiency was greatly improved from ~1% to 20%. The coding sequence was inserted directly behind the albumin start codon to ensure expression of HBx, and only then in differentiated hepatocytes (Fig 1b). The additional CAG-neo-IRES-puro-polyA sequence allows for antibiotic selection of positively transfected HUES8 clones. After positive clones have been identified, they are transfected with CAG-Cre to excise the loxp-flanked CAG-neo-IRES-puro-polyA sequence. Once screened with PCR to determine Cre excision, the cells are differentiated into hepatocytes and selected using puromycin via the IRES-puro-polyA sequence under the liver-specific albumin promoter. Due to prior excision of puromycin resistance under the CAG promoter, improperly differentiated cells will be selected against.

The BAC vector linearized with PI-Sce was transfected into HUES 8 cells and selected for with puromycin for two weeks before individual colonies were picked and cultured individually. 30 HUES 8 colonies were isolated in this step.

One disadvantage of the BAC recombineering system is screening for successful homologous recombination events, since PCR is not a viable option due to the larger sizes of the homologous arms. Thus, one arm is shortened via homologous recombination with an ampicillin selection marker to allow for screening via southern blot. Genomic DNA was extracted from positive clones and digested with NheI, which cleaved upstream of the inserted HBx construct and in the downstream endogenous sequence (Fig 1a, 1c). Thus, successfully recombined clones will have a larger band at 27 kb, which can be detected via southern blot (Fig 2). Out of the 30 surviving HUES 8 clones, 7 were positive for homologous recombination, indicating a targeting frequency of around 23%.

Once successfully recombined clones were identified, they were transfected with Cre plasmid, which excised the loxp-flanked CAG-Neo-puro sequence. Excision will be confirmed via PCR using primers flanking the loxp sites (Fig 1d).

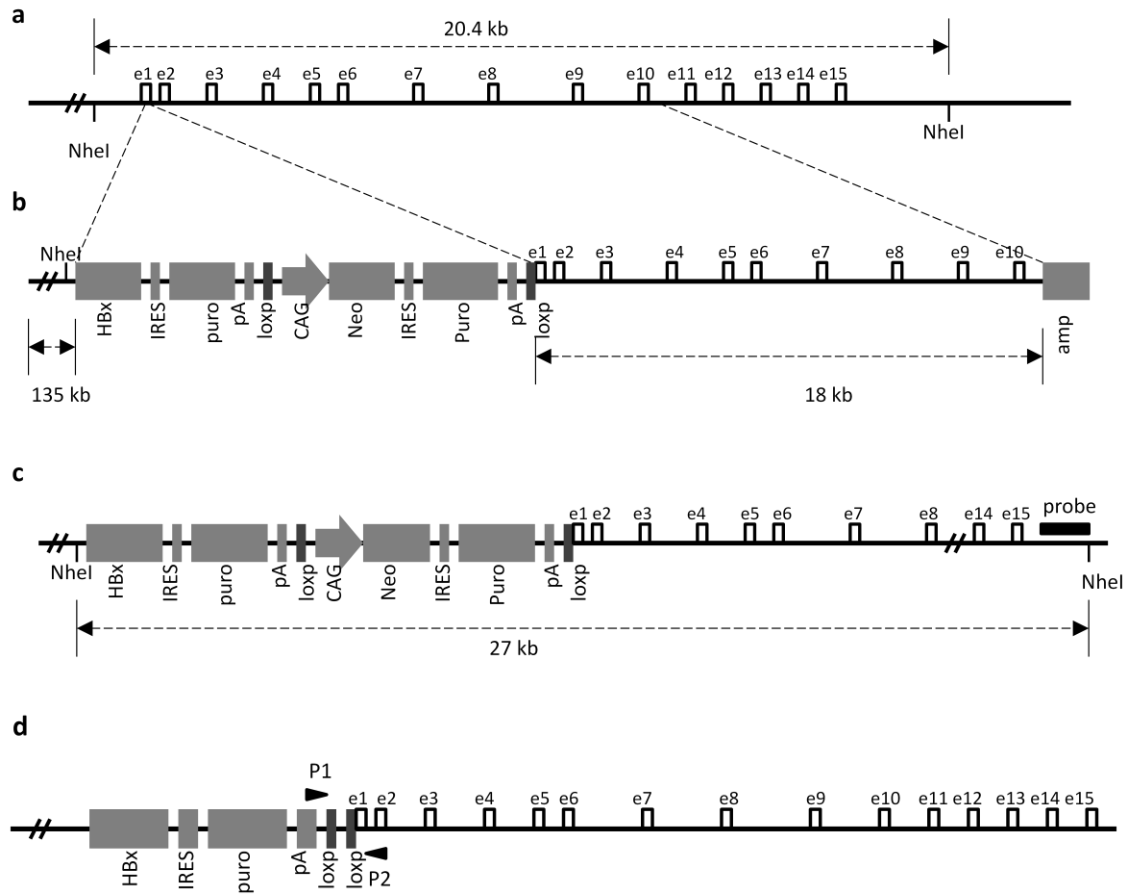

Figure 1: a) endogenous Albumin locus. **b)** targeting BAC vector with shortened arm. **c)** Albumin locus after homologous recombination with BAC. The probe used In the southern blot is indicated by a closed rectangle **d)** Albumin locus after Cre-mediated excision of the CAG-neo-IRES-puro-pA cassette. Arrows indicate location of primers, P1 and P2, used for PCR screening of positive Cre clones.

