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Abstract

Since telomerase expression is highly prevalent in human cancers, the quantitation of serum/plasma hTERT mRNA levels may be useful for early detection of pancreatic cancer (PCa). To analyze the correspondence between extracellular hTERT (exhTERT) mRNA levels and hTERT expression, we designed a cell culture system to investigate factors modulating the extracellular levels of hTERT mRNA in media conditioned by 8 PCa cell lines. We found that the level of exhTERT mRNA was dependent on cell growth rate. MIAPaCa-2, PANC-1, KLM-1 and PK-9 cells expressed high levels of exhTERT mRNA, independent of cell density, whereas proliferating PK-59, BxPC-3 and PK-45H cells released low levels of exhTERT mRNA. The augmented release of mRNA by spontaneous dead MIAPaCa-2 cells was further increased at post-confluence. In Capan-1 cells, low correspondence of marker was also due to RNase secretion. Upon reaching confluence, some PCa cell lines showed down-regulation of hTERT expression. Following cell–cell adhesion, as shown by E-cadherin engagement, PK-59 cells showed levels of extracellular message below the limits of detection, a loss not due to an increase in message degradation. These results suggest that the levels of exhTERT mRNA in the medium of PCa cell lines are altered not only in response to cell growth rate and cell destruction, but are responsive to extracellular cues such as RNases and cell density. A cell-free assay for exhTERT mRNA may therefore not be useful for early detection of PCa.

Keywords: Extracellular hTERT mRNA; cell-cell adhesion; pancreatic cancer; tumour marker; real-time RT-PCR; conditioned medium

Abbreviations: hTERT, human telomerase reverse transcriptase; ExhTERT, extracellular hTERT; PCa, pancreatic cancer; RT-PCR, reverse transcription-polymerase chain reaction; mAb, monoclonal antibody; IgG, immunoglobulin G; DMEM, Dulbecco’s modified Eagle’s medium; FBS, foetal bovine serum; SF, serum-free; PBS, Phosphate-buffered saline.

1. Introduction

Pancreatic cancer (PCa) has the lowest survival rate among human solid cancers. It is quite difficult to detect PCa in the early stages and to differentiate between PCa and other benign pancreatic diseases. Reliable diagnostic markers are one of highly potent requisites for detecting PCa. CA19-9, a conventional serum marker used at the advanced stage of PCa, often suffers from low diagnostic specificity (Sawabu et al., 2004). Human telomerase activity (Avilion et al., 1996) and its catalytic component, human telomerase reverse transcriptase (hTERT) (Chen et al., 2000; Dasi et al., 2001; Miura et al., 2006; Pelosi et al., 2006), are promising potential diagnostic markers for cancer screening. However, expression of hTERT has been found in some types of normal cells and activated lymphocytes (Kolquist et al., 1998) and about 20% of human cancers have been reported to have lost the telomerase activity (Bryan et al., 1995). Recently, Hiyama and Hiyama (2003) suggested that hTERT mRNA may be a better marker than telomerase activity. High levels of hTERT mRNA have been observed in more than 80% of cancers, with upregulation occurring during early stages of carcinogenesis. However, extracellular hTERT (exhTERT) mRNA showed low (Pelosi et al., 2006) or high (Miura et al., 2006) diagnostic sensitivity as a marker. The level of exhTERT mRNA in plasma/serum was not always corresponding to that in several cancer tissues (Chen et al., 2000; Dasi et al., 2001; Pelosi et al., 2006). Although the reasons for these disadvantages remain to be clarified, the disadvantages are often associated with low levels of circulating exhTERT mRNA in patients with hTERT-positive cancers. These low levels were thought to be due to the small degree of cancer cell destruction and release of RNases (Miura et al., 2006) or to cancer type (Pelosi et al., 2006).

Kopreski et al. (1999) suggested that the tyrosinase mRNA in sera from melanoma-bearing patients was not so unstable as had previously been thought. Previous reports (Stroun et al., 1978; Halicka et al., 2000; Tsui et al., 2002; El-Hefnawy et al., 2004; Garcia et al., 2008) also suggested that cell-free mRNA is stabilised in certain vesicular forms in culture supernatants or in plasma/serum and protected to a considerable extent from digestion by extracellular RNases. Both

passive (Halicka et al., 2000) and active (Stroun et al., 1978; Garcia et al., 2008; Skog et al., 2008; Hong et al., 2009) mechanisms of release of cell-free mRNA from cancers have been proposed.

In our preliminary studies, exhTERT mRNA was sometimes hardly detected in the conditioned medium of some hTERT-positive PCa cell lines. Further, studies with *in vitro* culture systems have been suggested to exclude some cancer characteristics such as lymphocytic infiltration and tumour-lymphovascular interactions (Tassi and Wellstein, 2006) and to introduce into quantitative analysis (Kulasingam and Diamandis, 2008) in cancer detection research. Thus, investigating the association of factors regulating exhTERT mRNA levels with cellular hTERT expression or with cell numbers in cell culture may provide insight into use of exhTERT mRNA as a diagnostic marker of PCa.

The purpose of this study was to investigate the factors that affect the detection of exhTERT mRNA marker in *in vitro* culture systems. Using real-time reverse transcription (RT)-polymerase chain reaction (PCR) detection, we quantified the amounts of exhTERT mRNA in cultured PCa cell lines with different growth rates and hTERT expression levels. Our results suggested that high cell density reduces the message release to the background level and results in the possibility of false negative detection in some low-release PCa cell lines. The results of the present study should help our understanding of exhTERT mRNA as a marker and its application for early PCa detection.

2. Material and methods

2.1. Cell lines and culture conditions

MIAPaCa-2, PANC-1, BxPC-3, KLM-1, PK-9, PK-45H and PK-59 cell lines were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. MIAPaCa-2 and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (FBS; Invitrogen). BxPC-3, KLM-1, PK-9, PK-45H and PK-59 cells were grown in RPMI 1640 medium

(Invitrogen) supplemented with 10% FBS. Capan-1 cells (a gift from Dr. Hiroyasu Esumi, National Cancer Center Research Institute East) were cultured in DMEM supplemented with 20% FBS.

2.2. Cell cultures in serum-free (SF) conditions

To avoid the effects of RNases in FBS, SF chemically defined media were used for some cells. Cosmedium 001 (Cosmo Bio Co., Tokyo, Japan) was used for PK-59 cells and Advanced DMEM/F12 medium (Invitrogen) supplemented with L-glutamine was used for MIAPaCa-2, PANC-1 and Capan-1 cells together with substrate coated with fibronectin (Sigma, St. Louis, MO). Both media contained insulin and transferrin.

2.3. Assay of cell growth rates and quantification of nonviable cells

Cells were seeded at a density of 1–3×10^5 cells per 60-mm dish (Falcon; Becton Dickinson, Franklin Lakes, NJ) and fresh serum-supplemented or SF media were supplied every 3 days after plating. The percentage of dead cells was quantified by the trypan blue dye-exclusion method, which can detect both apoptotic and necrotic cells. The average population doubling time was calculated from the viable cell number in exponential growth phase. Viable and nonviable cells were counted using a haemocytometer; a minimum of 500 cells were counted in total.

