

## PULSe Publication of the Year Application Cover Sheet

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## **Polo-Like Kinase 1 Inhibition Suppresses Hepatitis B Virus X Protein-Induced Transformation in an In Vitro Model of Liver Cancer Progression**

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Chronic hepatitis B virus (HBV) infection is linked to development of hepatocellular carcinoma (HCC). The HBV X protein (pX) is implicated in HCC pathogenesis acting as a weak oncogene or a cofactor in hepatocarcinogenesis. pX induces DNA re-replication, DNA damage, and partial polyploidy in a poorly differentiated, immortalized hepatocyte cell line. In this study we employed sorted, pX-induced polyploid cells to investigate their growth and oncogenic transformation potential over the course of 70 cell doublings. Immediately after live cell-sorting, nearly 40% of pX-induced polyploid cells undergo apoptosis, whereas the surviving cells exhibit proliferation sensitive to p53. After 40 cell generations the pX-expressing polyploid cultures exhibit loss of p53 function and become growth factor- and anchorage-independent, indicative of oncogenic transformation. The pX-induced polyploid cultures in the course of 70 cell generations undergo progressively increasing DNA damage, propagate damaged DNA to daughter cells, and display increased expression of a cluster of proliferation genes shown to be elevated in human HCC, including HBV-HCC. One of these genes is the mitotic kinase Polo-like kinase 1 (Plk1). Oncogenic transformation is suppressed in the absence of pX expression, and significantly, by inhibition of Plk1. These results identify Plk1 as crucial in pX-mediated oncogenic transformation. *Conclusion:* Partial polyploidy induced by pX is not immediately associated with oncogenic transformation. Continued DNA damage for 40 cell generations is reproducibly associated with loss of p53 function, enhanced expression of Plk1, and oncogenic transformation. Because Plk1 expression is also elevated in HBV-HCC tumors, this in vitro cellular model simulates liver cancer progression and pathogenesis in chronic HBV patients. Inhibition of Plk1 activity suppresses pX-mediated oncogenic transformation, identifying Plk1 as a promising therapeutic target for HBV-mediated HCC.

# Polo-Like Kinase 1 Inhibition Suppresses Hepatitis B Virus X Protein-Induced Transformation in an *In Vitro* Model of Liver Cancer Progression

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Abbreviations: BMEL, bipotential mouse embryonic liver; BrdU, bromodeoxyuridine; BubR1, budding uninhibited by benzimidazoles 1; H2AX, H2A histone family, member X; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MCM4-6, minichromosome maintenance; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; Plk1, Polo-like kinase 1; TYMS, thymidilate synthase; WCE, whole-cell extract.

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Chronic hepatitis B virus (HBV) infection is associated with increased risk of hepatocellular carcinoma (HCC) development.<sup>1</sup> HBV-HCC exhibit frequent and specific chromosomal aberrations<sup>2</sup> by mechanism(s) not yet understood. Studies have shown that preneoplastic human specimens exhibit markers of DNA damage,<sup>3-5</sup> that defective DNA repair causes chromosomal instability, accelerating liver carcinogenesis,<sup>6</sup> and genomic instability is likely the primary cause of carcinogenesis, based on the slow kinetics from carcinogen exposure to cancer development.<sup>7</sup>

The mechanism of HCC pathogenesis by chronic HBV infection involves effects of chronic inflammation and regeneration of the liver,<sup>8</sup> and effects of two HBV proteins, the X<sup>9</sup> and S<sup>10</sup> proteins. HBV DNA integrates into the host genome during early steps of tumor expansion,<sup>11</sup> and most tumors continue to express the X protein (pX). pX is multifunctional, essential for the viral life cycle,<sup>12</sup> and implicated in HCC pathogenesis,<sup>9,11</sup> acting

