Gene Expression Analysis of Immunostained Endothelial Cells Isolated From Formaldehyde-Fixated Paraffin Embedded Tumors Using Laser Capture Microdissection—A Technical Report

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ABSTRACT Laser capture microdissection (LCM) allows microscopic procurement of specific cell types from tissue sections that can then be used for gene expression analysis. In conventional LCM, frozen tissues stained with hematoxylin are normally used to the molecular analysis. Recent studies suggested that it is possible to carry out gene expression analysis of formaldehyde-fixated paraffin embedded (FFPE) tissues that were stained with hematoxylin. However, it is still unclear if quantitative gene expression analyses can be performed from LCM cells from FFPE tissues that were subjected to immunostaining to enhance identification of target cells. In this proof-of-principle study, we analyzed by reverse transcription-PCR (RT-PCR) and real time PCR the expression of genes in factor VIII immunostained human endothelial cells that were dissected from FFPE tissues by LCM. We observed that immunostaining should be performed at 4°C to preserve the mRNA from the cells. The expression of Bcl-2 in the endothelial cells was evaluated by RT-PCR and by real time PCR. Glyceraldehyde-3-phosphate dehydrogenase and 18S were used as house keeping genes for RT-PCR and real time PCR, respectively. This report unveils a method for quantitative gene expression analysis in cells that were identified by immunostaining and retrieved by LCM from FFPE tissues. This method is ideally suited for the analysis of relatively rare cell types within a tissue, and should improve on our ability to perform differential diagnosis of pathologies as compared to conventional LCM. *Microsc. Res. Tech. 72:908–912, 2009.*

INTRODUCTION

A method based on laser capture microdissection (LCM) that would allow for quantitative gene expression analyses of specific target cells in tissue sections could be very useful to several experimental and clinical fields. To date, formaldehyde as a 10% neutral buffered formalin is the most widely used as a fixative for various human tissues. As with DNA, formaldehyde reacts with RNA forming an *N*-methylol (*N*-CH_2OH) followed by an electrophilic attack to form a methylene bridge between amino groups. Adenine is the most susceptible nucleotide to electrophilic attack and it is likely that the adenines within the mRNA sequence and the poly(A) tail of mRNA will be modified in the formaldehyde-fixated paraffin embedded (FFPE) sections to varying degrees. Thus, it is normally considered that RNA isolated from FFPE sections are less suitable for reverse transcription (cDNA synthesis), as compared to RNA-isolated frozen tissue sections (Srinivasan et al., 2002). However, a recent report has suggested that it is possible to perform gene expression analysis from FFPE tissues (Pagedar et al., 2006).

The tumor microenvironment is composed of several cell types, of which the endothelial cells constitute a small fraction of the overall cell number. Therefore, if one needs to quantitatively analyze gene expression specifically in endothelial cells, they have to be somewhat selectively removed from the tissue, without contamination of other cell types. To overcome this

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technical problem, we developed a novel strategy of immuno-LCM in FFPE tissues, which may greatly facilitate the identification of the target cells (i.e., tumor-associated endothelial cells). Using such strategy, we have recently reported, using a severe combined immunodeficient (SCID) mouse model of human tumor angiogenesis, that Bcl-2 orchestrates a tumor-endothelial cell crosstalk that promotes tumor growth (Kaneko et al., 2007). In the analysis, we used the SCID mouse model of human tumor angiogenesis. It allows for the study of human tumors in murine hosts by implanting defined endothelial and tumor cell populations in biodegradable scaffolds (Nör et al., 1999, 2001a). We have demonstrated that this method allows for the development of functional human blood vessels that anastomize with the mouse vasculature (Nör et al., 2001a). Notably, it is suitable for studying both physiologic (when only human endothelial cells are implanted), or pathologic angiogenic processes (when both tumor cells and endothelial cells are coimplanted). Here, we used the SCID mouse model of human tumor angiogenesis as a platform to characterize in detail the method developed for quantitative gene expression analysis in LCM-retrieved endothelial cells after immunostaining from FFPE-engineered tumor tissues (Kaneko et al., 2007).

