Primary Culture of Breast Cancer: A Model System for Epithelial-Mesenchymal Transition and Cancer Stem Cells

TAKAHITO NISHIKATA[1,2], MAMI ISHIKAWA[1], TETSUHIRO MATSUYAMA[1], KAZUHIKO TAKAMATSU[1,3], TOSIYUKI FUKUHARA[4] and YUTAKA KONISHI[4]

[1]*Frontiers of Innovative Research in Science and Technology (FIRST), and*
[2]*Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, Kobe, Japan;*
[3]*Drug Safety Research Laboratories, Astellas Pharma Inc., Osaka, Japan;*
[4]*Kobe Urban Breast Clinic, Kobe, Japan*

Abstract. *Background: A novel model system to study the cellular, molecular, and genetic characteristics of breast cancer stem cells is needed. Personalized prognostic models are indispensable for assessing the effect of so-called tailor-made adjuvant therapy. Materials and Methods: Surgically-extirpated tissues were dispersed with Dispase and cultured in commonly used medium. The expression of tumor markers was detected with immunohistochemistry, and gene expression profiles of tissues and cells were analyzed using the Agilent Human Microarray. Results: We established a primary culture, which exhibited mesenchymal morphology. The gene expression profile of the primary culture revealed that the cells underwent epithelial-mesenchymal transition and had cancer stem cell properties. Conclusion: A primary culture of breast cancer cells can be easily established and could be used for studying breast cancer stem cells and assessing treatment of a patient.*

Metastasis control is an important issue for treating many types of cancer, including breast cancer. Establishment of a primary culture of cells from surgically-extirpated tumor tissue is a promising method for better designing suitable treatments for individual patients (1). Moreover, cancer stem cells (CSCs) are thought to be important targets for cancer therapy (2, 3). Previous researchers have established a primary culture of breast cancer cells with stem cell properties (4, 5). This system represents a suitable *in vitro* model for developing therapeutic strategies aimed at eradicating the CSCs in breast cancer.

In this study, we established primary cultured cells from a surgically-extirpated tumor. Comprehensive characterization of normal and malignant tissues, a normal breast epithelial cell line, and the primary cell culture with immunohistochemistry and gene chip analysis revealed that the established primary culture underwent epithelial-mesenchymal transition (EMT) and also had properties of CSCs. These characteristic features represent a good model system for studying breast cancer stem cells (BCSCs). Moreover, we used this method as a model system for evaluating the effects of hyperthermia as a possible therapeutic treatment. This system may help predict response-to-treatment.

Materials and Methods

Tissue samples. The normal and malignant tissues were extra portions of surgically-extirpated scirrhous carcinoma of the female breast and were obtained at the Kobe Urban Breast Clinic (Kobe, Japan). The patient was 55 years old and received neoadjuvant systemic chemotherapy with letrozole, tegafur/gimeracil/oteracil, and cyclophosphamide/docetaxel. The patient provided written informed consent, and this study was approved by the Human Ethics Committee of Konan University (11-02).

Cells and cell culture. The chemically-transformed normal mammary gland breast epithelial cell line CRL-8798 was obtained from the American Type Culture Collection (Manassas, VA, USA), and human dermal fibroblast cells (HDF) were purchased from Summit Pharmaceutical (Tokyo, Japan). The breast tissue was digested overnight at 37˚C with 1,000 pU/ml Dispase II (Eidia, Tokyo, Japan) or plated on a Petri dish and cultured for transmigration. The obtained primary cells were cultured in two different media: MCF medium [Earle's Eagle's Minimum Essential Medium, 1 mM sodium pyruvate, 10 μg/ml bovine insulin, 1× non-essential amino acids (Sigma, St. Louis, MO, USA), 10% Fetal Bovine Serum (EuroClone, Milano, Italy)] and a commercially available medium [designated ME medium in this study: Normal Human Mammary Epithelial Cell System (CC-3150; Lonza, Walkersville, MD, USA)].

Correspondence to: Takahito Nishikata, Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20 Minatojima-minamimachi, Chuo-ku, Kobe 605-0047, Japan. Tel: +81 783031349, Fax: +81 783031495, e-mail: nisikata@konan-u.ac.jp

Key Words: EMT, Epithelial-mesenchymal transition, CSC, cancer stem cell, CD44, musashi, cadherin.

