Mammary carcinogenesis in transgenic mice expressing a dominant-negative mutant of DNA polymerase β in their mammary glands

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DNA polymerase β (polβ) is a major contributor to mammalian DNA damage repair through its gap-filling DNA synthesis and 5'-deoxyribose phosphate lyase activities. In this way, polβ plays pivotal roles in the repair of oxidative DNA damage, replication, embryonic survival, neuronal development, meiosis, apoptosis and telomere function. A 36 kDa truncated polβ protein is expressed in human colorectal, breast, lung and renal carcinomas, but not in normal matched tissues. Interestingly, a binary protein–protein complex of polβΔ and X-ray cross-complementing group 1 acts as dominant-negative mutant. In this study, the potential tumorigenic activity of polβΔ was examined in nude and transgenic mouse models. Mouse embryonic fibroblasts (MEFs) expressing polβΔ in the absence of endogenous polβ exhibited increased susceptibility to N-methyl-N-nitrosourea (MNU)-induced morphological transformation as compared with cells expressing wild-type (WT) polβ. This was accompanied by reduced gap-filling DNA synthesis activity. Anchorage-independent transformed cells derived from polβΔ-expressing MEFs induced 100% tumor occurrence in nude mice. To support these data, we established transgenic mice expressing polβΔ specifically in the mammary glands from a whey acidic protein promoter-driven transgene. This is the first report of transgenic mice with tissue-specific expression of polβΔ. MNU-induced tumor formation was analyzed in transgenic mice expressing polβΔ together with endogenous WT polβ in their mammary glands and in normal control mice expressing only WT polβ. The latent period of tumor appearance was markedly shorter and tumor incidence was significantly higher in transgenic animals than in control animals treated under the same conditions. These results indicate that cells expressing the mutant polβΔ display an enhanced sensitivity to MNU that probably underlies an increased susceptibility to tumorigenesis.

Introduction

DNA polymerase β (polβ) is a major contributor to the repair of DNA damage via single-nucleotide gap-filling DNA synthesis in the mammalian short-patch base excision repair (BER) pathway (1,2). Polβ has been shown to play a pivotal role in repairing oxidative damage in DNA (3–5) and Sobol et al. (6) reported that BER activity was defective in a mouse embryonic fibroblast (MEF) cell line derived from a polβ knockout mouse. A DNA repair function for the 39 kDa polβ enzyme is consistent with its domain structure. An 8 kDa N-terminal domain has 5'-deoxyribose phosphate lyase and single-strand DNA-binding activities and a 31 kDa C-terminal domain has catalytic activity (1,7–9). Following from its repair function, polβ plays key roles in a number of cellular processes such as DNA replication, embryonic survival, neuronal development, meiosis, apoptosis, maintenance of chromosomal integrity and establishment of drug-resistant phenotype (10–15). In addition, interaction between polβ and telomere repeat-binding factor 2 has been shown to cause telomere dysfunction (16). Additionally, a high mutation frequency with lower fidelity of DNA synthesis has been reported (17), mediated by a variant of the polβ gene expressed in a colorectal adenocarcinoma (18).

We identified a specific 87 bp deletion encoding amino acid residues 208–236 in the polβ CDN in primary human colorectal, breast and lung tumors and in a primary culture of renal cell carcinoma (18,19–22). Both wild-type (WT) polβ protein and the mutant polβ protein (polβΔ) lacking 29 amino acids within the catalytic domain were expressed in colorectal and breast tumors and renal cell carcinoma (20,22,23). Other polβ variants have been shown to be expressed in prostate, bladder and gastric adenocarcinomas (19,24–26). Mutations in the polβ genomic sequence have also been reported in the early stages of serous ovarian cancer (27). Taken together with the known DNA repair function of polβ, the observation of polβ variants in tumors strongly suggests that polβ plays an important role in determining susceptibility to neoplasia.

Further results suggest that polβΔ inhibits functions of the WTpolβ protein in a dominant-negative manner (23,28). Additionally, a binary protein–protein complex of polβΔ and X-ray cross-complementing group 1 (XRCC1), a nuclear BER protein, mediates such dominant-negative activity (29). The survival and growth of cells expressing polβΔ were markedly reduced upon exposure to N-methyl-N-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea (MNU), two known DNA alkylating agents compared with cells expressing WTpolβ protein (23,28,30). These results suggest that the expression of polβΔ, a dominant-negative mutant, may contribute to hypersensitivity of these cells to N-methyl-N-nitro-N-nitrosoguanidine or MNU.