as a weak oncogene<sup>13</sup> or a cofactor in hepatocarcinogenesis.<sup>14</sup> pX increases the activity of the cellular mitogenic pathways<sup>15</sup> and enhances transcription of select viral and cellular genes.<sup>9</sup> Activation of mitogenic pathways by pX deregulates hepatocyte gene expression. Depending on growth conditions, this deregulation results in either accelerated cell cycle entry<sup>16</sup> or apoptosis.<sup>17</sup> Specifically, pX induces p53 apoptosis, only when pX-expressing cells are challenged with additional subapoptotic signals such as growth factor deprivation.<sup>17,18</sup> By contrast, in optimal growth factor conditions pX-expressing cells do not undergo apoptosis, but rather exhibit accelerated and unscheduled S phase entry, transient S phase pause, activation of the G<sub>2</sub>/M checkpoint, and eventual progression through mitosis.<sup>16</sup> Moreover, in optimal growth conditions pX induces DNA re-replication and DNA damage by deregulating expression of replication initiation factors Cdt1 and Cdc6, and geminin, the negative regulator of re-replication.<sup>19</sup> Despite pX-induced DNA re-replication and the ensuing DNA damage, these hepatocytes proceed through mitosis, propagating DNA damage to daughter cells and generating pX-expressing cells with aberrant DNA content (>4N DNA or partial polyploidy). Significantly, pX-induced partially polyploid cells have been isolated by live cell sorting.<sup>19</sup>

Herein, we investigate the growth properties and oncogenic transformation potential of pX-induced partially polyploid cells. Immediately after cell-sorting, 40% of pX-induced polyploid cells undergo apoptosis. The surviving cells, in the course of 70 cell generations, exhibit increasing DNA damage propagated to daughter cells, and progressively increasing expression of a cluster of proliferation genes that are elevated in human HCC tumors, including HBV-HCC.<sup>20</sup> This cluster includes replication factors minichromosome maintenance (MCM)4-6, proliferating cell nuclear antigen (PCNA), thymidilate synthase (TYMS), and Plk1. Importantly, Plk1 is overexpressed in many human tumors.<sup>21,22</sup> Constitutive Plk1 expression causes focus formation in NIH3T3 cells and tumors in nude mice,<sup>23</sup> whereas Plk1 silencing inhibits xenograft tumor growth.<sup>24</sup> Herein, we demonstrate that after 40 cell generations the polyploid cultures have characteristics of oncogenic transformation, dependent on pX expression. Significantly, inhibiting Plk1 suppresses pX-induced oncogenic transformation, suggesting Plk1 could provide a therapeutic target for HBV-HCC. These observations, together with accumulating evidence that genomic instability is the primary cause of carcinogenesis,<sup>3-7</sup> are congruent with the weak oncogenic potential of pX<sup>13,14</sup> and the slow progression of HBV-mediated HCC after chronic HBV infection.<sup>1</sup>

## Materials and Methods

**Cell Culture.** 4pX-1<sup>25</sup> and 4pX-1-p53<sup>kd19</sup> cell lines were grown in the presence of 5  $\mu$ g/mL tetracycline. pX expression was initiated by removal of tetracycline. Live cell-sorting of polyploid cells was performed as described.<sup>19</sup> Immediately after sorting, 30,000 cells containing >4N DNA, referred to as Passage 0 (P0), were plated in media containing 10% fetal calf serum<sup>25</sup> without tetracycline addition. On day 6, P0 cultures were replated (30,000 cells/dish), generating Passage 1 (P1). Similarly, Passage 2 through Passage 5 (P2-P5) cultures were generated consecutively by plating 30,000 cells from the previous day 6 culture.

**Apoptosis Assays.** Dual parameter flow cytometry for caspase 8 or 9 was performed using single-cell suspensions of P0 cells, employing Cytomics FC-500 (Beckman-Coulter). Fluorogenic substrates for caspase 8 (FAM-LETD-FMK) or caspase 9 (FAM-LEHD-FMK) were added to trypsinized cells (4,000 cells) per the manufacturer's instructions (Biocarta). Incubation was for 60 minutes at 4°C, followed by staining with propidium iodide (PI).