2.4. Extraction of cellular RNA and extracellular RNA fractions

Cellular RNA was extracted using ISOGEN-LS reagent (Nippon Gene, Tokyo, Japan). Prior to preparing conditioned media, cell cultures were washed once with prewarmed fresh medium (serum-containing or SF medium), 1.5 mL (60-mm dish) or 4 mL (100-mm dish) of fresh medium was added, and the cells were further incubated for 12 hours. Following collection of conditioned media, the supernatants were obtained after two steps of centrifugation as described previously (Tsui et al., 2002; Garcia et al., 2008). Extracellular RNA was extracted from samples of 0.35 mL to 0.7 mL of the cell-free media. RNA was stored at –70°C in 75% ethanol before use.

2.5. RT and PCR amplification

RNA was pre-treated with DNase 1 (Promega Corp., Madison, WI). RT reaction was performed using SuperScript III reverse transcriptase (Invitrogen) in a final volume of 20 μL in accordance with the manufacturer's instructions. PCR amplification was performed with AmpliTaq Gold (Roche Diagnostics, Mannheim, Germany).

2.6. Quantitative real-time PCR analysis

Real-time PCR was performed on a LightCycler thermal cycler system (Roche Diagnostics). Primers for hTERT, which produced an amplicon of 122 bp and did not target some splice variants, were as follows: forward, 5′-AACTCTTTGGGGTCTTGC-3′, reverse, 5′-GTGAAACCTGTACGCCTG-3′. The following primers for 18S rDNA, which produced an amplicon of 96 bp, were also applied: forward, 5′-CCAGTAAGTGCGGGTCATA-3′, reverse, 5′-GGGCCTCACTAAACCATCC-3′. cDNA was amplified with a pre-cycling hold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, combined annealing-extension at 64°C for 8 s, and 72°C for 12 s, followed by reading of fluorescence emission at 78°C for 2 s. Amplification was carried out in a final volume of 15 μL containing 5 pmol each primer, 4 mM $MgCl_2$, reverse-transcribed cDNA and 1× DNA master SYBR Green 1 mix. Amplified products were analysed using 3% NuSieve 3:1 agarose gels (Cambrex Bio Science, Rockland, ME) and visualised by staining with SYBR Gold (Molecular Probes Inc., Eugene, OR). The amplification specificity was also monitored by LightCycler melting curve analysis. Recombinant pGEM easy T-vectors (Promega) containing either hTERT cDNA or 18S rDNA were used as standards for calibration. The amplification ranges for hTERT cDNA and 18S rDNA were linear over 7 (10^2-10^9 copies per reaction) and 4 (10^3-10^7 copies per reaction) logarithmic dilutions, respectively. If required, the data for 18S rDNA obtained after RT and PCR amplification were used to normalise the sample-to-sample variation in the amount of input DNA. The hTERT mRNA levels were

determined from the relative ratio between the crossing point (Cp) of (hTERT:18S) sample divided by (hTERT:18S) calibrator.

2.7. Assay of message stability in the conditioned media of confluent PK-59 cells

RNA degradation was examined by monitoring the decay of exhTERT mRNA obtained from SF cultures. A combination of 0.35 mL of conditioned medium ($4.2–4.8\times10^4$ copies of exhTERT mRNA) derived from proliferating PK-59 cells and 0.35 mL of that from confluent cells was applied for incubation at 37°C. After incubation, RNA was extracted using ice-cold Isogen-LS reagent, reverse-transcribed and quantified by real-time PCR assay.

2.8. Treatment with E-cadherin neutralising antibody

Exponentially growing cells were harvested and washed in phosphate-buffered saline (PBS). The cells (10^5 cells/mL) were seeded on non-coated or fibronectin-coated 6-well plates (Falcon) and cultured in the presence or absence of 10 μg/mL of mouse anti-E-cadherin monoclonal Ab (mAb) HECD-1 (Takara Bio, Otsu, Japan) for 12 hours under SF conditions. Mouse immunoglobulin G (IgG) (Dako, Carpinteria, CA) was used as a control.

2.9. Immunoblotting analysis

Cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail (Sigma). Cell lysates (20 μg of protein) were fractionated by 7.5% SDS-PAGE. The proteins blotted onto polyvinylidene difluoride membranes were analysed using HECD-1 or anti-actin antibody. The bound antibody was detected using peroxidase-conjugated secondary reagents and an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

2.10. Statistical analysis

Student's *t* test was used for statistical analyses and $P < 0.05$ was considered significant.

3. Results

3.1. Quantification of hTERT expression in 8 PCa cell lines

The doubling time of 8 PCa cell lines varied from 27 to 48 hours (Fig. 1A). Cellular levels of hTERT expression, normalised by 18S rRNA, were higher in the cells with a shorter doubling time. The rapidly growing cells, such as MIAPaCa-2, PANC-1, KLM-1 and Capan-1 cells, with doubling times of 27 to 31 hours, showed high levels of hTERT expression over a range of $3–5\times10^{-3}$. The hTERT expression levels of slow-growing BxPC-3 and PK-45H cells were less than 2×10^{-3}, and cells with moderate growth rates, PK-9 and PK-59, showed moderate hTERT expression levels ($2–4\times10^{-3}$). Thus, hTERT expression was correlated with the growth rates of PCa cell lines *in vitro*. In contrast, quantitative analyses showed high $\mathrm{mean} \pm \mathrm{SD}$ levels of expression of 18S rRNA in MIAPaCa-2 ($2.68 \pm 0.15 \times 10^9$ copies/μL) and PANC-1($2.53 \pm 0.20 \times 10^9$) cells, intermediate levels of expression in KLM-1($1.50 \pm 0.15 \times 10^9$) and BxPC-3 ($1.71 \pm 0.25 \times 10^9$) cells and lower levels ($<1.15 \times 10^9$ copies/μL) in the other PCa cell lines, indicating that the expression of 18S rRNA was unrelated to the doubling potentials of PCa cell lines.

3.2. Time course of exhTERT mRNA accumulation in the conditioned media

To examine the relationship between exhTERT mRNA level and cell growth rate, mid-log phase (day 5) cultures of MIAPaCa-2, KLM-1 and PK-59 cells were analysed (Fig. 1B). The exhTERT mRNA levels for MIAPaCa-2 and KLM-1 cells were always higher than those of PK-59 cells, suggesting that exhTERT mRNA accumulation is related to cell growth. In contrast, the levels of extracellular 18S rRNA in the conditioned media of KLM-1 and PK-59 cells ranged from 0.6 to 2.0×10^9 copies, and that from MIAPaCa-2, in which the extracellular 18S rRNA level increased with cell density, ranged from 0.6 to 7.5×10^{10} copies (Fig. 1C). Thus, cell-free 18S

rRNA was accumulated in KLM-1 and PK-59 cells in a manner unrelated to cell growth.

3.3. Influence of cell density on exhTERT mRNA levels in serum-supplemented cultures

The exhTERT mRNA levels were compared between cultures at two cell densities: low cell density (L), under which the cells continued to proliferate, and high cell density (H) in stationary phase (Fig. 1D). No significant differences according to cell density were found in the media of MIAPaCa-2, PANC-1, KLM-1 or PK-9 cells, which showed high exhTERT mRNA levels (average 0.5–1.2×10^6 copies/10^6 cells, L and H) ($P > 0.05$). In contrast, exhTERT mRNA was almost undetectable in the media of confluent PCa cells that showed low message levels of less than 3×10^5 copies/10^6 cells under conditions of cell proliferation (L).