We hypothesize that cells expressing polβΔ protein will display diminished DNA repair activity. Consequently, due to the persistence of lesions in DNA, error-prone replication will occur that may initiate a chain of events leading to expression of a tumorigenic phenotype. To test this hypothesis, we took two approaches. First, we examined survival, morphological transformation and gap-filling DNA synthesis activity of MNU-treated cells expressing polβΔ in the absence of WTpolβ. The ability of these cells to form tumors in nude mice was also investigated. In a second approach, we established a transgenic mouse line expressing the polβΔ specifically in the mammary glands. In this model, endogenous WTpolβ was expressed in the same cells as the transgene-encoded polβΔ protein, allowing the dominant-negative function of polβΔ to be expressed. Tumor formation in transgenic and control mice treated with MNU was evaluated. The data from both of these experimental approaches provide evidence that polβ expression potentiates MNU-induced cellular transformation and tumor formation. Moreover, the increased susceptibility of polβΔ transgenic mice to MNU-induced mammary carcinogenesis supports a dominant-negative role for the polβΔ mutant.

Materials and methods

Cell lines

The stable MEF 19.4AP cell line was established in this laboratory by transfecting 19.4 MEF derived from a polβ knockout mouse (6) with a polβΔ208–236 plasmid (23). G418-resistant colonies were selected by growth in Dulbecco’s modified Eagle’s medium-glutamax. The 19.4AP cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 80 µg/ml hygromycin, 700 µg/ml G418, 10% fetal bovine serum and penicillin and streptomycin. To obtain average activity, a pooled cell line named 19.4AP was made by mixing an equal number of cells from 30 individual expanded colonies. The 16.3 MEF cell line (6) expressing WT polβ was used as a control (23,29).

Abbreviations: BER, base excision repair; MEF, mouse embryonic fibroblast; MNU, N-methyl-N-nitrosourea; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; polβ, polymerase β; WAP, whey acidic protein; WT, wild type; XRCC1, X-ray cross-complementing group 1.

1These authors contribute equally to this work.

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Western blot analysis
To determine expression of polβ in cells or tumor tissues, cell extracts were prepared (23) and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins were transferred to membrane and detected by a purified anti-polβ antibody.

Gap-filling DNA synthesis activity assay
The gap-filling DNA synthesis activity in nuclear extracts was determined essentially as described in (23,28). A 51 bp double-stranded DNA with a G-U mismatch at position 22 was used as the substrate. The reaction mixture consisted of 10 U creatine phosphokinase, 2 μg bovine serum albumin, 500 mM Na+2-Hydroxyethylpiperazine-1-ethanesulfonic acid, pH 7.9, 100 mM MgCl2, 600 mM KCl, 20 mM dithiothreitol, nuclear extract and 32P-dCTP (3000 Ci/mmol) in a volume of 50 μL. It was incubated at 37°C for 30 min and cooled on ice to terminate the reaction. The product was separated by 6% polyacrylamide gel electrophoresis and the radioactivity was detected on the X-ray film at –80°C.

Cell survival assay
A colony-forming assay (23) was used to quantitate the survival of MNU-treated cells. A freshly made stock solution of MNU, >99% pure (NCI Chemical Carcinogen Reference Standard Repository, St Louis, MO), in dimethyl sulfoxide was serially diluted with medium and used immediately. Prior to the treatment, 400 cells were grown in a 60 mm dish for 18 h. Cells were exposed to MNU for 1 h at 37°C, washed with phosphate-buffered saline (PBS) buffer, and were allowed to grow for 7–10 days. Cells were then fixed with 70% methanol and stained with 10% Giemsa. Colonies containing >30 cells were counted.

Morphological transformation assay
Two thousand cells were treated with MNU at various doses for 1 h (31,32). Cells were also incubated in the medium containing dimethyl sulfoxide (final concentration: 0.005%), which served as a solvent control. The MNU–dimethyl sulfoxide was removed through multiple washings with PBS and cells were allowed to grow for 6 weeks with a change of medium twice a week. Treated colonies were then fixed with 70% methanol, stained with Giemsa and scored as either type II or type III (31,32). The foci index was calculated as the number of foci per dish. For transfer into nude mice (see below), type III foci were isolated from unstained dishes using cloning cylinders.

Tumorogenic potential of transformed cells
Type III transformed cells were grown in mass culture for three passages. To determine whether growth was anchorage independent or dependent in soft agar, cells were seeded in 0.33% agar on a 5% agar base layer (31,32). Untreated cells were used as a control. Colonies of transformed cells that formed in the soft agar layer were isolated using Pasteur pipettes and grown in mass culture. Cells (5 × 104) from these cultures were suspended in PBS and were injected subcutaneously into the backs of male athymic Nu/Nu mice (32). Animals were examined for tumor appearance every third day. Resulting tumors were excised, fixed in 4% formalin, paraffin blocked and stained with hematoxylin and eosin for histopathological evaluation. Injected mice were observed closely for body weight loss, abnormal behavior such as poor physical activity, heavy breathing, poor eating or drinking or serious scratching or for any signs of pain. Mice approaching one of these states were humanely euthanized in a CO2 chamber.