**Real-Time Polymerase Chain Reaction (PCR) and Clustering Analysis.** Real-time PCR quantifications were performed as described.<sup>16</sup> PCR primers for Plk1: Fwd 5' AACCAAAGTGGAAATATGAAGGG 3'; Rev 5' GAGGTCTCAAAGGGAGGCT 3'. PCR primers for pX have been described,<sup>18</sup> MCM4-6, PCNA, and TYMS,<sup>19</sup> and the 16-gene signature set.<sup>26</sup> For unsupervised clustering analysis using the 16-gene expression profile, mouse fetal liver microarray data<sup>27</sup> and real-time PCR data from 4pX-1, bipotential mouse embryonic liver (BMEL) cells, and postnatal mouse livers were normalized using dChip v. 1.6 software.<sup>26</sup>

**Antibodies Used in Western Blots.** p53 (CM5, 1:1000) from Vector Laboratories; H2A histone family, member X (H2AX) and  $\gamma$ H2AX (1:1,000) from Cell Signaling; Plk1 (1:1,000) Abcam; budding uninhibited by benzimidazoles 1 (BubR1; 1:500) Affinity Bioreagents; and MRE11 (1:500) Abcam.

**Soft Agar Assays.** 10<sup>5</sup> cells of P1-P5 cultures were plated per 6-cm dish on a layer of 1% agarose in 2 mL growth media.<sup>25</sup> Cultures were fed with fresh media every 4 days for 14 days, with or without addition of 5  $\mu$ g/mL tetracycline, or 20  $\mu$ M BTO-1 (Sigma).

## Results

To study the role of pX in hepatocyte transformation, we employ the tetracycline-regulated pX-expressing 4pX-1 cell line.<sup>25</sup> The 4pX-1 cell line is immortalized and