3.4. Density-dependent down-regulation of hTERT expression in PANC-1, PK-59 and Capan-1 cells cultured in SF media

Under SF culture conditions, the average cellular levels of hTERT expression in PANC-1 and PK-59 cells were 1.38 and 1.32×10^{10} copies/10^6 cells at low cell density (L), respectively (Fig. 2A). At high cell density (H), both of these cell lines and Capan-1 cells showed significant decreases in hTERT expression, with levels of 43%, 42% and 50% of those under conditions of cell proliferation, respectively. In contrast, hTERT expression in MIAPaCa-2 cells was augmented by increasing cell density ($P < 0.05$). These results suggest that hTERT expression in some PCa cell lines is down-regulated in a density-dependent manner.

3.5. Density-dependent message loss in the media of PK-59 and Capan-1 cells of SF cultures

ExhTERT mRNA was undetectable in confluent cultures of PK-59 and Capan-1 cells (H; Fig. 2B, right), even under culture conditions where serum RNases were excluded. In contrast, the exhTERT mRNA level of confluent PANC-1 cells (H) was similar to that under low-density culture conditions (L) ($P > 0.05$), whereas that of MIAPaCa-2 cells was augmented by cell density

(Fig. 2B, left) ($P < 0.01$). We next estimated the apparent release rate of exhTERT mRNA from each type of proliferating cells into the culture media (L). Apparent release rate was calculated by division of extracellular hTERT mRNA by intracellular hTERT mRNA per hour. The release of cellular hTERT mRNA into the media of MIAPaCa-2 and PANC-1 cells was $1.1–1.4\times10^7$ copies/hour, whereas that for PK-59 cells was about 9×10^5 copies/hour, indicating a low release in PK-59 cells.

3.6. Density-dependent message loss in response to cell–cell adhesion interactions in PK-59 cells

To directly confirm the density-dependent changes in exhTERT mRNA, its levels were analysed in cultures of PK-59 cells at 4 different growth phases, *i.e.*, 2, 4, 6 and 8 days after cell seeding. The 122-bp hTERT cDNA product was detected in the first 3 phases depending on cell growth in culture (lanes 1 to 3, Fig. 3), whereas no product was observed in the media of cultures in stationary phase carrying 1.3×10^5 cells/cm^2 (lane 4). These results suggested the reduction of exhTERT mRNA to the background level (< 30 copies/10^6 cells) at confluence in cultures of PK-59 cells. In contrast, the level of extracellular 18S rRNA was not altered during the experimental periods.

3.7. Influence of cell density on MIAPaCa-2 and PK-59 cell viability

Spontaneous cell death can also be found in cancer cells at the post-confluent stage (Hosick, 1976). A mechanism by which RNA is packaged and separated from dead cells has been suggested (Halicka et al., 2000). We examined whether changes in exhTERT mRNA level in MIAPaCa-2 and PK-59 cells may respond to this in our culture system. Approximately 3.4% of confluent PK-59 cells (H) were positive on trypan blue dye-exclusion test (Fig. 4). The frequencies of dead cells did not increase in proliferating PK-59 cells (L), which were exhTERT-positive. In contrast, approximately 4.3% and 11.8% of MIAPaCa-2 cells showed spontaneous cell death in cultures before (L) and after (H) reaching confluence, respectively. The

results suggested that high cell density causes a significant increase in spontaneous cell death in MIAPaCa-2 cells ($P < 0.01$), resulting in an increase in exhTERT mRNA.

3.8. Little augmentation of message degradation outside confluent PK-59 cells

The stability of cell-free RNA has been examined by monitoring its degradation on incubation with plasma/serum (Tsui et al., 2002; El-Hefnawy et al., 2004). We examined the possible mechanism of message loss due to possible increases in extracellular RNase levels. When the stability was monitored on incubation with media (Fig. 5), about 5%–8% message loss was observed even on prolonged exposure for 2 hours, with stability almost identical to that in the unconditioned control medium. As a control, the media derived from confluent Capan-1 cells, a PCa cell line that shows RNase outside the cells (Fernandez-Salas et al., 2000) were combined with the exhTERT mRNA of PK-59 cells. Message degradation, with a half-life of about 80 min, was observed (Fig. 5). These results suggested that the density-dependent message loss in PK-59 cells was not attributable to an increase in RNA degradation in culture medium.

3.9. Cell–cell adhesion accompanied by message loss

Cell–cell adhesion is maintained by E-cadherin, which mediates epithelial adherens junction organisation. Anti-E-cadherin antibody mimicked the formation of cadherin-mediated cell–cell interactions *in vitro* (St. Croix et al., 1998; Symons et al., 2002). We examined whether the antibody treatment resulted in changes in exhTERT mRNA levels. PK-59 and MIAPaCa-2 cells were treated with anti-E-cadherin antibody (mAb as in Fig. 6A) or control immunoglobulin for 12 hours, leading to loss of message from the cultured medium of PK-59, but not MIAPaCa-2, cells (Fig. 6A). There was no significant difference in the levels of extracellular 18S rRNA, suggesting that the antibody treatment had no effect on RNA metabolism in the two PCa cell lines. To assess whether E-cadherin expression contributed to the observed differences in antibody response, we assayed E-cadherin expression in these cell lines by Western blotting (Fig.6B). MIAPaCa-2 cells

showed no expression of E-cadherin, consistent with observations reported previously (Nakajima et al., 2004), whereas it was detected in PK-59 cells. These results suggested that E-cadherin-mediated cell–cell adhesion may be involved in message loss outside the cells.

4. Discussion

There have been little preclinical studies regarding the relationship between the levels of hTERT expression and exhTERT mRNA. In this study, using four culture conditions, including medium with and without serum, in addition to different conditions from logarithmic growth to confluence, we investigated what factors determine the levels of exhTERT mRNA in the conditioned medium of cultured PCa cells. The present study suggested the involvement of at least four factors that influenced *in vitro* modulation of exhTERT mRNA levels: cell growth rate, cell density, RNase and cell destruction.

First, our results revealed high exhTERT mRNA levels in cultures of rapidly growing PCa cells, such as MIAPaCa-2, PANC-1 and KLM-1 cells, whereas low levels were observed in slow-growing BxPC-3 and PK-45H cells. Under SF culture conditions, apparent release rates were approximately 10-fold higher in proliferating MIAPaCa-2 and PANC-1 cells than in proliferating PK-59 cells. Thus, cell growth rate is one of the factors determining exhTERT mRNA levels *in vitro*.

Second, the hTERT expression was down-regulated in confluent cultures of PANC-1, PK-59 and Capan-1 cells by about 50% to 58% of the levels found in the corresponding proliferating cells. Although the down-regulation of hTERT activity has been reported in quiescent mouse cells (Holt et al., 1996), to our knowledge, this is the first report of the down-regulation of message levels in human cancer cells at confluence. The observation that this effect is mediated by cell density is interesting because down-regulation was often accompanied by message loss in low-release PCa cells. Although there is no direct evidence linking both observations, the

message loss appears to be closely coupled with cell–cell adhesion mediated by E-cadherin and growth arrest in PK-59 cells. We postulated that cell–cell adhesion may then cause a decrease in message release to below the limit of detection, resulting in message loss.