MATERIALS AND METHODS

Cells

Human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) stably transduced with Bcl-2 (HDMEC-Bcl-2) and empty vector controls HDMEC-LXSN were cultured in EGM2-MV (Cambrex), as described previously (Kaneko et al., 2007; Karl et al., 2005; Nör et al., 1999, 2001a,b). Oral squamous cell carcinoma cells (OSCC3; gift of M. Lingen, University of Chicago) were cultured in DMEM supplemented with 10% fetal bovine serum.

Severe Combined Immunodeficient (SCID) Mouse Model of Human Angiogenesis

The care and treatment of experimental animals were in accordance with University of Michigan institutional guidelines. Porous poly-L-lactic acid (PLLA; Boehringer Ingelheim, Germany) scaffolds (6 mm $\times$ 6 mm $\times$ 1 mm biodegradable) with an average pore diameter of 180 μm were fabricated, as previously described (Nör J.E., 2001). Just before implantation, scaffolds were seeded with 0.9×10^6 HDMEC and 0.1×10^6 tumor cells (OSCC3) in a 1:1 Matrigel/EGM2-MV mix. Five- to nine-week-old male SCID mice (n = 5) (CB-17, Chales River Laboratories, Wilmington, MA) were anesthetized with ketamine and xylazine, and two scaffolds were implanted subcutaneously in the dorsal region of each mouse. Twenty-eight days after transplantation, mice were euthanized, and the scaffolds were retrieved, fixed for 10 h in 10% buffered formaldehyde at 4°C. Then, the samples were cut in two pieces, one for paraffin blocks, and the other for frozen blocks. Eight micrometer serial sections from each piece were cut, and mounted on PEN foil slides (Leica Microsystems, Bannock Burn, IL) for LCM.

Immunostaining for Paraffin Sections

To stain the microvascular networks formed by the implanted human endothelial cells, immunoperoxidase staining was conducted using a mouse antihuman Factor VIII monoclonal antibody (1:100 dilution; Lab Vision, Fremont, CA). Slide-mounted tissue sections were deparaffinized twice with 3 min xylene washes at room temperature, and rehydrated through a series of graded ethanol at 4°C. A working dilution of 0.125% trypsin was used to pretreat these sections for 30 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide with methanol for 3 min at 4°C. Immunostaining was performed as described earlier (Kaneko et al., 2007, 2001a,b). A first incubation was made with mouse anti-Factor VIII (diluted 1:100) for 16 h at 4°C. Sections were then incubated with biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA; diluted 1:500) for 1 h at 4°C and incubated with avidin-biotin-peroxidase complex (Elite ABC kit; Vector) at 4°C. Peroxidase activity was developed in diaminobenzidine-H_2O_2 solution (DAB substrate kit; Vector) for 3 min at room temperature. Sections were then air-dried at 4°C. Negative control staining was always conducted in parallel by the omission of the primary antibody, antimouse IgG, or avidin-biotin-peroxidase complex.

Immunostaining for Frozen Sections

Frozen sections were cut in a cryostat (Leica, Germany), and were incubated with anti-Factor VIII antibody (1:500 dilution). This was followed by the same steps of immunostaining for paraffin sections.

LCM

After immunostaining, a three-step dissection strategy was performed using a LCM microscope (Leica AS LMD; Leica Microsystems) with a pulsed 337 nm UV laser (Leica Microsystems). Blood cells in Factor VIII-positive capillaries were excluded first. Then Factor VIII-positive endothelial cells (~1,500) were dissected and collected into individual tubes filled with RNAlater® (Ambion, Austin, TX) and immediately placed on ice. Cells without a nucleus were excluded from the cell count. Then, tumor cells surrounding the blood vessels were collected similarly. Experiments were always carried out in duplicate. The FFPE blocks used in this study were ~1-year-old.