Construction of the polδA transgene
To establish a transgenic human polβA mouse line, we generated a 2.9 kb transgene construct consisting of a 5′ whey acidic protein (WAP) promoter followed by the polβA208–236 coding sequence, WAP exon 1 (partial), WAP intron C, WAP exon 4 and 70 bp of the WAP 3′ untranslated region including its polyadenylation signal. The polβA coding sequence was amplified from cDNA (23) with SpeI/polβ forward (5′-ATCTAGTACCGAGGTGTTGCTCTG-3′) and HindIII/polβ reverse (5′-ATACCCCAGCCATTCCTGGCTCCTTG-3′) primers. The polymerase chain reaction (PCR)-amplified product was cloned into a pBluescript plasmid containing a SacII cleavage site at the 3′ end (5′-ATATCCCGCGGTTATGCGCAAATTGGGTTCCAGG-TACC-3′). The amplified product was then cloned into the SpeI–SacI II sites of the plasmid pB103, 3′ to the WAP 5′ sequence. The orientation of the polβA/WAP insert and the cloning junctions were confirmed by sequencing. The transgene was excised from the plasmid for microinjection using BssHII.

Microinjection and generation of transgenic mice
The purified 2.9 kb WAP-polβA construct was microinjected into the pronuclei of B6CBA mice by our Transgenic Core Facility. Sixteen pups were born. Tail clips were taken from the pups at 7–10 days of age. DNA was isolated from the tail clips and analyzed by PCR and Southern blot for the presence of the transgene.

DNA PCR
DNA was initially characterized by PCR using a WAP+1 forward (5′-ATCACGTCACACTTG CCTGCCGCGG-3′) and a polβ reverse primer (5′-TCTTTCACAAATGTTTGGAG-3′).

Southern blot assay
DNA samples from all 16 mice were digested with SpeI and HindIII and analyzed by Southern blot using an ~960 bp SpeI + HindIII-digested polβA cDNA transgene probe. The probe was labeled with α-32P-dCTP by the random priming method.

Treatment of polδA transgenic mice with MNU
To induce WAP-polβA transgene expression in the mammary glands, timed 7-day pregnant female transgenic mice (8 weeks old) were used in these experiments. Mice were injected intra-peritoneally with 50 mg/kg MNU in PBS once a week for 7 weeks. A control group of pregnant transgenic mice received weekly injections of PBS only. An additional control group of pregnant, female non-transgenic Balb/c animals of similar genetic background and age were treated identically in a manner identical to the transgenic animals. This group represented induction of tumors by MNU in normal animals. Another group of normal control mice was given injections of PBS only. This group controlled for spontaneous occurrence of tumors. Animals were examined for tumor appearance by palpation once a week for 3 months and then every third day. The latency period before the first tumor appearance and the tumor index (the number of tumor bearing mice divided by the total number of mice given MNU) were recorded. Animals were watched closely for weight loss, abnormal behavior such as slow mobility, heavy breathing, poor eating or drinking or serious scratching or for any signs of pain. Animals showing any of these signs were humanely euthanized and were subjected to a complete necropsy. Tumors or organs were fixed in Histochoice solution, paraffin embedded, sectioned and stained with hematoxylin and eosin.

All studies involving mice were performed in accordance with National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines for the humane care and use of research animals.

Results
Characterization of polβ knockout MEFs with stable expression of the polδA mutant
In order to examine the biological relevancy of the expression of polβA208–236 variant found in a number of cancers, we established a stable MEF cell line that lacks WTpolβ expression, but expresses the polβA208–236 mutant. This cell line, 19.4AP, was generated by transfection of the 19.4 MEF cell line that was originated from a polβ knockout mouse (6). The well-characterized 19.4 cells do not express WT polβ and are lacking in DNA repair activity (6). The expression and function of polβ in the newly generated 19.4AP cells were characterized by western blot analysis and gap-filling DNA synthesis activity. As expected, the 19.4AP cells expressed only a 34 kDa form of polβ protein, corresponding to the deletion mutant (Figure 1A, lane 2). A 39 kDa polypeptide corresponding to WTpolβ was expressed in 16.3 cells used as a positive control (Figure 1A, lane 3). No polβ protein was detected in extracts from untransfected 19.4 cells. A 41 kDa actin expression indicates equal loading of all samples.