Third, the results of combination experiments indicated that the exhTERT mRNA of PK-59 cells was substantially stable. We did not directly address RNases, but it was unlikely that the exhTERT mRNA loss in PK-59 cells was due to an increase in degradation by RNases. The cell-free RNA was also shown to be stable for several hours, as reported previously (Tsui et al., 2002; El-Hefnawy et al., 2004). In contrast, the instability observed in Capan-1 cells may be due to RNases, although the mechanism by which they cause lability of enclosed mRNA is not yet known. The exhTERT mRNA levels may depend on both the rate of message supply and rate of degradation in the medium of RNase-secreting cancer cells. Our results indicating a change in positivity in Capan-1 cells under SF condition in culture support this suggestion.

Fourth, MIAPaCa-2 cells have a defect in adhesion-regulated control, known as contact inhibition, loss of which is often found in E-cadherin-deficient, poorly differentiated cancer cells (St. Croix et al., 1998; Motti et al., 2005). Among the 8 PCa cell lines examined, only MIAPaCa-2 cells showed high message levels in post-confluent cultures. It has been suggested that extracellular mRNA is released from nonviable (Halicka et al., 2000) as well as viable (Stroun et al., 1978; Garcia et al., 2008; Skog et al., 2008; Hong et al., 2009) cells. The augmentation of exhTERT mRNA levels by high cell density may be caused by dead cells during persistent cell proliferation of MIAPaCa-2 cells, as suggested by the measurement of cell viability and extracellular 18S rRNA level, a putative indicator of cell destruction in cultures (Bottcher et al., 2006).

Quantitative analysis indicated that PK-59 cells appeared to be low-release cancer cells, the extracellular levels of which remained low irrespective of RNase and unaltered under alternate culture conditions between serum-supplemented and SF medium. When the extracellular levels were compared in cultures of 10^6 serum-fed cells, an average of 1.6×10^5 copies of exhTERT

mRNA, representing about one-eighth to one-third of the levels found in MIAPaCa-2, PANC-1, KLM-1 and PK-9 cells, were found in the media of PK-59 cells. The results suggested that the extracellular levels may not be indicative of the number of the cells conditioning the medium, as compared with the relevant levels found in high-release cells. Further, PK-59 cells showed message loss at confluence, indicating that there may be false negative results on analysis of exhTERT mRNA from low-release cells *in vitro*. Low exhTERT mRNA levels in BxPC-3 and PK-45H cells also remained unaltered regardless of RNase and alternate culture conditions (data not shown), suggesting that BxPC-3 and PK-45H cells also have a low-release phenotype. Further studies in large samples of PCa cells are required to confirm the phenotype.

Previous studies (Dasi et al., 2001; Miura et al., 2006) have reported that the levels of cell-free hTERT mRNA did not always correlate with clinical parameters including tumor grade. We examined the levels of exhTERT mRNA in 8 PCa cell lines histologically classified as well (Capan-1 and PK-45H), moderately (PK-9, PK-59 and BxPC-3), and poorly (MIAPaCa-2, PANC-1 and KLM-1) differentiated (Amikura et al., 1995; Kimura et al., 1996; Furukawa et al., 1998; Sipos et al., 2003). We found that the three poorly differentiated PCa cell lines exhibited high levels of exhTERT mRNA whereas the well differentiated PK-45H cell line showed low levels of exhTERT mRNA. However, some differences of exhTERT mRNA levels were also observed among moderately differentiated PCa cell lines.

Cell growth-related mRNAs are reported contained in, and released from, microvesicles (Skog et al., 2008; Hong et al., 2009). ExhTERT mRNA appears to be released from proliferating PCa cells through microvesicles. Tyrosinase mRNA released by proliferating melanoma cells can be detected in cell-free RNA fractions (Kopreski et al., 1999). We found that the exhTERT mRNA release was dependent on cell growth rate, suggesting that the RNA marker may not be spontaneouly released but actively liberated from proliferating PCa cells. The manner of marker liberation made it possible to develop quantitative approaches in cell cultures. Analysis of two types of PCa cells which have different capacities for active liberation of exhTERT mRNA will

help us to understand the mechanisms of release and the association of the marker secretion in the malignant progression of PCa. Our studies suggested that only a minor fraction of cellular hTERT mRNA was released as exhTERT mRNA. The amount of exhTERT mRNA released in the SF medium was about 0.028%, 0.025% and 0.0017% of cellular hTERT mRNA present in proliferating MIAPaCa-2, PANC-1 and PK-59 cells, respectively. Since the amount of extracellular 18S rRNA due to cell destruction was 0.2% to 0.88%, that of cellular 18S rRNA present in SF cultures of MIAPaCa-2 cells (data not shown), the amounts of exhTERT mRNA released by proliferating cells were lower than those of ‘passively released’exhTERT mRNA (1/517 to 1/8) expected from cancer cell disruption. Though the release was much lower, changes in exhTERT mRNA level were nevertheless detected during proliferation of PK-59 cells. Further, low exhTERT mRNA released by some well and moderately differentiated PCa cell lines could also be detected in cell-free RNA fractions. Thus, even a minor but substantial fraction of exhTERT mRNA is useful as a marker *in vitro*.

Microvesicles in conditioned medium have also been shown to contain 18S rRNA (Hong et al., 2009). In general, the cellular levels of 18S rRNA are independent of cell proliferation status and are used as an internal control for gene expression in pancreatic tissues (Rubie et al., 2005). Since its cellular levels are high, cares must be taken in quantitative analysis (Rubie et al., 2005). Our findings also suggest a lack of correlation between culture volume and 18S rRNA release in microwell analyses. It is likely, however, that products of the housekeeping gene may be released regardless of exhTERT mRNA release. Our preliminary results from three independent experiments showed that extracellular levels of 18S rRNA remain almost unchanged, even down-regulation of hTERT expression reduced the level of exhTERT mRNA to lower than background (data not shown). Moreover, the levels of 18S rRNA release were almost unaltered during the proliferation of KLM-1 and PK-59 cells, in which the release of exhTERT mRNA was augmented. Further, KLM-1 cells showed low levels of extracellular 18S rRNA and high exhTERT mRNA release, whereas BxPC-3 cells showed high levels of extracellular 18S rRNA

and low exhTERT mRNA release (data not shown), suggesting that different mechanisms may be responsible for the release of exhTERT mRNA and 18S rRNA.

In a clinical setting, the most important question is whether PCa cells with low exhTERT mRNA release may be present in pancreatic lesions *in vivo*. If present, this could result in overdiagnosis in association with infiltrated lymphocytes. Correlation of marker analysis with cytological assessment could minimise the risk of false diagnosis (Hiyama and Hiyama, 2003). Although the modality may suffer from low specificity, Dasi *et al.* (2001) reported that the mean values of plasma exhTERT mRNA in cancer patients with colorectal cancer and follicular lymphoma were significantly higher than those in healthy controls, but no significant correlation was observed between the clinical tumour stage and the amount of plasma exhTERT mRNA. Analysis of cell-free exhTERT mRNA prior to dissemination of cancer cells could provide a promising non-invasive means of early detection of cancer cells (Chen et al., 2000; Dasi et al., 2001). We found, however, that factors such as RNases and cell density, in addition to cell growth rate and cancer cell destruction, influenced the release of exhTERT mRNA from PCa cells. Moreover, *in vivo*, exhTERT mRNA is produced not only by cancer cells but also by lymphocytic cells. These findings suggest that assays of plasma/serum exhTERT mRNA levels for the early detection of PCa or as trace maker during therapy would be inapplicable clinically.