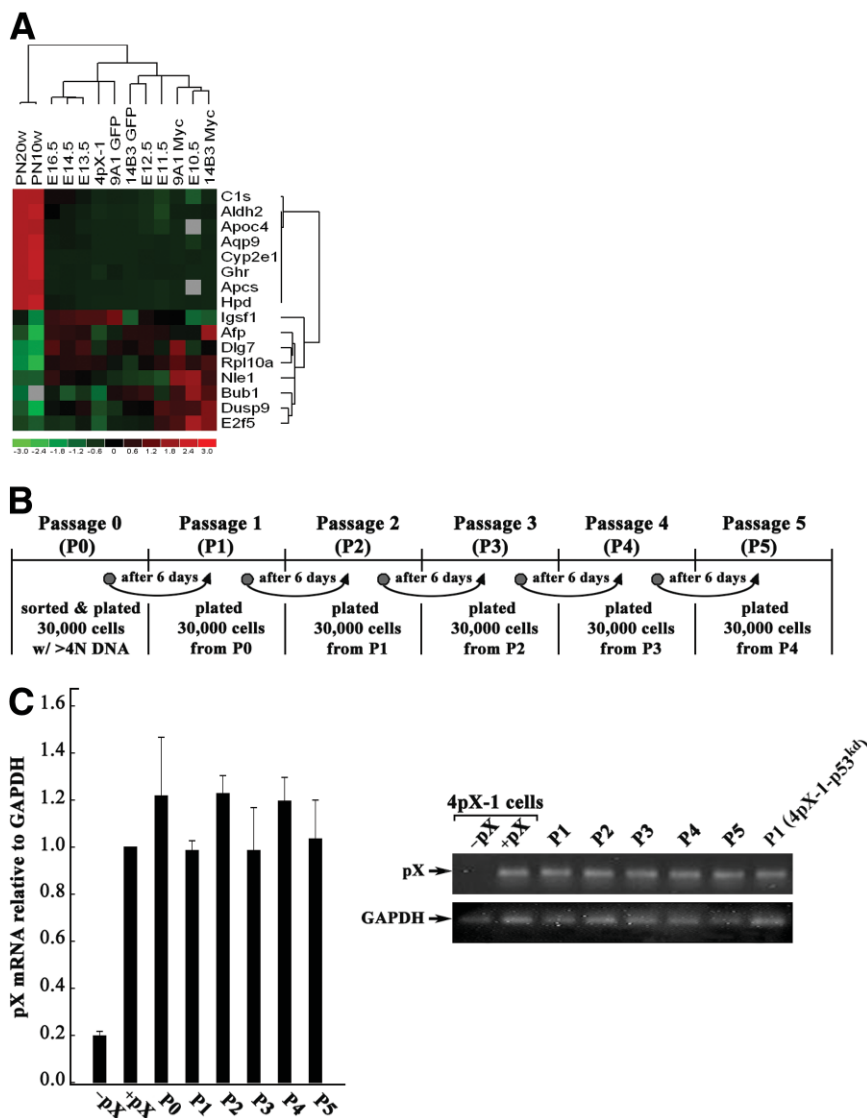


Fig. 1. (A) Unsupervised hierarchical clustering of 4pX-1 cells, BMEL cells, and mouse livers at different developmental stages using the recently described 16-gene signature.<sup>26</sup> PN10w and PN20w, mouse liver at 10 and 20 days after birth; embryonic mouse liver, E10.5-E16.5; and BMEL 9A1 and 14B3 cell lines<sup>28</sup> transduced with GFP or c-myc.<sup>26</sup> (B) Generation of consecutive P0-P5 cultures from sorted pX-induced polypliod cells. (C) Left panel: real-time PCR quantification of X mRNA using RNA from 4pX-1 cells grown with (+) pX for 10 hours or without (-) pX, and day 6 P1-P5 cultures. Quantification, using GAPDH as internal control, is relative to 4pX-1 cells. Right panel: agarose gel electrophoresis of PCR reactions employing primers for pX and GAPDH.

resembles less-differentiated hepatocytes.<sup>19,25</sup> Employing the expression profile of 16 marker genes that recognize liver developmental stage,<sup>26</sup> we determined that the 4pX-1 cell line co-clusters with fetal hepatocytes between embryonic day (E)12.5 and E13.5, and BMEL cells<sup>28</sup> (Fig. 1A), thus modeling poorly differentiated hepatocytes. Because hepatic progenitors participate in liver regeneration after chronic liver injury,<sup>29</sup> and are likely precursors of HBV-HCC,<sup>30</sup> the 4pX-1 cell line is physiologically relevant to study pX-mediated oncogenic transformation. Moreover, the 4pX-1 cell line expresses low level of pX,<sup>25</sup> resembling pX levels in natural infection.<sup>12</sup> 4pX-1 cells grown in optimal conditions with pX expression generate a partially polypliod cell population (6%-8% of total cells) containing >4N DNA.<sup>19</sup> pX-induced polypliod cells have been isolated by live cell-sorting<sup>19</sup> (Supporting Fig. 1), replated, and propagated generating the consecutive P1-P5 cultures (Materials and

Methods and Fig. 1B). P1-P5 cultures continue to express pX (Fig. 1C), thus enabling study of the role of pX in oncogenic transformation.

#### **Growth Properties of pX-Induced Polypliod Cells.**

Because p53 maintains genomic stability,<sup>31</sup> and pX sensitizes cells to p53 apoptosis,<sup>18</sup> we quantified the apoptotic cells in the P0 population immediately after sorting, until 24 hours after replating. Dual parameter FACS analysis, employing fluorogenic substrates for activated caspase 8 or 9 and PI staining (Supporting Fig. 2), demonstrates 45% of P0 cells undergo apoptosis immediately after cell-sorting (Fig. 2A). Apoptosis was decreased to less than 10%, 24 hours after replating (Fig. 2A). Fluorescence microscopy using fluorogenic caspase 9 substrate, confirmed these results (Fig. 2B). Interestingly, apoptosis increased in the absence of pX, suggesting pX rescues a small, but significant number ( $P < 0.005$  for indicated pairs) of P0 cells from apoptosis.

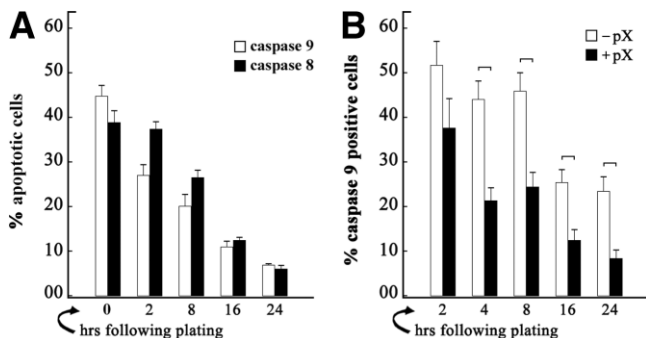


Fig. 2. (A) Quantification of apoptotic P0 cells by dual parameter FACS analyses, using fluorogenic substrates for caspase 8 (FAM-LETD-FMK) or caspase 9 (FAM-LEHD-FMK) and PI;  $n = 3$ . (B) Quantification of caspase 9-positive P0 cells by fluorescence microscopy using ImageJ software; 400 cells were counted per timepoint. Statistical significance of indicated pairs is  $P < 0.005$ , using Student's  $t$ -test.