To characterize the activity of the polβ mutant expressed in the 19.4AP cells, DNA repair activity was measured in nuclear extracts using gap-filling DNA synthesis assay. The repair activity was reduced substantially in the 19.4AP cells (Figure 1B, lane 2) as compared with the 16.3 cells (Figure 1B, lane 3). These results indicate that the deletion of 29 amino acid residues in the catalytic domain of polβ results in an enzyme (polδA) that has impaired DNA repair capability.

Expression of polδA adversely affects survival of MEFs exposed to the mutagen, MNU
To determine the effect of polδA expression on the survival of cells exposed to a DNA damage-inducing mutagen, 19.4AP cells and 16.3
(WTpolβ) cells were treated with increasing concentrations of MNU from 1 to 100 nM. Cell survival, expressed as relative cloning efficiency, decreased from 53 to 6% for 19.4ΔP cells treated with 1–100 nM of MNU (Table I). At a concentration of 300 nM, MNU completely shut off growth of these cells. The survival of 16.3 cells used as a positive control also decreased in a dose-dependent manner, although the survival rates of 19.4ΔP cells were markedly reduced compared with the survival rates of equivalently treated 16.3 cells. For example, following treatment with 1 nM MNU, 100% of the 16.3 cells survived, but only 53% of the 19.4ΔP cells survived. Although none of the 19.4ΔP cells treated with 300 nM MNU survived, similarly treated 16.3 cells still had a relative cloning efficiency of 37%. The 19.4ΔP cells devoid of any polβ expression were used as a negative control and these cells were found to be the most sensitive to MNU. These results indicate that the lack of efficient DNA repair activity due to expression of a polβ deletion mutant causes 19.4ΔP cells to be more sensitive to MNU. Thus, WTpolβ protein plays an important role in the survival of cells following exposure to DNA-damaging agent.

**Table I. Survival of cells treated with MNU**

<table>
<thead>
<tr>
<th>Cells</th>
<th>MNU (nM)</th>
<th>No. of colonies (mean ± SE)</th>
<th>Survival (RCE, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.3</td>
<td>DMSO control</td>
<td>38 ± 1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>38 ± 1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>38 ± 2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30 ± 3</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>19 ± 1</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>14 ± 1</td>
<td>37</td>
</tr>
<tr>
<td>19.4ΔP</td>
<td>DMSO control</td>
<td>34 ± 1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>18 ± 2</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6 ± 2</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2 ± 1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19.4</td>
<td>DMSO control</td>
<td>44 ± 2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5 ± 0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4 ± 1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Notes:
- Results are the mean ± SE of three dishes.
- Relative cloning efficiency (RCE, %) is expressed as average number of colonies in treated dishes per number of colonies in dimethyl sulfoxide (DMSO) control × 100.
- Maximum concentration of DMSO in medium was 0.005%.

Morphological transformation of 19.4ΔP cells treated with MNU

We next examined how susceptible 19.4ΔP cells were to transformation as compared with 16.3 cells. Having established the relationship between cell growth and doses of MNU, we used MNU as the transforming agent in these experiments. Transformed colonies or foci when unstained were easily visible in Petri dishes by the naked eye. Foci were scored as type II and type III (31,32). Type II foci cells were characterized as a piled cluster of markedly basophilic cells, moderately polar, in which criss-crossing was not pronounced. Type III foci cells were intensely basophilic and highly polar, showing marked piling with disorientation, criss-crossing, occasional chording and invasiveness to neighboring normal cells’ monolayers. The morphology of a typical type III transformed focus, generated in MNU-treated 19.4ΔP cells, is shown in Figure 2. Type III foci appeared in MNU-treated 19.4ΔP cultures earlier than in similarly treated 16.3 cultures. Type III foci were observed 2 weeks after treatment of 19.4ΔP with 50–100 nM MNU. The foci index for both 19.4ΔP and 16.3 cells increased with increasing the MNU concentrations (Table II). However, the foci index was consistently significant in 19.4ΔP than in 16.3 cells. For example, following treatment with 50 nM MNU, the foci index for the 19.4ΔP cells was 15 times higher than that of the 16.3 cells. These results indicate that expression of the mutant polβ protein in the absence of WTpolβ makes cells more vulnerable to morphological transformation mediated by a DNA-damaging agent such as MNU.