In conclusion, a quantitative approach using cell culture systems provided useful information regarding the marker exhTERT mRNA. The applicability of the marker to some well and moderately differentiated PCa cell lines may be almost dependent upon the feasibility to detect low exhTERT mRNA release under conditions of cell proliferation. The RNA-based marker in microvesicles may have clinical utility at higher stages of the asymptomatic cancer. Further studies are required to assess the clinical relevance of our findings.

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Figure Legends

Fig. 1. Levels of hTERT expression and exhTERT mRNA in serum-fed PCa cell lines. (A) Cellular levels of hTERT expression were normalised relative to 18S rRNA. Doubling Times (h), representing the means of three independent experiments, are shown at the top of the panel. (B and C) Time course of extracellular accumulation of (B) exhTERT mRNA and (C) extracellular 18S rRNA in the conditioned media. Following the inoculation of 1×10^5 cells/60-mm dish, the cells were fed with fresh media on days 3, 5 and 7. A 0.35 mL aliquot of medium was withdrawn after 12-h and analyzed. (D) Effects of cell density on exhTERT mRNA levels. Cells were seeded at $2.5–3\times10^6$ (L) and $6–9\times10^6$ (H) cells per 100-mm dish. Columns, means of three independent experiments; Bar, SD of mean. * $P > 0.05$; ** $P < 0.05$

Fig. 2. Levels of hTERT expression (A) and exhTERT mRNA (B) under SF conditions in cultures. Cells were inoculated at a cell density of either 3×10^5 cells (L) or $3–4.5\times10^6$ cells (H) per 60-mm dish, washed once with prewarmed SF medium and incubated for an additional 12-h in fresh SF medium. Both cellular hTERT and exhTERT mRNA levels are presented as the means ± SD of three independent experiments. * $P > 0.05$; ** $P < 0.05$.

Fig. 3. Changes in exhTERT mRNA levels during the growth of PK-59 cells. PK-59 cells were inoculated at 3×10^5 cells per 60-mm dish containing SF medium. After washing once with prewarmed SF medium, fresh SF medium was fed to the cells to obtain the 12-h incubation medium at each of 2 (lane 1), 4 (lane 2), 6 (lane 3) and 8 (lane 4) days. Extracellular RNA was extracted from 0.35 mL of conditioned or unconditioned medium (lane 5). The products obtained by the ordinary RT- PCR assay were stained with SYBR-Gold. Markers (M) were DNA fragments of *Hinc*II-digested ΦX174.

Fig. 4. Effects of cell density on viability of MIAPaCa-2 and PK-59 cells. Cells were seeded at a density of 3×10^5 cells per 60-mm dish containing SF media. Media were replenished every 3 days after cell inoculation. Dead cells were measured by trypan blue dye-exclusion method on days 4 (L) and 8 (H) of parallel culture. The viability was assayed in triplicate examinations (bars ± SD). ∗ $P > 0.05$; ∗∗ $P < 0.01$

Fig. 5. Changes in exhTERT mRNA levels after combination with conditioned media. The conditioned medium ($4.2–4.8\times10^4$ copies of exhTERT mRNA) from proliferating PK-59 cells was incubated at 37°C with those of confluent counterparts (○-○), unconditioned medium control (●-●) or of confluent Capan-1 cells (X-X) as a positive control. After incubation (h), the residual amounts of exhTERT mRNA were quantified and represented as the log-input percentage of mRNA. The half-lives were calculated from the slopes of the regression lines of the data, which represent the means ± SD of three independent experiments.

Fig. 6. Treatment with anti-E-cadherin mAb (A) and E-cadherin analysis (B) in MIAPaCa-2 and PK-59 cells. (A) Conditioned media were obtained after incubation with mouse IgG (Co; control) or anti-E-cadherin mAb (10 μg/mL) for 12-h. Samples of 0.35 mL (for MIAPaCa-2 cells) or 0.7

mL (for PK-59 cells) were extracted and the levels of exhTERT mRNA and extracellular 18S rRNA were determined as described in Fig. 3. (B) Analysis of E-cadherin and actin. Cell extracts were electrophoresed and subjected to immunoblotting with the corresponding antibodies.

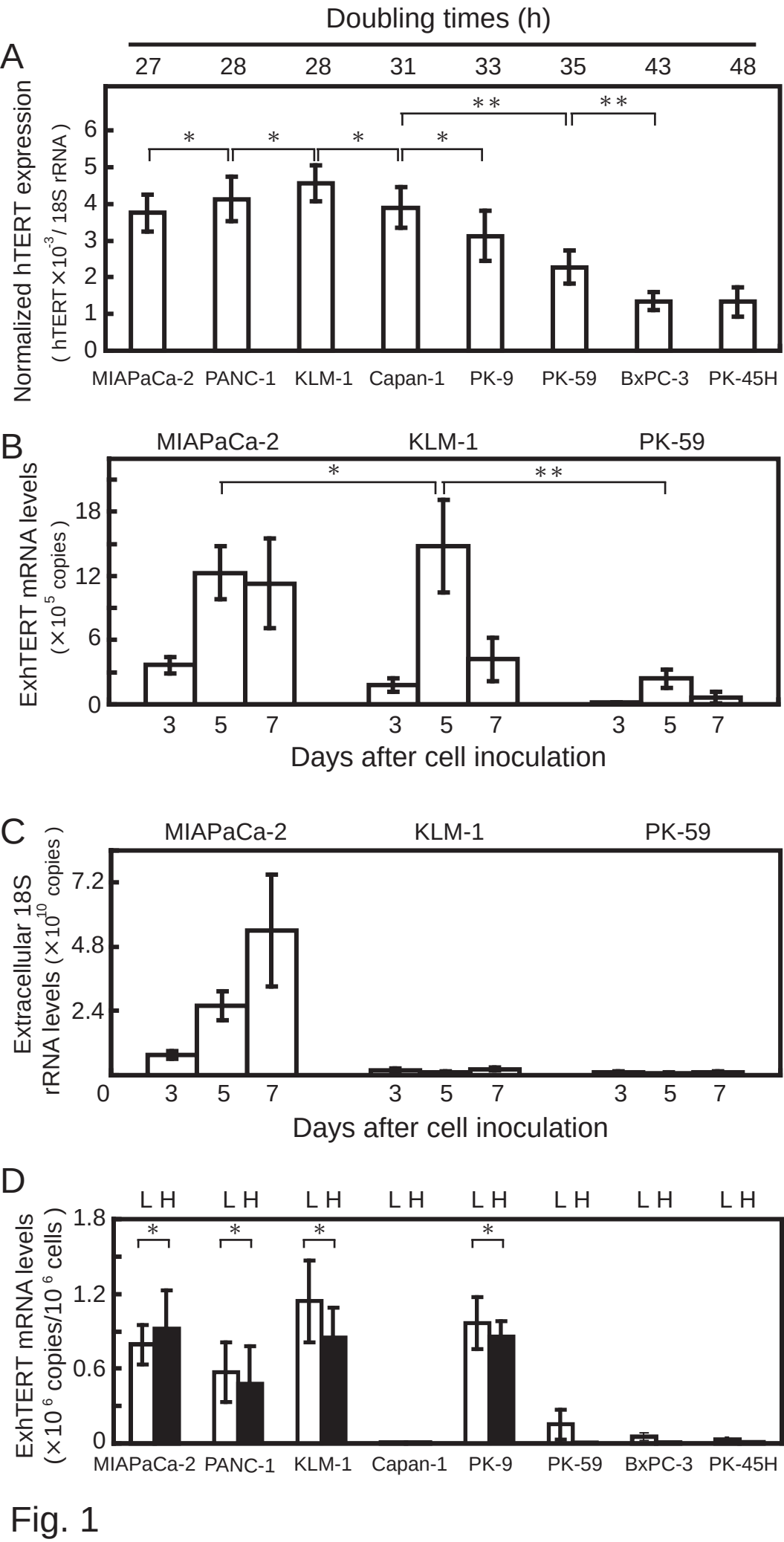
Doubling times (h)
A
27
28
28
31
33
35
43
48
Normalized hTERT expression
(hTERT ×10-3 / 18S rRNA)
MIAPaCa-2
PANC-1
KLM-1
Capan-1
PK-9
PK-59
BxPC-3
PK-45H
B
MIAPaCa-2
KLM-1
PK-59
ExhTERT mRNA levels
(×10 5 copies)
Days after cell inoculation
C
MIAPaCa-2
KLM-1
PK-59
Extracellular 18S
rRNA levels (×10 10 copies)
Days after cell inoculation
D
L H
ExhTERT mRNA levels
(×10 6 copies/10 6 cells)
MIAPaCa-2
PANC-1
KLM-1
Capan-1
PK-9
PK-59
BxPC-3
PK-45H