Table I. *Expression profiles of characteristic genes in MCKH cells.*

Gene name	Gene	Malignant tissue	CRL-8798 (normal cell line)	MCKH	Change*
Hormone receptor					
ESR	Estrogen receptor 1	1.771±0.080	0.036±0.089	0.040±0.069	Down
PGR	Progesterone receptor	6.927±1.337	0.052±0.028	0.106±0.018	Down
ERBB2	erb-b2	0.980±0.024	0.355±0.010	0.302±0.013	Down
Keratin					
KRT12	Keratin 12	3.083	1.021	2.560	None
KRT19	Keratin 19	4.044	0.009	0.243	Down
KRT37	Keratin 37	17.341±2.306	2.236±1.117	2.741±0.514	Down
Tumor marker					
BCAR1	Hreast cancer anti-estrogen resistance 1	1.520	3.231	2.676	None
BCAS4	Breast carcinoma amplified sequence 4	4.205	8.733	5.893	None
CEACAM5	Carcinoembryonic antigen-related CAM5	0.737	0.824	3.033	Up
MUC1	Mucin 1	3.916±0.588	3.681±2.022	0.510±0.030	Down
MUC16	Mucin 16	0.519±0.369	0.837±0.766	1.097±0.904	Up
NOTCH signaling					
CTNND2	Catenin delta 2	0.152±0.021	0.028±0.012	0.053±0.020	Down
DLL3	Delta-like 3	16.285±1.956	168.485±9.937	18.655±2.259	None
NOTCH1	NOTCH 1	0.529±0.187	2.984±0.808	0.528±0.553	None
WNT signaling					
CTNNA1	Catenin alpha 1	0.833±0.045	1.227±0.067	1.815±0.200	Up
WNT1	WNT1	0.488±0.085	1.115±0.506	0.869±0.130	None
WISP1	WNT1-inducible signaling pathway	44.875	1.023	14.912	Down
Stemness					
CD24	CD24	1.330	0.384	0.001	Down
CD44	CD44	1.045	3.996	2.186	Up
CDKN1A	p21, CIP1	3.459	7.805	19.861	Up
MSI1	Musashi homolog 1	0.863	6.472	6.206	Up
Others					
MMP1	Matrix metallopeptidase 1	1.049±0.550	52.622±19.158	2942.302±1107.466	Up
PROM1	CD133	0.015	0.017	0.042	Up
RB1	Retinoblastoma 1	1.154±0.065	0.997±0.035	0.842±0.046	None

Total RNAs were extracted from the extirpated normal and malignant tissues, CRL-8798, and MCKH primary cultured cells were analyzed with Agilent SurePrint G3 Human Gene Expression 8X60k. Expression profiles were calculated as fold changes compared to the normal tissue. Each value is the average of more than two features from the gene chip. Data obtained from more than three features are described with±SD. *Expression profile in MCKH cells, which increased/decreased more than two-fold compared to that in malignant tissue, is indicated by 'Up' and 'Down', respectively. 'None' indicates less than two-fold change.

Histochemistry. The extirpated tissues were fixed with 4% formalin and embedded in paraffin. Cultured cells were grown on coverslips and fixed with 4% formalin followed by fixation in 100% methanol.

Antibodies. The antibodies for the selected tumor markers were as follows: anti-estrogen receptor (ER) (Ab-14; Thermo Scientific, Cheshire, UK), anti-cytokeratin-19 (CK19) (BioLegend, San Diego, CA, USA), anti-CD66/carcinoembryonic antigen (CEA) (CEACAM5; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mucin 1 (MUC1; Abcam, Cambridge, UK), anti-breast cancer antigen 225 (BCA225; Abcam), anti-tumor associated glycoprotein 72 (TAG72; Abcam), anti-cancer antigen 125 (CA125; Abcam), anti-beta-tubulin (T5293; Sigma), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (American Qualex, San Clemente, CA, USA).

Gene expression profiles. The total RNAs of extirpated tissues and cultured cells were extracted with RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The gene expression profiles were analyzed with Hokkaido System Science (Sapporo, Japan) using the SurePrint G3 Human GE Microarray Kit 8X60k (Agilent, Santa Clara, CA, USA). These data were compared to data obtained from normal tissue adjacent to the tumor, extirpated from the same patient.