**Figure 1.** (A) The upper panel shows western blot analysis of expression of the polβ protein in cell extracts of 19.4 (lane 1), 19.4ΔP (lane 2) and 16.3 (lane 3) cells. Protein (25 μg) was loaded in each lane. The bottom panel shows the same membrane reprobed with an anti-β-actin antibody to confirm that each lane contained equivalent amounts of protein. (B) Gap-filling DNA synthesis activity (51 bp repair product) in nuclear extracts of 19.4 (lane 1), 19.4ΔP (lane 2) and 16.3 (lane 3) cells.

**Table II. Morphological transformation of 19.4ΔP and 16.3 cells treated with MNU**

<table>
<thead>
<tr>
<th>Cells</th>
<th>MNU (nM)</th>
<th>No. of type II foci</th>
<th>No. of type III foci</th>
<th>Total No. foci</th>
<th>Foci index No. of foci per dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.4ΔP</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4/6</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35</td>
<td>8</td>
<td>43/6</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
<td>9</td>
<td>59/6</td>
<td>9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>49</td>
<td>11</td>
<td>60/6</td>
<td>10 ± 1.1</td>
</tr>
<tr>
<td>16.3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1/6</td>
<td>0.2 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>6/6</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1</td>
<td>2</td>
<td>3/6</td>
<td>0.6 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
<td>3</td>
<td>5/6</td>
<td>0.8 ± 0.12</td>
</tr>
</tbody>
</table>

Total numbers of foci are from two individual assays using six dishes per assay. The number of foci is the average of both assays.

**Figure 2.** Morphology of a representative type III colony of 19.4ΔP cells treated with MNU using ×10 magnification.
growth of many normal eukaryotic cells is dependent upon attachment to a matrix, transformed cells are often able to grow in suspension in semi-solid media such as agar. Hence, anchorage-independent growth is considered a marker of transformed fibroblast and epithelial cells (31,32). We randomly selected four 19.4AP MNU-transformed cell lines and two 16.3 MNU-transformed cell lines to test in an anchorage-independent growth assay. All of these cell lines had formed type III foci following MNU exposure as described in the preceding section. Large distinct colonies were formed in agar dishes with all four 19.4AP transformed cell lines. In contrast, although few small colonies were detected in agar dishes with the 16.3 transformants, these colonies disappeared after 3–5 days. After 7 days of growth in agar, 19.4AP colonies were picked from agar dishes and grown in mass culture for injection into nude mice. To minimize the numbers of mice required, we injected cells from just one of the lines, 19.4AP#53 (from #53 colony) into nude mice. Tumors appeared at multiple sites in these mice within 4 weeks (Figure 3); tumors were observed in 100% of the mice injected with 19.4AP#53 cells (Table III). Histopathological examination revealed that the tumors that developed were fibrosarcomas (cells were fibroblasts). These results demonstrate that MNU-treated 19.4AP cells that display anchorage-independent growth in culture have neoplastic activity in vivo. As expected, no tumors formed in nude mice inoculated with 16.3 cells expressing WTpol protein even after 4 months. Since MNU-transformed 16.3 cells did not survive in agar, these cells were not injected into mice. Interestingly, tumors did not develop in nude mice injected with parental 19.4AP cells that were not exposed to MNU. These data suggest that polβ expression alone is not sufficient to cause tumor induction in this model system; another factor, e.g. MNU or another DNA-damaging agent, is required. This is consistent with the findings of Bergoglio et al. (33) who suggested a single polβ over-expression event was not sufficient to initiate tumorigenesis in vivo.

Table III. Tumorigenic activity of transformed 19.4AP cells in nude mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth in soft agar</th>
<th>Tumorigenicity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>–</td>
<td>0/5</td>
</tr>
<tr>
<td>Untreated 16.3</td>
<td>–</td>
<td>0/5</td>
</tr>
<tr>
<td>Transformed 16.3</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Untreated 19.4AP</td>
<td>–</td>
<td>0/5</td>
</tr>
<tr>
<td>Transformed 19.4AP</td>
<td>+</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Tumorigenicity in nude mice injected subcutaneously with soft agar-selected transformed 19.4AP cells. Results are expressed as number of mice with tumors per total number of mice injected with control or transformed cells.

aExpressed as number of mice with tumors per total number of mice injected with control or transformed cells. ND: not determined.