***p53-Independent Growth of pX-Induced Polyploid Cultures.*** Next, we investigated whether p53 modulates the proliferation of pX-expressing polyploid cells. P0-P5 cultures were treated with or without the p53-specific inhibitor PFT- $\alpha$ <sup>32</sup> and counted on day 6. Cell doublings on day 6 were estimated based on the consideration that 30,000 cells were seeded for each consecutive culture (Fig. 3 and Supporting Fig. 3). We observed a small but progressive increase in cell number of P0-P2 cultures (Fig. 3A). Inhibition of p53 by PFT- $\alpha$  further increased the cell number of P0-P2 cultures (Fig. 3A). Interestingly, the cell number of P3-P5 cultures increased by 50%, even without PFT- $\alpha$  addition, indicating loss of p53 function (Fig. 3A; Supporting Fig. 4).

Because growth of P3-P5 cultures was insensitive to PFT- $\alpha$  (Fig. 3A), we quantified by real-time PCR the expression of p53 mRNA in P0-P5 cultures (Fig. 3B). P0 cells immediately after cell-sorting have the highest level of p53 mRNA, in agreement with the increased apoptosis for that interval (Fig. 2), and the enhanced p53 protein level in P0 lysates (Fig. 3C). The mRNA level of p53 in P1-P3 cultures is similar to the p53 mRNA level in 4pX-1 cells, 10 hours following pX expression. Interestingly, the mRNA of p53 decreased in P4 and P5 cultures ( $P < 0.05$ ), suggesting either defects in p53 transcription after 40 cell doublings, or posttranscriptional mechanisms such as microRNA expression targeting p53 mRNA.

***DNA Re-replication and Propagation of DNA Damage in pX-Induced Polyploid Cultures.*** To confirm the growth profiles of P1-P5 cultures (Fig. 3A), we quantified on day 6 the percent of P1-P5 cells that synthesize DNA by monitoring incorporation of bromodeoxyuridine (BrdU) (Fig. 4A), and the mitotic index by immunostaining with  $\alpha$ -tubulin to detect mitotic spindles (Fig. 4B). In P1-P3 cultures 25%-35% of cells incor-

porate BrdU. Interestingly, in P4 and P5 cultures as well as in P1 cultures from the 4pX-1-p53<sup>kd</sup> cell line nearly 60% of the cells incorporate BrdU. Likewise, the percent of cells undergoing mitosis (Fig. 4B) increased by 2.5-fold from P2 to P3 cultures ( $P < 0.05$ ) and five-fold from P3 to P5 cultures ( $P < 0.005$ ).

Next, we investigated whether BrdU incorporation in P1-P5 cultures is associated with DNA damage (Fig. 5A). Phosphorylated H2AX ( $\gamma$ H2AX) is a marker of DNA damage induced by DNA re-replication.<sup>19,33</sup> The number of  $\gamma$ H2AX-positive cells progressively increased ( $P < 0.05$ ) from P1 to P5 cultures. Immunoblots of  $\gamma$ H2AX confirmed this increase (Fig. 5C). To investigate whether DNA damage occurs in cells that synthesize DNA, P1-P5 cultures were costained for BrdU and  $\gamma$ H2AX. Nearly 50% of  $\gamma$ H2AX-positive cells were also BrdU-positive. Compared to P1 cultures, the number of P5 cells positive for both markers displayed a statistically significant ( $P < 0.05$ ) increase (Fig. 5A). Based on our earlier studies,<sup>19</sup> the BrdU-positive and  $\gamma$ H2AX-positive cells are undergoing DNA re-replication.