RT-PCR

Total RNA from paraffin-embedded tissue sections was extracted using TRIzol reagent (Invitrogen), and purified with RNeasy Mini kits (Qiagen), as we described (Kaneko et al., 2007, 2008). Its purity (Mo, SG_{man}: $OD_{260/280}$ = 1.9–2.1) was determined photometrically (Ultrospec 2000, Pharmacia Biotech, Germany). cDNA synthesis and PCR amplification were done in single tubes with SuperScript one-step reverse transcription-PCR (RT-PCR) and Platinum Taq kit (Invitrogen) using simultaneously a human Bcl-2 primer set and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer set. The sequence of the primers used here were as follows: Bcl-2, sense, CTGCGAA GAACCTTGTGTGA and antisense TGTCCCTAC CAACCAGAAGG; and GAPDH, sense, CATGGCCTC

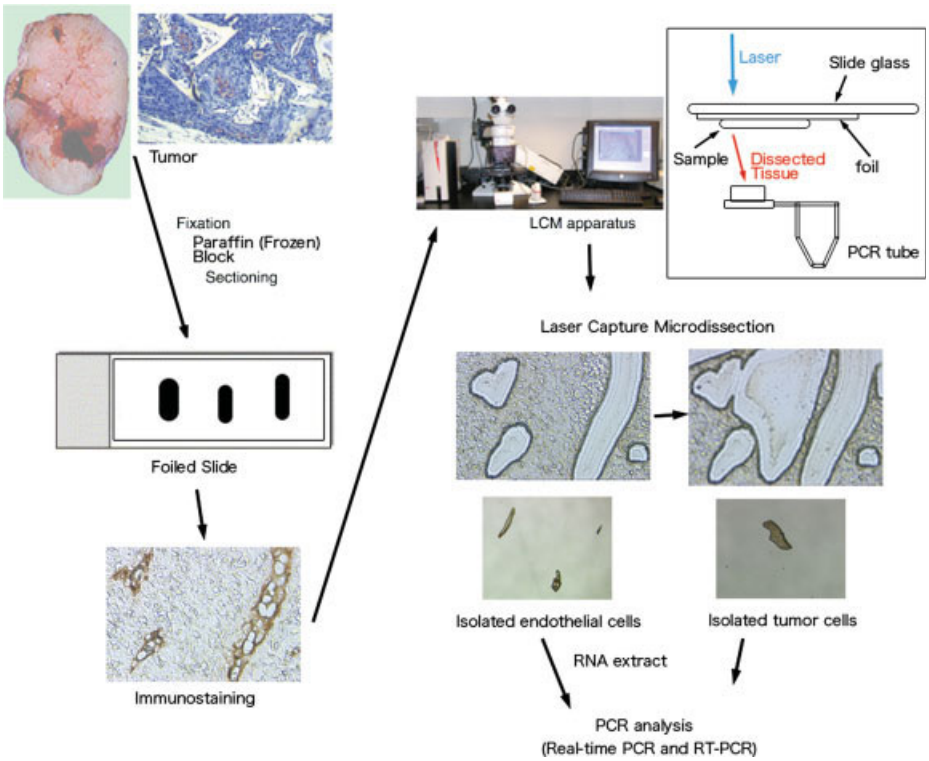

Fig. 1. The overall strategy used here for quantitative gene expression analysis of immunostained FFPE tissue sections. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

CAAGGAGTAAG and antisense, AGGGGTCTACAGG CAACTG. Negative control reaction was always conducted in parallel by using water instead of RNA. The PCR products were detected by standard agarose gel with 1.5% ethidium bromide.

Real-Time PCR

Total RNA was extracted and purified from LCM-retrieved samples, as described above. First-strand complementary DNA (cDNA) synthesis was performed with TaqMan® reverse transcription reagents (Applied Biosystems). Probe and primer sets of TaqMan Gene Expression Assays (Hs00608023_m1; Bcl-2, Hs00174103_m1; and Hs99999901_s1; 18S) were obtained from Applied Biosystems. Total RNA at 0.02 μg per 30 μL of reaction mixture was prepared by TaqMan Universal PCR Master Mix (Applied Biosystems). For standard RNA, we used TaqMan One-step RT-PCR Master Mix Reagents (Applied Biosystems). The reac-