Polβ expression and DNA repair activity in transformed 19.4AP cells and the tumors that they form in nude mice

To evaluate whether the transformation or tumor formation processes modified the expression or function of polβ, we performed western blot analysis using extracts from 19.4AP anchorage-independent MNU-transformed cells (clone #53) and two tumors that grew in nude mice inoculated with 19.4AP#53 cells (#29 and #53). Cell extracts from 16.3 and 19.4AP (non-MNU treated) cells were used as controls. As shown in Figure 4A, a 36 kDa protein corresponding to the polβ deletion mutant was observed in the 19.4AP#53 cell extract (lane 2) and there was no expression of WTpol protein. This was expected since these cells originated from untransformed 19.4AP cells that do not express WTpol protein (lane 1). The 36 kDa mutant protein was also expressed in extracts from tumor tissues #29 and #53 (lanes 3 and 4) along with the 39 kDa endogenous polβ protein. As expected, the 39 kDa WTpol protein and the 36 kDa mutant polβ protein were expressed in 16.3 (lane 5) and 19.4AP cells (lane 1), respectively. The bottom panel demonstrated equal loading by 41 kDa β-actin protein expression. These data indicate that expression of the deleted form of polβ is maintained in the 19.4AP cells throughout their transformation and growth as tumors in nude mice.

To test polβ function, we measured gap-filling DNA synthesis activity in nuclear extracts from the 19.4AP#53 cell lines and tumor#29. Nuclear extracts from 16.3 cells were used as a positive control. A 51 bp repaired product was identified in all tested nuclear extracts (Figure 4, panel B). The intensity of the repaired product in tumor#29 was comparable with that generated by the 16.3 nuclear extract. This result suggests that the endogenous WTpol protein expressed in tumor#29 provides sufficient catalytic activity to generate normal levels of DNA repair activity. In contrast, decreased gap-filling DNA synthesis activity was observed in the 19.4AP and 19.4AP#53 nuclear extracts. This is expected since these cells express only the mutant polβ protein that has a deletion of 29 amino acid residues within the catalytic domain. Since we reported previously that MCF-7 breast cancer cells expressing 39 kDa WT polβ protein exhibit efficient repair activity (34), we used a nuclear extract from MCF-7 cells as an additional control in this experiment. The data indicate that polβ-mediated DNA repair is deficient in 19.4AP cells due to their expression of the deleted form of polβ. This deficiency is maintained through the transformation process as demonstrated by the 19.4AP#53 cell line. Tumors derived from these cells, however, express endogenous as well as mutant polβ and therefore demonstrate WT gap-filling DNA synthesis activity.

![Fig. 3. Representative appearance of multiple tumors in nude mice injected with transformed 19.4AP cells.](https://academic.oup.com/carcin/article-abstract/28/6/1356/2476250/fig3)

![Fig. 4. (A) Western blot analysis of polβ and polβΔ proteins in cell extracts (100 μg per lane) from 19.4AP and 19.4AP#53 cells and nude mouse tumors. Lane 1, untransformed 19.4AP cells; lane 2, #53 clone of transformed 19.4AP cells selected in soft agar assay; lanes 3 and 4, tumor#29 and tumor#53 from nude mice and lane 5, 16.3 positive control. Upper panel shows 36 and 39 kDa polβ proteins. Lower panel shows equal loading by expression of 41 kDa β-actin. (B) Gap-filling DNA synthesis activity assay. Nuclear extracts (50 μg) from 19.4AP cells, 19.4AP#53 cells, tumors #29 and #53 and 16.3 cells were tested for their ability to generate a 51 bp repaired DNA product. Lane MCF-7, nuclear extract of MCF-7 cells used as marker.](https://academic.oup.com/carcin/article-abstract/28/6/1356/2476250/fig4)
Generation of transgenic mice expressing mutant polβ in the mammary glands

To direct expression of polβD208–236 in transgenic mice, we chose the WAP promoter (35–37). The advantage of using the WAP promoter is that it is highly expressed in the mammary glands of pregnant mice (35–37). Our strategy was to establish transgenic mice expressing both endogenous polβ and polβD that would mimic expression of polβ identified in human tumors (18–23). The transgenic construct is illustrated in Figure 5A. Digestion of the plasmid polβD/WAP3’/WAP5’ by BssHII separated the 2.9 kb transgene from the 3 kb vector (Figure 5B) prior to microinjection. Sixteen founder pups were born and DNA prepared from tail clips was analyzed for the presence of the transgene using DNA PCR with a WAP+1 forward primer and a polβ reverse primer. A 245 bp PCR product of the expected size was identified in two founder mice (Figure 5C, lanes 4 and 5). The PCR product was not detected in the remaining 14 animals (represented by two mice, lanes 2 and 3), indicating that the transgene was not transmitted to the DNA of these animals. DNA samples from all 16 mice were further analyzed by Southern blot by digestion with SpeI and HindIII and hybridization to a polβD cDNA probe. Figure 5D shows a hybridizing fragment of ~960 bp in two transgenic mice shown in lanes 5 and 6 (shown by an arrow). DNA samples shown in lanes 4 and 5 (PCR) and lanes 5 and 6 (Southern) were from same positive mice, respectively. These results confirm that the polβD transgene was transmitted to two founder mice whereas the other 14 animals were non-transgenic. The Southern blot also shows fragments >2 kb that hybridized to the transgene probe. These fragments might be due to incomplete digestion of the genomic DNA. Lane 8 shows the ~960 bp probe used as a marker.