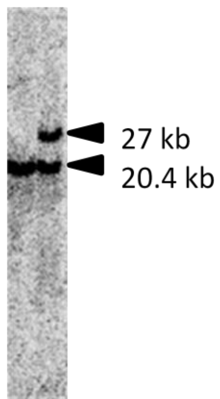

Figure 2: Southern blot confirming homologous recombination of targeting BAC vector in albumin locus. Positive clones have an additional band at 27 kb, while unsuccessful events have only one 20.4 kb.

Discussion

Genetic manipulation of hESCs via BAC recombineering is a promising technique for developing more relevant and efficient human disease models. Here, a HBV-dependent HCC cell line model was created by transfecting HBx into HUES8, which was then differentiated into liver cells.

BAC recombineering technology is more practical primarily due to its nearly twentyfold success rate over plasmid recombination in hESCs. In addition, commercially available BAC is sufficient for homologous recombination, in comparison to the necessity for isogenic plasmid constructs. The only drawback of the BAC system is the difficulty in screening for homologous recombination due to the larger size of the homologous arms. This can be overcome by shortening one of the arms to ~20 kb and selecting the appropriate restriction enzyme for genomic digestion during southern blotting. By having one site upstream of the modified construct and the other within the downstream endogenous genome, positive clones can be identified by the larger band.

The prevalence of HCC due to HBV makes this model valuable for future mechanistic and drug discovery studies both in vivo and in vitro. There are certain complications in current HBV disease models. Most cell culture models in literature involve HBx transfection into hepatocellular carcinoma lines. However, these models cannot give us the full scope of HBx-dependent tumorigenesis, since they were cancerous prior to the modification. Immortalized hepatocyte lines do not have this complication, but the act of immortalization itself is a significant characteristic of cancer. For instance, many immortalized lines are

established by hTERT activation, a protein HBx has been shown to upregulate as well. Lastly, it is difficult to transfect primary human hepatocyte cells; the success rate is around 3% with the common plasmid-based recombination system (Diao et al, 2000). In addition, the cells have a finite lifespan, making transfection of new batches necessary if one chooses to consistently use this model, whereas stem cells do not need to be replenished.

Reprogramming diseased somatic cells into induced pluripotency stem cells (iPSCs) has also received attention as a stem cell-based disease model. However, reprogramming factors are often oncogenic and successful iPSCs frequently downregulate tumor suppressor genes. In addition, genetic instability and genomic variability between clones are frequent and an issue still investigated today (review in Rodolfa and Eggan, 2006; Fu et al, 2012). While some successful disease models have been constructed using iPSC technology, this is currently not an option for cancer models. The genetic instability and oncogenic potential of this technology hampers the reliability of iPSCs as a cancer disease model.

Another emerging technique in the genetic modification of stem cells is zinc-finger nucleases, which use significantly shorter homologous sequences in the DNA recognition domain and the donor plasmid. The zinc finger nuclease creates a sequence-specific double-stranded break in the DNA, which then recombines with a donor plasmid containing the genetic modifications. It has been shown to be very effective in altering stem cells, with a targeting efficiency of ~50%. However, the possibility of random integration in other parts of the

genome is problematic, as it is impossible to detect and can have adverse side effects (Hockemeyer et al, 2009).

Transfecting human embryonic stem cells with HBx and differentiating positive clones into liver cells is an efficient method of creating a more accurate HCC cell culture model. Though the differentiated hESCs are not immortal, their progenitors are. Thus, transfection and selection of positive clones is only performed once.

Mice, one of the most versatile disease models in the laboratory, cannot be infected with the virus; HBx mouse studies involve the use of transgenic organisms. In addition, mice and humans have differing physiological characteristics. The differentiated hepatocytes created here can be utilized to create humanized mice models for HCC via transplation into immunodeficient mice. A similar chimeric system has been constructed by Bissig *et al* (2010) where the livers of immunodeficient mice were repopulated with human hepatocytes, before HBV infection. However, only chronic HBV infection can cause HCC, as exhibited in transgenic mice models (review in Koike, 2002). Thus, the duration of HBV infection must be verified before this particular system can be accepted as a HCC disease model. The model created here forgoes this problem due to constitutive activation of the albumin promoter and thus, expression of HBx, in the differentiated hepatocytes.

In the future, the Cre-positive clones will be differentiated into liver tissue and HBx expression will be confirmed via Western blot. For in vivo studies, completely differentiated cells can be injected into immunodeficient mice for

development of human liver tumors.

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