Heat-shock experiment and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Cells were plated onto 35-mm culture dishes (2.5×10^5 cells/dish), and heat shocked by incubation at 40˚C for 1 h, 43˚C for 10 min, or 43˚C for 1 h. Heat shock was carried out three times. The cell survival rate was measured with the 3-(4, 5-dimethylthiazolyl-2)–2,5-diphenyltetrazolium bromide (MTT) method. Total RNAs were extracted 1 h after the heat shock, and cDNAs were obtained with reverse transcription. The PCR program was 94˚C for 2 min, followed by 19 cycles of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 1 min, followed by a final cycle of 72˚C for 4 min. Under these conditions, amplification was not

Table II. *Expression profiles of tumor antigens*.

Tumor antigen	Antibody	Malignant tissue	Normal tissue	MCKH (PDL=1.68)*	MCKH (PDL=9.43)*	CRL-8798
Estrogen receptor	Ab-14	(+)**	(-)**	-	-	-
Cytokeratin-19	C19	+	-	+	-	-
CD66/CEA	CEACAM5	+	-	-	-	-
MUC1	115D8	+	-	-	-	+
BCA225	CU18	+	-	-	-	+
TAG72	B72.3	+	-	-	-	-

*PDL: Population doubling level; **expression of estrogen/progesteron receptors was detected in the clinical test. +: Positive expression was observed by immunohistochemical staining. -: Not expressed.

saturated. The amounts of mRNAs were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA, and the expression profiles are shown as the ratio to that of control cells, which were cultured at 37˚C.

Results

Establishment of primary cultures from the breast tumor. Primary cultures were established using two different methods: a tissue-fragment culture and a dispersed cell culture. Cells were grown in two different media, MCF and ME medium. In this study, we used Dispase II for cell dispersal and obtained primary cultures from all plated dishes. On the other hand, the tissue-fragment culture method was less effective for establishing a primary culture. We found that the MCF medium was more suitable for establishing a primary culture from the breast tumor.

Both the tissue-fragment culture and the dispersed cell culture methods produced similar populations of primary cultured cells. At the initial phase of cell spreading, morphological heterogeneity was observed in which both a long, flattened mesenchymal phenotype and a columnar epithelial phenotype were present. However, several passages later, all primary cultures exhibited a uniform mesenchymal phenotype. In this study, these cell lines were deemed equivalent and were designated as MCKH (primary culture of mammary carcinoma from KH) primary cultured cells.

The primary culture recapitulated some original tumor characteristics but also exhibited a characteristic expression pattern including stemness. To characterize the primary cultured cells, we carried out gene expression analyses and performed immunocytochemical detection of selected tumor markers in the normal and malignant tissues, and CRL-8798 and MCKH cells. The tumor of this patient was originally characterized in a clinical test as ER/progesterone receptor-positive, human epidermal growth factor receptor-2-negative. In addition, the levels of CK19 in the patient's peripheral blood (3.7 ng/ml) were slightly higher than the normal levels, whereas levels of B-type natriuretic peptide (BNP), CA125, CA19-9, CEA, and squamous cell carcinoma antigen (SCC) were normal.

Table I summarizes the gene expression analyses. The RNA expressions of *ER*, progesterone receptor, *CK19*, *MUC1*, and some other genes were dramatically lower in MCKH cells compared to the malignant tissue. Some tumor markers [breast cancer anti-estrogen resistance 1 (*BCAR1*) and breast carcinoma amplified sequence 4 (*BCAS4*)], some genes in the NOTCH and WNT signaling pathways [*NOTCH1*, delta-like (DLL) 3, and *WNT1*], and a keratin gene (*KRT12*) maintained a normal expression pattern.

Immunocytochemical analyses showed that ER, CK19, CD66/CEA, MUC1, BCA225, and TAG72 were clearly expressed in the extirpated malignant tissue, whereas their expression was lost in the primary culture (Table II, Figure 1). Only CK19 was expressed faintly in early passages of the primary culture (Figure 1J and Table II). These observations suggest down-regulation of these genes during establishment of the primary culture.