Fig. 1

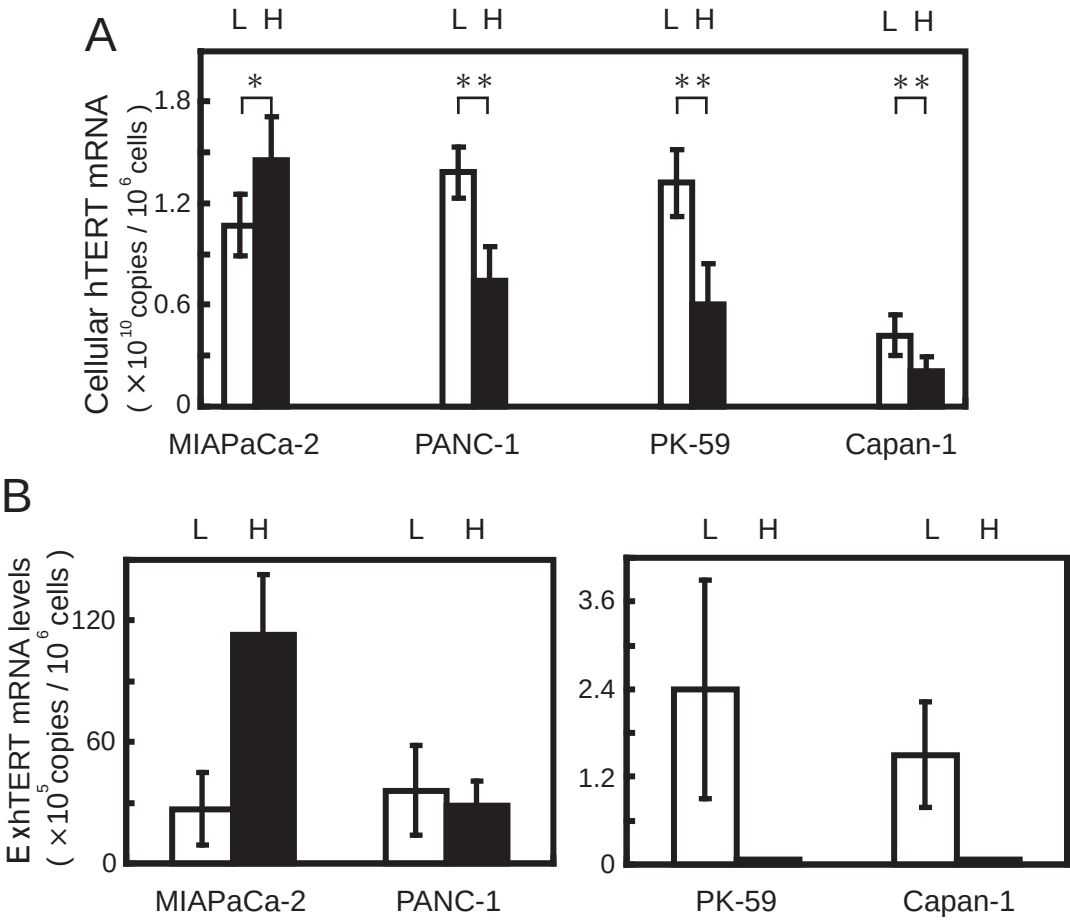
A
L H
L H
L H
L H
*
**
**
**
1.8
1.2
0.6
0
Cellular hTERT mRNA
(×10^10 copies / 10^6 cells)
MIAPaCa-2
PANC-1
PK-59
Capan-1
B
L H
L H
L H
L H
120
60
0
3.6
2.4
1.2
0
ExhTERT mRNA levels
(×10^5 copies / 10^6 cells)
MIAPaCa-2
PANC-1
PK-59
Capan-1