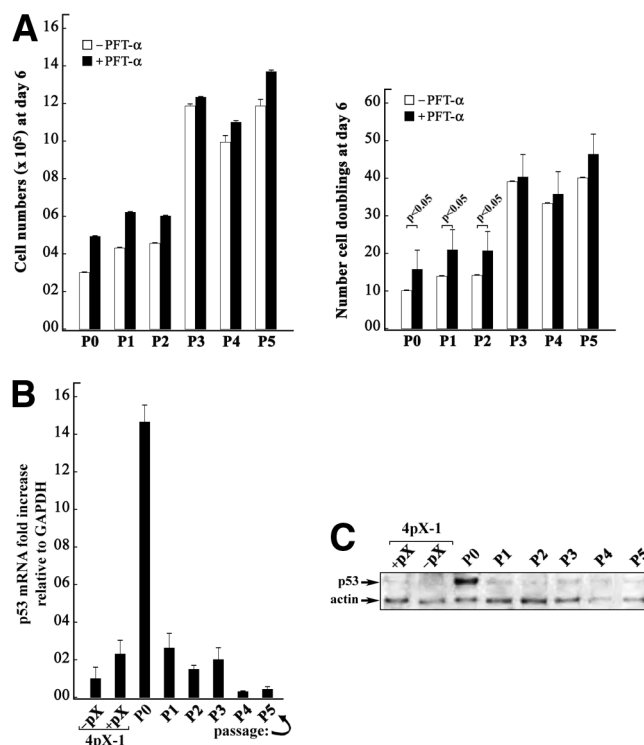


Fig. 3. (A) Growth curves of day 6 P0-P5 cultures grown as in Fig. 1B. Cells were counted in triplicate. Left panel: 4pX-1 cells grown with (+) or without (-) 10  $\mu$ M PFT- $\alpha$ . Right panel: cell doublings of P0-P5 cultures, estimated on 30,000 cells seeded per dish;  $n = 3$ . (B) Real-time PCR quantification of p53 mRNA and (C) immunoblot of p53. RNA for PCR reactions in (B), and WCE for immunoblots in (C), from 4pX-1 cells grown with (+) pX for 10 hours or without (-) pX, P0 cells immediately after sorting, and day 6 P1-P5 cultures. PCR quantifications (B), using GAPDH as internal control, is relative to 4pX-1 cells grown without pX.