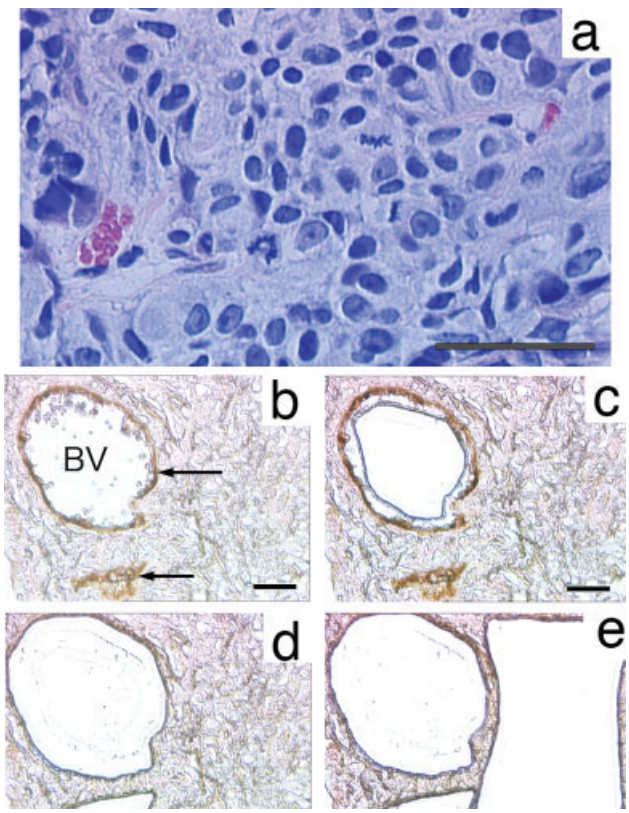

Fig. 2. Step-by-step characterization of the technique based on LCM used for retrieval of either endothelial cells or tumor cells from FFPE tissue sections. (**a**) H&E staining of this engineered tumor tissue that was coimplanted with OSCC3 (oral squamous cell carcinoma cells) and HDMEC-Bcl-2 (endothelial cells stably transduced with Bcl-2). (**b**) Factor VIII+ endothelial cells (arrows). There are both well-vascularized and unclearly vascularized endothelial cells. BV: blood vessel. (**c**) Removal of blood cells. (**d**) Retrieval of factor VIII+ endothelial cells. (**e**) Retrieval of tumor cells adjacent to blood vessels. Bar = 500 μm. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

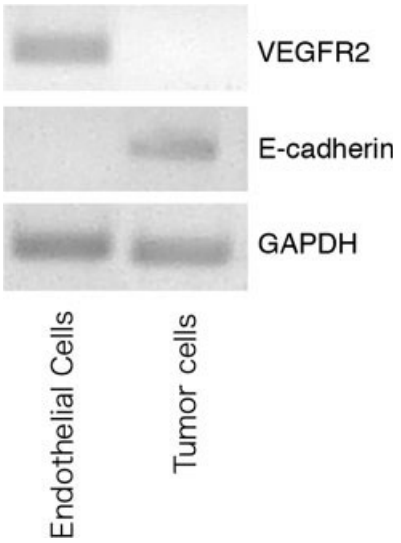

Fig. 3. Quality control check of RNA from endothelial cells or tumor cells from FFPE tissues using VEGFR2 (a specific marker for endothelial cells) and E-cadherin (a specific marker for cells of ectodermal origin).

tions were performed in 96-well clear optical reaction plate using ABI7700 Sequence Detection System (Applied Biosystems), and the data were normalized by the data of 18S controls. All reactions were performed in triplicate, each run contained at least one negative and one positive controls, and three independent experiments were performed to confirm reproducibility of results.

The overall strategy used here for quantitative gene expression analysis of immunostained FFPE tissue sections is summarized in Figure 1. After tumor retrieval, samples were processed for FFPE, sectioned, mounted on LCM slides, and immunostained using the avidin-biotin-peroxidase method. Following the LCM, RNA extraction and PCR analysis were performed.

Statistical Analysis

For statistical comparison of two experimental groups, we used Student's *t*-tests using Prism 5.0 (Graphpad, San Diego, CA).

RESULTS

The step-by-step LCM procedure is shown in Figure 2. We first retrieved blood cells, and then Factor VIII-immunostained endothelial cells, and lastly the surrounding tumor cells. Notably, Factor VIII immunostaining allowed for clear identification of the endothelial cells.