Balb/c mice have been reported to be susceptible to MNU-induced mammary tumorigenesis (38,39). Hence, the two positive founder animals were mated with normal Balb/c mice to establish a transgenic mouse line. Eighteen pups were born from these matings. Tail DNA from the F1 pups was examined by Southern blot to confirm that the transgene had been transmitted. As shown in Figure 5E, the 960 bp band indicating the presence of the transgene was observed in samples from pups #1–3, 5, 8, 10, 11 and 16–18. F1 animals #4, 6, 7, 9 and 12–15 were transgene negative. These results demonstrate that the WAP-polβD construct is not lethal during mouse embryogenesis.

To evaluate whether the WAP-polβD transgene was expressed in the mammary glands of transgenic mice, cell extracts were made from the mammary glands of two F1 mice (#1 and #2), 1 week after delivery of littersm as WAP is expressed when prolactin and progesterone are at high levels in serum. Figure 6A shows expression of both the transgene 36 kDa polβD protein and the endogenous 39 kDa polβ protein in the mammary glands of both F1 mice (lanes 1 and 2). Positive controls included 39 kDa WTPolβ protein expressed in the 16.3 cell line (lane 3) and a 39 kDa polβ recombinant protein (lane 4) purified in our laboratory (29). Figure 6B shows that only WTPolβ (39 kDa) and not the transgene-encoded deleted form is expressed in lung, liver, stomach, heart, kidney and spleen (lanes 1–6, respectively). These results strongly suggest that expression of the transgene is limited to the mammary glands, as expected based upon the known tissue specificity of the WAP promoter. Breeding to Balb/c mice was...
repeated six times to generate F6 transgenic mice that were at 98.2% of Balb/c background. Animals were maintained in our institution’s animal facility strictly according to National Institutes of Health–AAALAC guidelines.

Mammary carcinogenesis in transgenic mice expressing the polβ deletion mutant

In order to examine tumorogenesis in transgenic mice expressing polβΔ in the mammary glands, we first established the LD₅₀ of MNU in these mice. Transgenic animals were given MNU at a dose of 25–200 mg/kg body wt intra-peritoneally once a week for 3 weeks (at 7 and 14 days of pregnancy and 1 week after litters were born). MNU at a dose of 100 mg/kg body wt was found to be lethal, establishing 50 mg/kg body wt as the LD₅₀ (data not shown).

To compare the susceptibility of transgenic and WT mice to MNU-induced tumorigenesis, pregnant female transgenic OCF6 generation (~98% Balb/c background) and normal pregnant female Balb/c mice (of similar age and genetic background as transgenic animals) were treated on identical schedules with MNU at 50 mg/kg body wt. Intraperitoneal injections were given for 7 weeks, starting at day 7 of pregnancy and continuing to the fourth week of the lactation period. Pups were kept with their mothers so that nursing would maintain high levels of prolactin in the serum important for expression of the WAP-driven polβΔ transgene. Mammary tumors were identified by palpation and were first detected in MNU-treated transgenic mice at 12 weeks after MNU injection. In contrast, tumors did not appear in similarly treated control mice until 28 weeks (Figure 7). Thus, transgene expression is correlated with an earlier appearance of MNU-induced mammary tumors.

Moreover, the percentage of transgenic mice that developed MNU-induced mammary tumors (70%) was significantly higher than for control mice (22%) (Table IV). Mammary tumors were identified at both upper right and left sides of upper and lower body of transgenic mice treated with MNU. All mammary tumors were identified by the Mouse Phenotyping Service, Ohio State University, Columbus, OH, mice treated with MNU. All mammary tumors were identified by the Mouse Phenotyping Service, Ohio State University, Columbus, OH, as adenosquamous carcinomas (Figure 8, lower panel). The histology of a normal mammary gland from an untreated Balb/c mouse is shown in Figure 8, upper panel, used as a control. In addition to mammary tumors, MNU-treated transgenic mice also had enlarged spleens and bronchioalveolar carcinomas with intra-pulmonary metastases and adenomas in their lungs. Hemangiosarcoma in spleens and carcinomas in ovaries were found in transgenic mice treated with MNU. Interestingly, Balb/c control mice treated similarly did not develop any tumor in the mammary glands or any other organ until 28 months after treatment. Strikingly, on average, two to four adenomas were found in lungs of each of control mouse, whereas 10–20 adenomas were identified in lungs of each transgenic mouse. Neither transgenic nor control mice treated with PBS developed tumors as of 16 months after treatment. Taken together, these results indicate that expression of the polβΔ transgene in the mammary glands potentiates MNU-induced tumorigenesis as evidenced by a shortened latency period and increased tumor incidence.