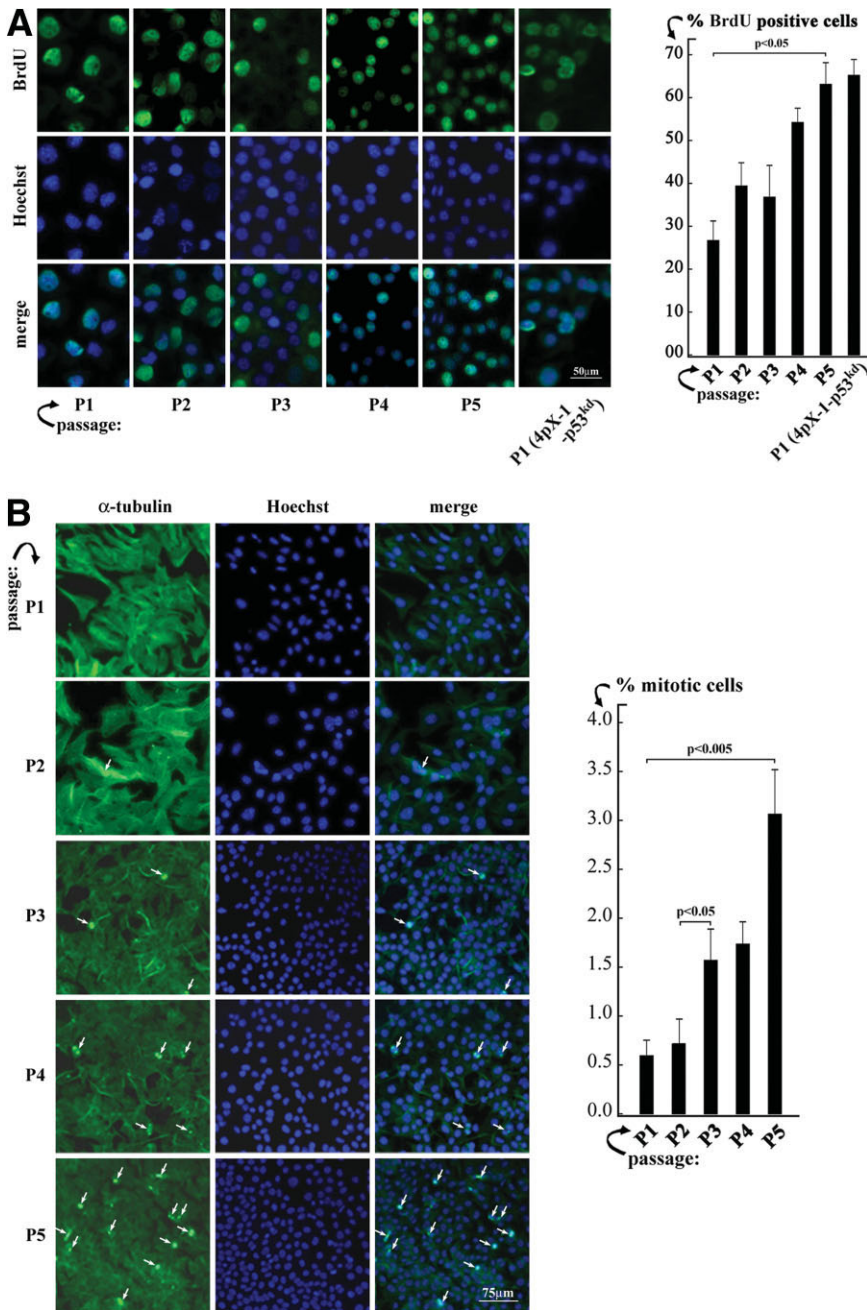


Fig. 4. Immunofluorescence microscopy of day 6 P1-P5 cultures stained for: (A) BrdU and (B)  $\alpha$ -tubulin. BrdU added 30 minutes prior to fixation. Histograms in (A) and (B) are quantifications of BrdU-positive and mitotic cells, respectively. One thousand cells were counted per histogram ( $n = 2$ ). Statistical significance determined by Student's  $t$  test of indicated pairs.

To determine whether P1-P5 cells propagate re-replication-induced DNA damage, day 6 cultures were coimmunostained for  $\gamma$ H2AX and  $\alpha$ -tubulin to detect mitotic cells. The number of P4 and P5 cells immunostained for  $\gamma$ H2AX while undergoing mitosis was significantly ( $P < 0.05$ ) increased (Fig. 5B), demonstrating enhanced propagation of DNA damage. Because DNA damage induces a cellular response that initiates DNA repair,<sup>34</sup> we investigated the potential of pX-expressing polyploid cells to repair DNA damage. Immunoblots of the DNA repair protein MRE 11<sup>35</sup> demonstrate a progressive decrease in MRE11 level from P2 to P5 cultures (Fig. 5D), indicating defective DNA repair.

Because propagation of DNA damage is linked to genomic instability, we determined the DNA content of P0-P5 cultures by FACS. Nearly 16% of P5 cells contained  $>4N$  DNA, representing a 2-fold increase in polyploidy from P0 to P5 cultures (Fig. 6A and Supporting Fig. 5). Although pX induces DNA re-replication (Fig. 5A), a distinct peak of cells with 8N DNA was not observed in the polyploid cultures, suggesting the DNA is partially re-replicated and/or mitotic defects contribute to aberrant DNA content. Employing confocal microscopy, we examined the mitotic spindles of P3-P5 cultures. The most frequent mitotic aberrations are improperly formed mitotic spindles, associ-







Fig. 8. (A) Phase contrast images of day 6 polyploid cultures grown in 10% or 2% fetal bovine serum, soft agar, with (+) or without (-) pX, and with (+) BTO-1 (20  $\mu$ M), as indicated. (B) Quantification of the size of P4 and P5 colonies was relative to P3 colonies, employing ImageJ software; n = 3. (C) Quantification of colonies formed on soft agar. One thousand cells were counted per histogram, considering as colonies clusters of cells of various sizes.

damage for  $\approx 40$  cell doublings precedes pX-induced oncogenic transformation. As in human HCC tumors,<sup>20</sup> our *in vitro* cellular model exhibits enhanced expression of a cluster of genes involved in proliferation, including Plk1. Significantly, Plk1 inhibition suppresses pX-mediated oncogenic transformation. Because Plk1 inhibitors are currently in clinical trials for various human cancers,<sup>43</sup> Plk1 could be a target for therapy of HBV-HCC. Thus, the significance of this study is establishment of an *in vitro* liver cancer progression model that simulates liver cancer pathogenesis in chronic HBV patients.