To confirm if the tissues dissected by LCM were without contamination, we performed RT-PCR analysis for vascular endothelial cell growth factor receptor 2 (VEGFR2: a specific marker for endothelial cells) and E-cadherin (a specific marker for cells of ectodermal origin). RT-PCR analysis showed that mRNA from dissected tissues was adequate for PCR analysis for target genes. Furthermore, dissected endothelial cells did not express E-cadherin, and tumor cells did not express VEGFR2, confirming the quality and origin of the RNA (Fig. 3). As shown in Figure 4a, Bcl-2 and GAPDH

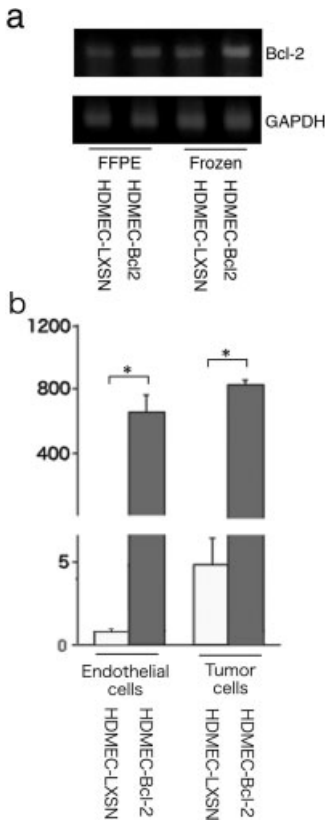

Fig. 4. PCR analysis for LCM samples. (**a**) Reverse transcription/polymerase chain reaction (RT-PCR) to evaluate levels of Bcl-2 and GAPDH mRNA expression in endothelial cells in FFPE or frozen tissue sections that were engineered in SCID mice. (**b**) Real-time PCR used to quantify Bcl-2 expression in endothelial cells and tumor cells retrieved from FFPE tissue sections. Specimens were obtained from engineered tumor tissues. Data presented from real-time PCR experiments reflect the expression level of Bcl-2 normalized by 18S. *, $P < 0.001$ (*t*-test).

gene expression in both FFPE and frozen tissues was detected by RT-PCR. Notably, we were able to compare the expression levels between tumor cells and endothelial cells in paraffin samples by real time PCR (Fig. 4b).

DISCUSSION

LCM is normally used to demonstrate the presence of specific gene transcripts in cryosectioned tissues (Cristobal et al., 2004, 2005). LCM of immunostained frozen tissue sections has been used successfully for gene expression analysis (Fend et al., 1999; Stoebner et al., 2008). We have also reported that gene expression of some antigen presenting cell-related molecules, such as HLA-DR α-chain, CD83, and CD86, can be analyzed in LCM-retrieved HLA-DR-expressing cells from immunostained frozen tissue sections (Kaneko et al., 2008). RNA recovery from several kinds of FFPE tissues is now possible (Su et al., 2004), although frozen tissue sections may allow us to retrieve better quality and more amount of mRNA, as compared to mRNA from the FFPE tissues. However, no technical reports have described quantitative gene expression analysis after LCM of immunostained FFPE tissues. In this report, we describe a method for gene expression anal-

ysis of Factor VIII-expressing endothelial cells microdissected from FFPE tissues. This method allowed us to retrieve mRNA from a predetermined cell type in histologically defined areas of routinely prepared FFPE tissues. We have also reported that this method can be used in FFPE human surgical materials such as those from head and neck carcinomas (Kaneko et al., 2007).

We here demonstrated that formaldehyde, a cross-linking fixative, do not prevent us from extracting enough RNA for RT-PCR or real time PCR, at least for Bcl-2 and house keeping gene amplification. These results are in agreement with previous reports assessing PCR amplication of RNA in tissues recovered from paraffin blocks by other methods (Ben-Ezra et al., 1991; Koopmans et al., 1993).

The present method of selective LCM-retrieval may be useful to compare gene expression patterns in tumor cells versus endothelial cells during tumor progression or tumor angiogenesis. This method could also be useful for quantitative gene expression analysis in blood vessels from tumors of patients that have been treated with antiangiogenic drugs, allowing for validation of the effect of drug on the expected targets. Such capability might be exceedingly useful for the evaluation of the bioactivity of new drugs.