Fig. 2

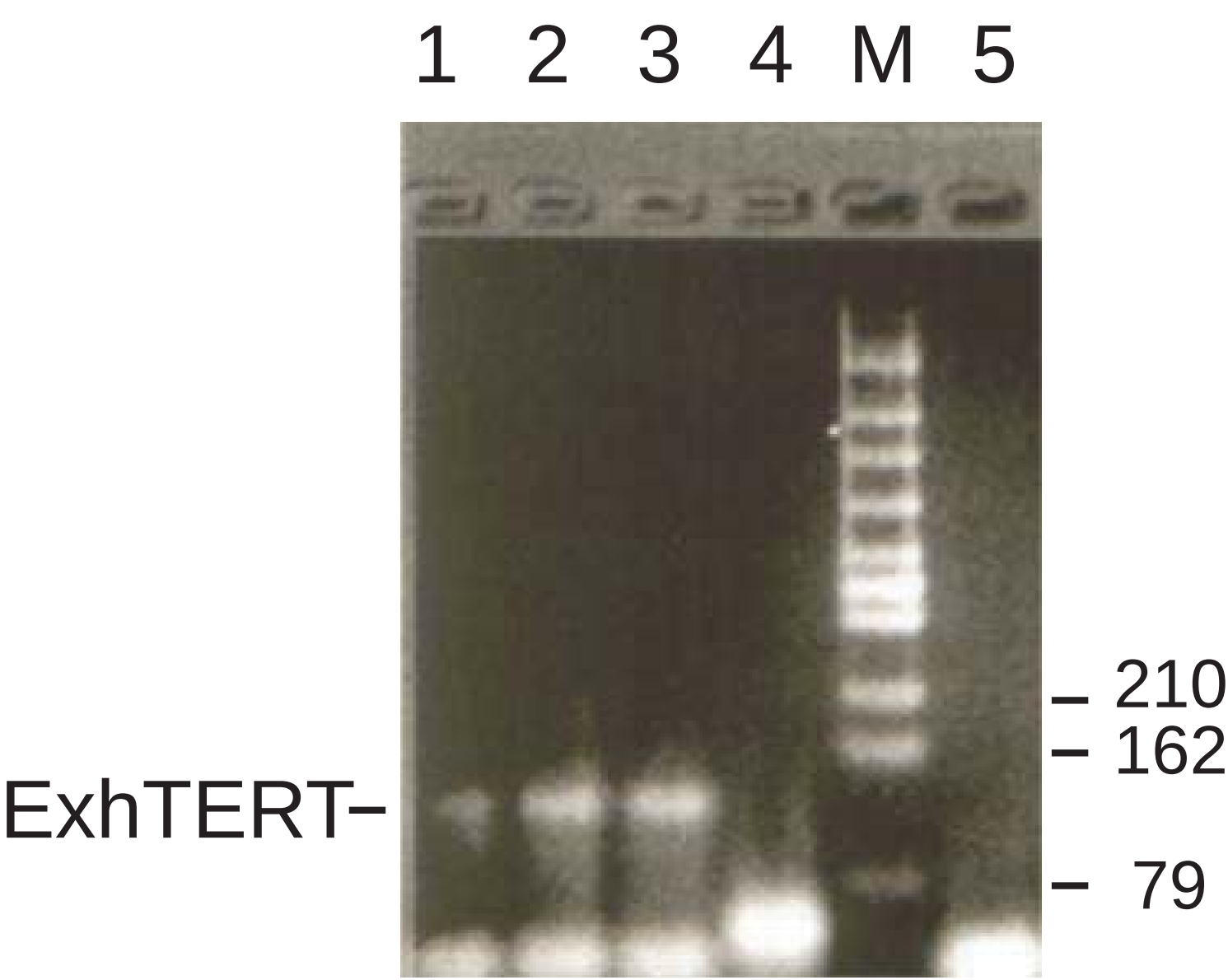

Fig. 3

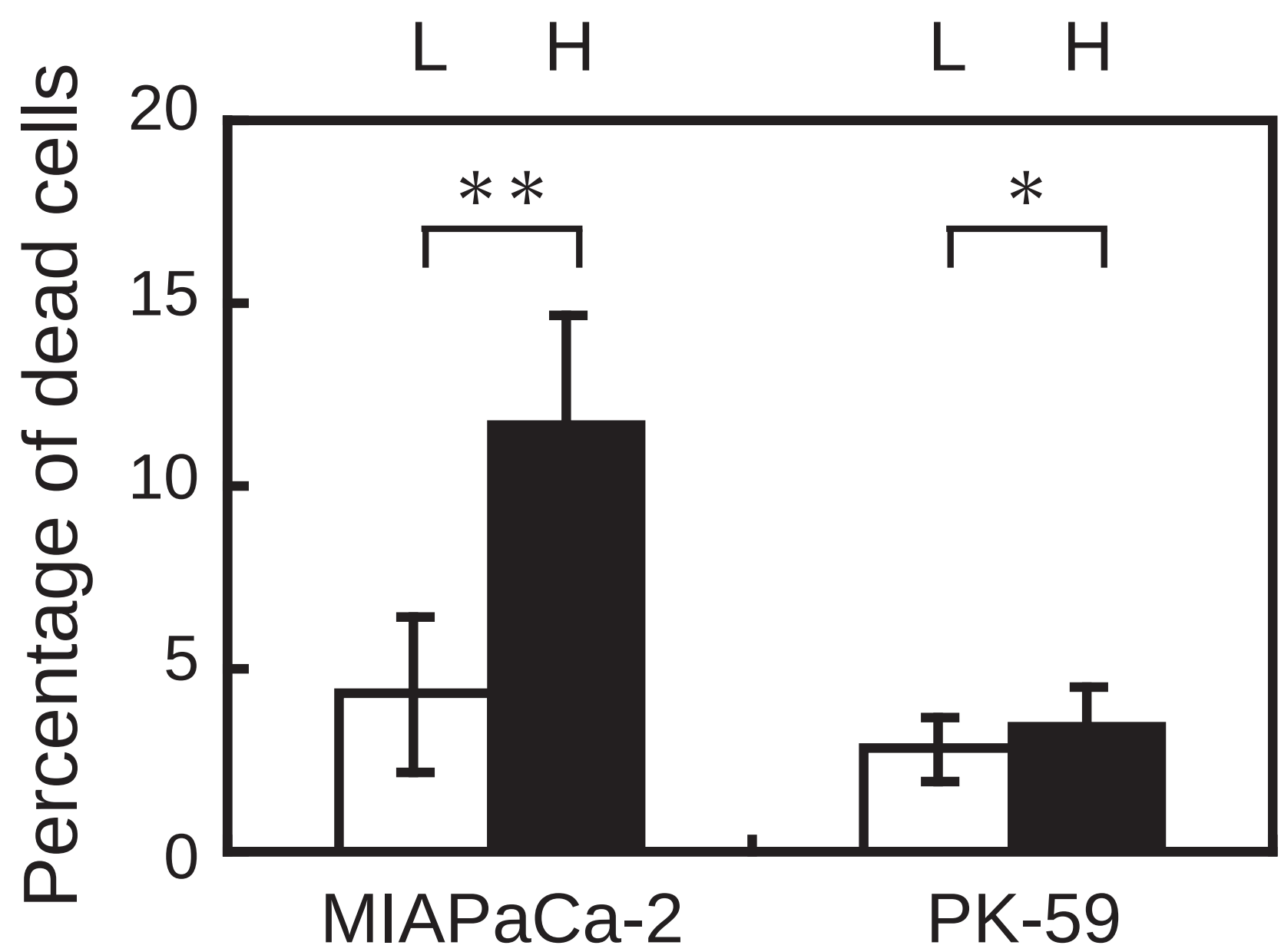

Fig. 4

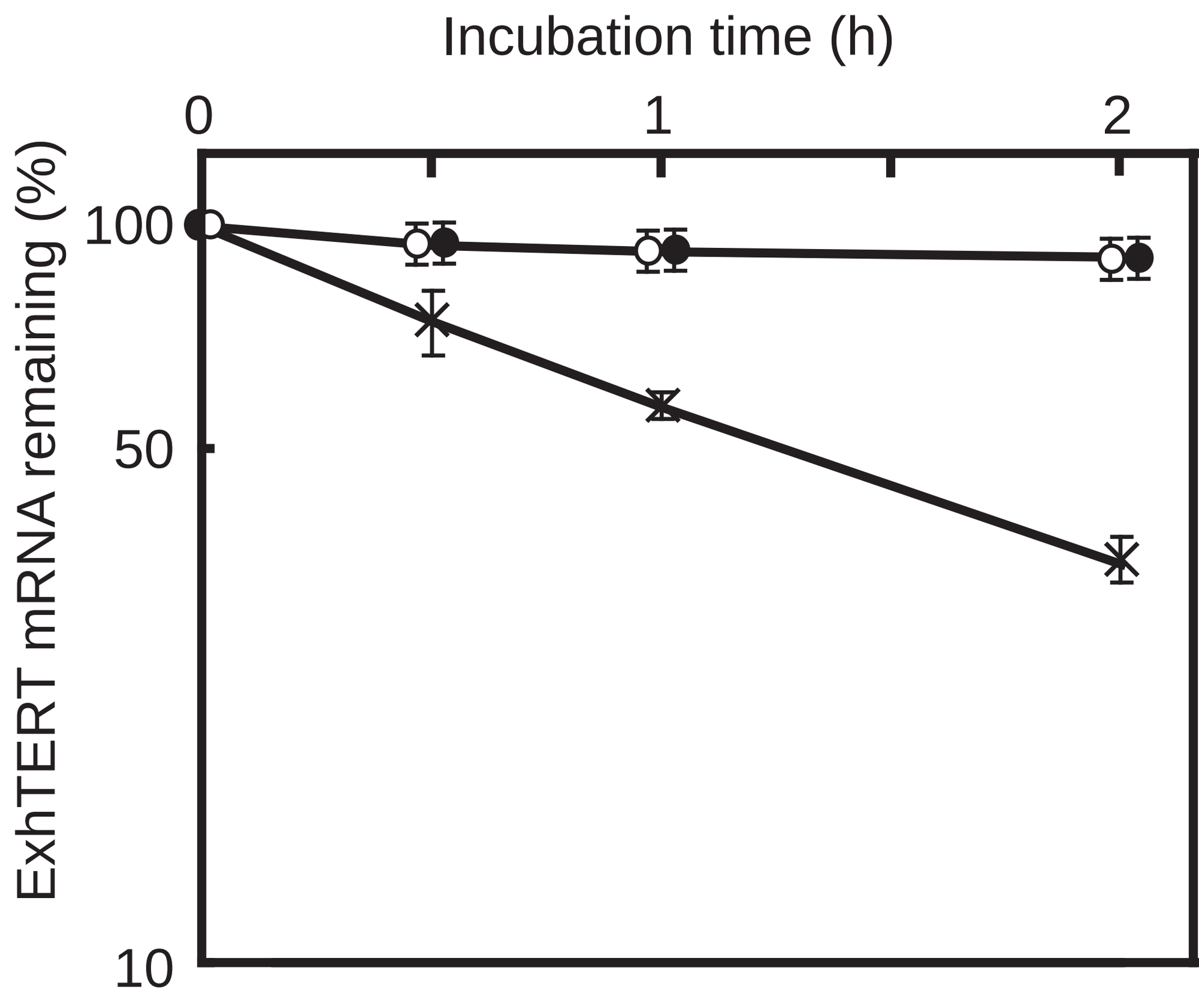

Fig. 5