Recently, CSCs have become an intriguing cell population within the tumor (5). Markers for human BCSCs are $CD44^{+}$ and $CD24^{-/low}$ (6). As shown in Table I, MCKH cells lost CD24 expression and had up-regulated CD44 expression. Moreover, *p21* (*CIP1*) and musashi homolog 1 (*MSI1*) have also been suggested to be putative stem cell markers in the breast (7). In MCKH cells, *p21* was up-regulated more than five-fold than in the malignant tissue, and *MSI1* was up-regulated more than 7-fold. All of these changes in the expression of stemness marker genes clearly show the stem cell characteristics of MCKH cells.

Moreover, the expression of a metallopeptidase gene (matrix metallopeptidase 1, *MMP1*) was dramatically increased (more than 2,800-fold) in MCKH cells, suggesting invasive characteristics of MCKH cells. CD133 expression may be related to metastatic localization in the bone (8). Slight up-regulation of CD133 in MCKH cells suggests high invasiveness into bone. In fact, bone metastasis was found six months after surgery in the patient who donated the

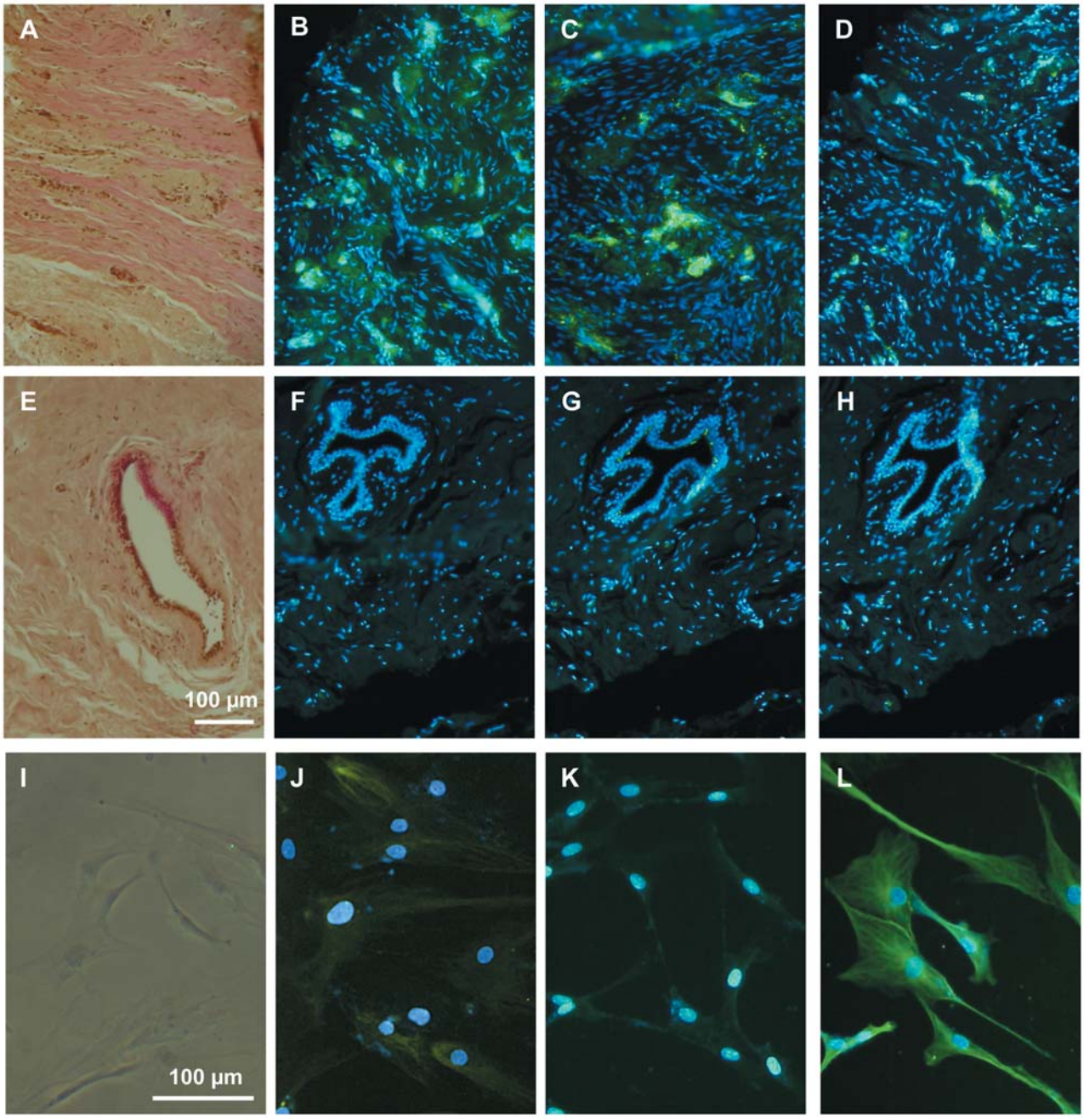

Figure 1. *Immunocytochemical detection of some tumor markers. A-H: Histochemistry of malignant tissue (A-D) and normal tissue (E-H). Sections were stained with hematoxylin and eosin (A and E), or immunohistochemically stained for cytokeratin-19 (CK19; B and F), mucin-1 (MUC1; C and G), and breast cancer antigen 225 (BCA225; D and H). Immunostained tissues were double stained with DAPI (2 µg/ml). Mammary ducts were rich in nuclei (F-H) and negative for the proteins examined. I-L: Primary cultured MCKH cells were photographed under phase-contrast microscopy (I) or immunohistochemically stained for CK19 (J and K) and tubulin (L). CK19 staining was positive in early-passage cells (J: population doubling level=1.68) and negative in aged cells (K: population doubling level=9.43). Scale bar=100 µm.*

tissue. Loss or reduced retinoblastoma expression is seen in high-grade breast adenocarcinomas (9), but no significant change in retinoblastoma gene expression was observed in MCKH cells.

Primary cell culture exhibited the EMT phenotype. EMT is characterized by down-regulation of epithelial proteins including E-cadherin, epithelial cytokeratins (KRT8, KRT18, and KRT19), junctional adhesion molecules including