**pX Initiates Genomic Aberrations Leading to Oncogenic Transformation.** The pathogenesis of HBV-HCC in chronic HBV patients proceeds slowly, involving effects of chronic inflammation of the liver<sup>8</sup> as well as effects of the weakly oncogenic pX.<sup>11,13,14</sup> Moreover, genomic aberrations occur frequently in HBV-HCC,<sup>2</sup> but mechanisms that initiate and propagate these genomic changes remain to be understood. We have shown that pX induces DNA re-replication and DNA

damage, resulting in partial polyploidy.<sup>19</sup> Herein, we demonstrate that pX continues to propagate re-replication-induced DNA damage to successive generations of polyploid cells (Fig. 5), resulting in oncogenic transformation after 40 cell generations (Fig. 8). We conclude that pX-induced DNA re-replication initiates genomic aberrations leading to oncogenic transformation. These results agree with observations that pX potentiates c-myc-induced liver carcinogenesis in transgenic mice,<sup>13</sup> because c-myc overexpression has been linked to DNA damage,<sup>44</sup> and pX likely amplifies c-myc-induced DNA damage, thereby accelerating liver carcinogenesis. Whether continuing liver inflammation in chronic HBV patients synergizes with pX to promote re-replication-induced DNA damage, polyploidy, and oncogenic transformation remains to be determined. This mechanism of re-replication-induced DNA damage is relevant to HBV-HCC pathogenesis. We base this conclusion on microarray analyses (<http://genome-www.stanford.edu/hcc>), which revealed genes involved in DNA synthesis (MCM4-6,

PCNA, and TMYS) and G<sub>2</sub>/M phase progression (Plk1) to be elevated in human HCC tumors including HBV-HCC.<sup>20</sup> These genes are also up-regulated by pX in 4pX-1 cells progressing through S and G<sub>2</sub> phases (19) and in pX-expressing P1-P5 cultures (Fig. 7). Importantly, genes up-regulated in conditions of DNA damage are the same as those induced in normal S phase progression.<sup>45</sup>

**pX Propagates Genomic Aberrations Resulting in Oncogenic Transformation.** Thorgeirsson and Grisham,<sup>46</sup> based on clinical data of preneoplastic lesions and various types of HCC, concluded that loss of mechanism(s) that maintain genomic integrity propagates chromosomal aberrations randomly, while also selecting for genetic changes that support high proliferation rates. Our results support these conclusions. Specifically, more than 50% of P0 cells undergo apoptosis within 24 hours after replating (Fig. 2). Interestingly, pX rescued a statistically significant number of cells from apoptosis (Fig. 2), suggesting pX down-regulates mechanisms that maintain genomic integrity. Furthermore, loss of p53 function coincides with a statistically significant increase in the mitotic index (Fig. 4B), suggesting that abrogation of p53 function and deregulation of mitosis occur by the same mechanism. A candidate molecule for this mechanism is Plk1, known to mediate recovery from the G<sub>2</sub>/M checkpoint.<sup>36</sup> Plk1 is clearly increased in G<sub>2</sub> phase of P3 cultures, and intriguingly, the reverse occurs for BubR1 (Fig. 7). Because BubR1 is necessary for p53 activation in mitosis,<sup>47</sup> we propose that loss of BubR1 results in misalignment of chromosomes as well as absence of p53 activation, resulting in cell survival (despite DNA damage) and oncogenic transformation. The mechanisms mediating the decrease of BubR1 and MRE11 in the polyploid cultures are currently under investigation. Because Plk1 inhibition suppresses oncogenic transformation in our cellular model (Fig. 8) and Plk1 is also up-regulated in HBV-HCC,<sup>20</sup> inhibition of Plk1 could provide a target for prevention and/or therapy of HBV-HCC in chronic HBV patients.

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