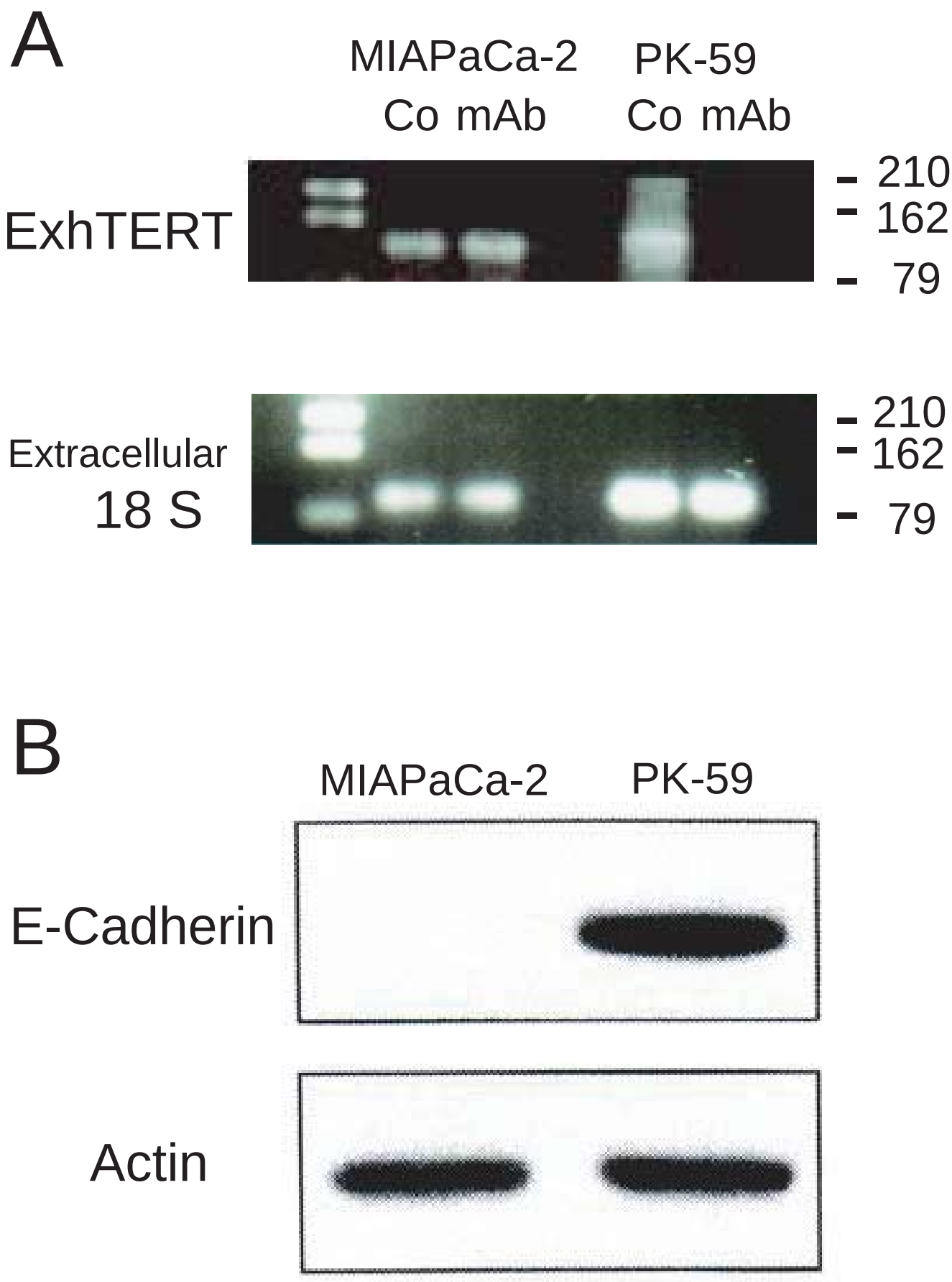
A
MIAPaCa-2
PK-59
Co mAb
Co mAb
ExhTERT
210
162
79
Extracellular
18 S
210
162
79
B
MIAPaCa-2
PK-59
E-Cadherin
Actin

Fig. 6