Discussion

DNA polβ is essential for filling the one-nucleotide gap at apurinic–apyrimidiné sites in damaged DNA via the short-patch BER pathway. In contrast, the long-patch BER pathway is largely dependent on polβ in cell extracts, but can be reconstituted with polδ, polε, proliferating cell nuclear antigen and flap endonuclease1 (1,2). A deletion variant of polβ encoding a 36 kDa protein (polβΔ) with diminished DNA repair activity is expressed in various human cancers. The molecular details of how the deleted form of polβ impacts DNA repair are not completely understood. The polβΔ mutant like WTpolβ has been shown to interact with XRCC1, poly(ADP-ribose) polymerase, apurinic endonuclease and MGC5306 in vitro and in vivo (29,40). MGC5306 is a recently identified novel protein that binds not only to polβΔ but also to WTpolβ (40). Although the purified polβΔ protein is capable of efficiently repairing apurinic–apyrimidiné sites, a presumed binary complex of polβΔ and XRCC1 is incapable of filling these gaps. This complex binds more strongly to a gapped
DNA strand than a complex of WTpolβ and XRCC1. Thus, polβA bound to XRCC1 inhibits functions of the WT polβ in a dominant-negative fashion (29).

To investigate the biological consequences of expression of the dominant-negative deletion mutant of polβ, we used two animal models: nude and transgenic mice. Results from both studies demonstrated that cells expressing the polβ mutant protein displayed increased sensitivity to MNU, an environmentally related DNA-damaging agent, which resulted in enhanced tumorigenesis in vivo. When exposed to MNU, MEF cells expressing only the mutant form of polβ protein exhibited altered morphology. Cells further selected for anchorage-independent growth in soft agar induced multiple tumors in 100% of injected nude mice. Two other polβ variants identified in colorectal and prostate tumors (18,24) were expressed in murine mammary tumor cells and found to induce focus-forming transformation and anchorage-independent growth (41).

The effect of polβA on tumor formation was also evaluated in a transgenic mouse model of MNU-induced mammary carcinogenesis. For this purpose, we created a transgene construct in which polβA208–236 expression is controlled by the strong mammalian tissue-specific promoter, the WAP promoter (35–37). The construct also uses an 843 bp of 3’ WAP sequence that contains a 70 bp untranslated region including a poly A region. These sequences have been shown to determine tissue specificity and integration site, independent of transgene expression (35,42). The advantage of using the WAP promoter for our study is that it is highly expressed in mammary glands of pregnant transgenic mice (35–37). The prolactin hormone secreted during pregnancy stimulates the ovaries to secrete progesterone and estradiol. This combination of hormones in pregnant mice causes proliferation of mammary epithelial cells that enhances chemical- or oncogene-mediated mammary tumorigenesis (35–37,39,42). In addition, a number of transgene constructs driven by the WAP promoter have been successfully used to study mammary carcinogenesis (36,37). The data presented in this study demonstrate that the polβA208–236 transgene was effectively expressed in the target organ of the WAP-driven polβA transgene, the mammary glands. Since the transgenic mice express endogenous WTPolβ in addition to the transgene-encoded mutant polβ, this transgenic model mimics the observed expression of polβ (WT and mutant) in many human tumors when normal control mouse expressing WTPolβ mimics normal matched tissues (20,22,23). Our analysis of control and transgenic pregnant mice exposed to MNU showed that transgene expression resulted in a higher incidence of tumor formation, with tumor forming in multiple mammary glands of each animal. Furthermore, the latent period prior to tumor appearance was significantly shorter in transgenic animals than in control mice.

Taken together, these accumulated results unequivocally demonstrate that expression of the polβA protein enhances acquisition of neoplastic phenotype following exposure of cells to a DNA-damaging agent. Furthermore, since the polβA protein has this effect despite expression of WT endogenous polβ in the same cells, these data support the hypothesis that polβA acts as a dominant-negative mutant. This transgenic model expressing a mutant repair gene will be useful for future studies at improving our understanding of the mechanisms underlying human oncogenesis.

Based upon the impaired DNA repair activity of the polβA mutant protein, it is tempting to speculate that expression of polβA might lead to persistence of unrepaird MNU-induced DNA lesions, resulting in mutagenesis that might lead to cellular transformation. In this way, polβA expression might have significant effects on the development of human cancers.

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References


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