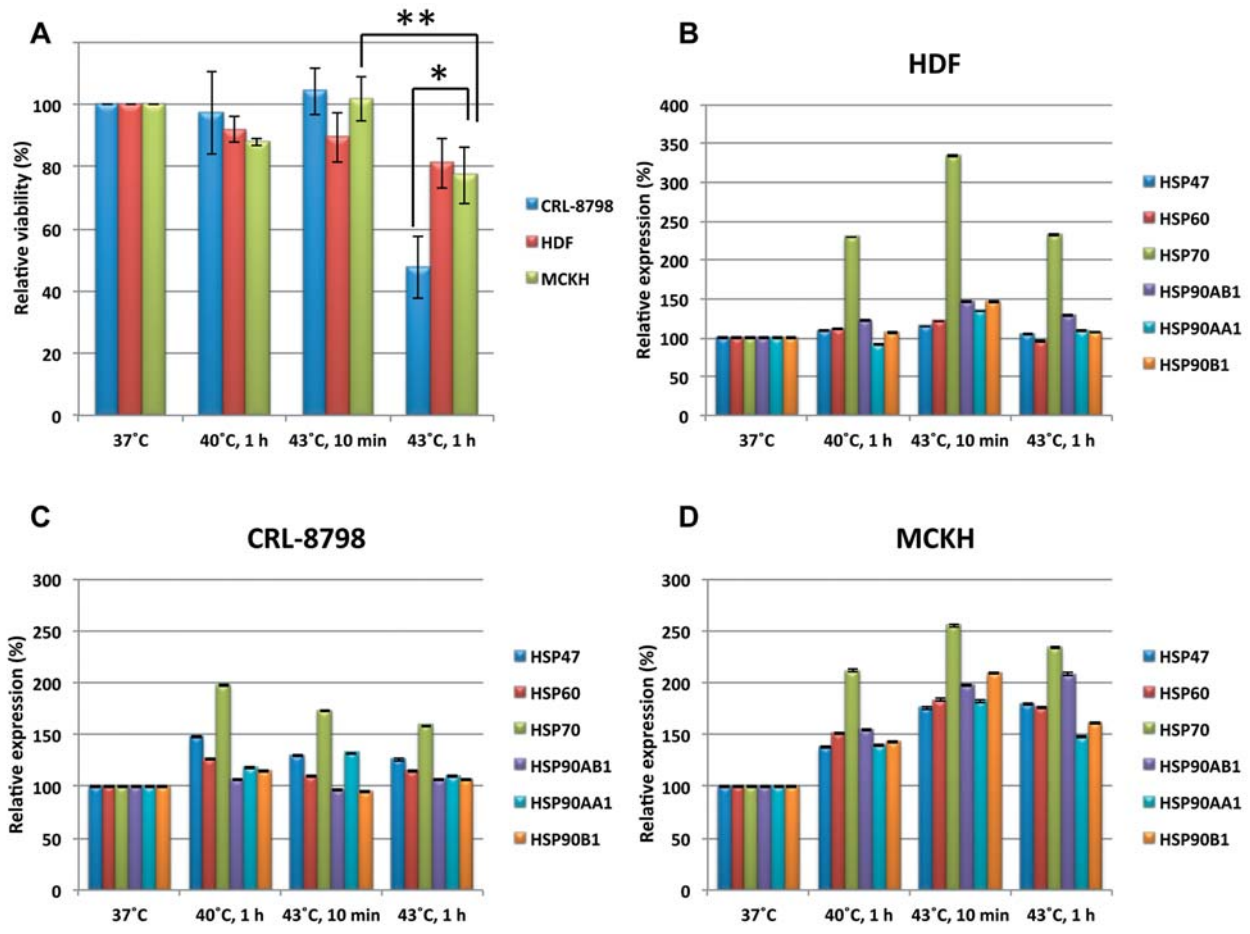

Figure 2. *Survival rate and heat shock protein gene expression after heat shock treatment. A: Relative viability 24 h after heat-shock. After heat shock at 43˚C for 1 h, the difference between CRL-8798 and MCKH cells was significant (*p=0.036). For the MCKH cells, the difference between heat shock at 43˚C for 10 min and 43˚C for 1 h was also significant (**p=0.039). Significant differences were calculated with Student's t-test. B-D: Relative expression of heat-shock protein genes, 1 h after heat shock in HDF (B), CRL-8798 (C), and MCKH (D) cells.*

occludins (OCLN), claudins (CLDN), and desmoplakin (DSP); and by up-regulation of mesenchymal proteins including vimentin (*VIM*), fascin-1 (FSCN), α-smooth muscle actin (ACTA2), N-cadherin (CDH2), cadherin-11 (CDH11), TGFβ-1 (TGFB1), and transcription factors related to cadherin gene expression including forkhead box C1 and C2 (FOXC1 and -*2*), goosecoid homeobox (GSC), snail homolog 1 and 2 (SNAI1 and -*2*), E12/E47 (TCF3), twist homolog 1 and 2 (TWIST1 and -*2*), and Zinc finger E-box binding homeobox 1 and 2 (ZEB1 and -*2*) (10).

As shown in Table III, MCKH cells exhibited down-regulation of epithelial markers and up-regulation of mesenchymal markers. In particular, E-cadherin was down-regulated 400-fold, and N-cadherin almost 200-fold compared to those in malignant tissue. These changes were not seen with CRL-8798 cells. Moreover, both *CLDN* genes were dramatically reduced, and ACTA2 and several transcription factors (SNAI1, SNAI2, and TWIST2) were increased by more than 4 times. These data strongly suggested the presence of EMT in MCKH cells.

Primary culture as an assay system for adjuvant treatment. Because MCKH cells retained some original tumor characteristics, we heat-shocked the MCKH cells to examine the usefulness of this model system for testing possible treatments for the patient. As shown in Figure 2A, although more than 50% of CRL-8798 cells died after heat shock at 43˚C for 1 h, about 80% of HDF and MCKH cells survived after the same heat shock. HDF cells highly-overexpressed heat-shock protein (HSP)70, and this overexpression was present even after the 40˚C heat shock (Figure 2C). In MCKH cells, in addition to overexpression of *HSP70*, all HSP genes examined in this experiment were overexpressed (Figure 2D) compared to the CRL-8798 cells (Figure 2B).