In conclusion, we demonstrated that immunostaining-based LCM method presented here allowed us to quantify Bcl-2 and housekeeping gene mRNAs from endothelial cells in FFPE tissue sections. This method may be suited for the analysis of relatively rare cell types within a tissue, and should improve on our ability to perform differential diagnosis of pathologies as compared to conventional LCM.

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REFERENCES

Ben-Ezra J, Johnson DA, Rossi J, Cook N, Wu A. 1991. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. J Histochem Cytochem 39:351–354.

Cristobal R, Wackym PA, Cioffi JA, Erbe CB, Popper P. 2004. Selective acquisition of individual cell types in the vestibular periphery for molecular biology studies. Otolaryngol Head Neck Surg 131: 590–595.

Cristobal R, Wackym PA, Cioffi JA, Erbe CB, Roche JP, Popper P. 2005. Assessment of differential gene expression in vestibular epithelial cells types using microarray analysis. Mol Brain Res 133:19–36.

Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, Raffeld M. 1999. Immuno-LCM: Laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 154:61–66.

Kaneko T, Okiji T, Kan L, Takagi M, Suda H. 2001a. Ultrastructural analysis of MHC class II molecule-expressing cells in experimentally induced periapical lesions in the rat. J Endod 27:337–342.

Kaneko T, Okiji T, Kan L, Suda H, Takagi M. 2001b. An immunoelectron-microscopic study of class II major histocompatibility complex molecule-expressing macrophages and dendritic cellsin experimental rat periapical lesions. Arch Oral Biol 46:713–720.

Kaneko T, Zhang Z, Mantellini MG, Karl E, Zeitlin B, Verhaegen M, Soengas MS, Lingen M, Strieter RM, Nunez G, Nör JE. 2007. Bcl-2 orchestrates a cross-talk between endothelial and tumor cells that promotes tumor growth. Cancer Res 67:9685–9693.

Kaneko T, Okiji T, Kaneko R, Nör JE, Suda H. 2008. Antigen presenting cells in human radicular granulomas. J Dent Res 83:553–557.

Karl E, Warner K, Zeitlin B, Kaneko T, Wurtzel L, Jin T, Chang J, Wang S, Wang CY, Strieter RM, Nunez G, Polverini PJ, Nör JE. 2005. Bcl-2 acts in a proangiogenic signaling pathway through nuclear factor-κB and CXC chemokines. Cancer Res 65:5063–5069.

Koopmans M, Monroe SS, Coffield LM, Zaki SR. 1993. Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. J Virol Methods 43:189–204.

Nör JE, Christensen J, Mooney DJ, Polverini PJ. 1999. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 154:375–384.

Nör JE, Peters MC, Christensen JB, Sutorik MM, Linn S, Khan MK, Addison CL, Mooney DJ, Polverini PJ. 2001a. Engineering and characterization of functional human microvessels in immunodeficient mice. Lab Invest 81:453–463.

Nör JE, Christensen J, Liu J, Peters M, Mooney DJ, Strieter RM, Polverini PJ. 2001b. Up-regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth. Cancer Res 61:2183–2188.

Pagedar NA, Wang W, Chen DH, Davis RR, Lopez I, Wright CG, Alagramam KN. 2006. Gene expression analysis of distinct populations of cells isolated from mouse and human inner ear FFPE tissue using laser capture microdissection—A technical report based on preliminary findings. Brain Res 1091:289–299.

Srinivasan M, Sedmak D, Jewell S. 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 161:1961–1971.

Stoebner PE, Le Gallic L, Berthe ML, Boulle N, Lallemant B, Marque M, Gaspard C, Delfour C, Lavabre-Bertrand T, Martinez J, Meunier L. 2008. Decreased expression of thymidine phosphorylase/platelet-derived endothelial cell growth factor in basal cell carcinomas. Exp Dermatol 17:908–915.

Su JM, Perlaky L, Li XN, Leung HC, Antalffy B, Armstrong D, Lau CC. 2004. Comparison of ethanol versus formalin fixation on preservation of histology and RNA in laser capture microdissected brain tissues. Brain Pathol 14:175–182.