Table III. *Expression profiles of the epithelial-mesenchymal transition (EMT)-related genes in MCKH cells.*

Gene name	Gene	Change in EMT	Malignant tissue	CRL-8798 (normal cell line)	MCKH
Cell-cell adhesion & junctional complex					
CDH1	E-Cadherin	Down	1.530±0.060	1.752±0.059	0.004±0.001
CLDN3	Claudin 3	Down	1.466	0.002	0.002
CLDN4	Claudin 4	Down	1.928	0.054	0.001
DSP	Desmoplakin	Down	0.921±0.040	0.607±0.052	0.167±0.008
OCLN	Occludin	Down	0.919±0.128	0.730±0.150	0.483±0.216
Epithelial keratins					
KRT8	Keratin 8	Down	2.162±0.138	3.083±0.429	0.226±0.052
KRT18	Keratin 18	Down	1.265±0.092	5.222±0.853	0.275±0.052
KRT19	Keratin 19	Down	4.044	0.009	0.243
Mesenchymal transition markers					
ACTA2	Actin, alpha 2	Up	0.433	0.042	2.095
CDH2	N-Cadherin	Up	2.592±0.548	0.580±0.169	496.748±187.900
CDH11	Cadherin 11	Up	5.432	0.005	10.574
FSCN1	Fascin 1	Up	2.311	16.110	8.330
SPARC	Secreted protein, acidic, cysteine-rich	Up	7.179	0.123	9.801
VIM	Vimentin	Up	0.639	0.038	2.375
Cadherin expression					
TGFB1	Transforming growth factor-β1	Up	3.585±0.197	6.308±0.278	10.043±0.541
FOXC1	Forkhead box C1	Up	0.134	0.191	0.204
FOXC2	Forkhead box C2	Up	0.869	4.303	2.993
GSC	Goosecoid homeobox	Up	0.455	0.007	0.032
SNAI1	Snail homolog 1	Up	1.807	0.245	13.918
SNAI2	Snail homolog 2	Up	0.740	3.982	3.251
TCF3	E12/E47	Up	1.250	1.255	0.919
TWIST1	Twist homolog 1	Up	1.281±0.188	0.628±0.159	1.254±0.820
TWIST2	Twist homolog 2	Up	2.121	0.870	10.256
ZEB1	Zinc finger E-box binding homeobox 1	Up	1.844±0.097	0.041±0.004	1.310±0.070
ZEB2	Zinc finger E-box binding homeobox 2	Up	0.659±0.012	0.028±0.004	0.186±0.045

Total RNAs extracted from the extirpated normal and malignant tissues, CRL-8798, and MCKH primary cultured cell were analyzed with Agilent SurePrint G3 Human Gene Expression 8X60k. Expression profiles were calculated as fold changes compared to the normal tissue. Each value is the average of more than two features from the gene chip, except for GSC. Data obtained from more than three features are described with SD. Up: Up-regulated; Down: down-regulated.

This overall overexpression of HSP genes suggests resistance by MCKH cells to heat shock. Thus, the MCKH model system shows that heat shock may only be minimally effective in treating this patient.

Discussion

In this study, the most suitable method for establishing a primary culture from a surgically-extirpated breast tumor was the dispersed cell culture method using Dispase. MCKH cells exhibited EMT, which may represent the metastatic state of this tumor. As EMT is thought to be a crucial event for carcinoma progression, basic studies of EMT using model systems are still important for understanding how to control the metastatic progression of tumors (1).

The other important feature of MCKH cells is their stemness. CSCs exist within many types of cancer and are thought to play pivotal roles in both tumor initiation and maintenance of tumor growth (11). However, the origin of these CSCs remains unknown. Two hypotheses have been proposed suggesting that CSCs originate from normal tissue stem cells or arise from differentiated cancer cells through EMT (12). MCKH cells clearly exhibited both EMT and stemness, strongly suggesting a close relationship between EMT and CSCs.

BCSCs have been well-studied. For example, Ponti *et al.* established $CD44^{+}/CD24^{low}$ BCSCs (4), which can be propagated *in vitro* as non-adherent mammospheres. Li *et al.* carried out proteomic analyses using mammospheres as a model system (13). Although we could not culture MCKH cells as mammospheres or demonstrate their tumorigenic activity, primary culture of breast cancer cells with a mesenchymal phenotype and stemness, as was seen with MCKH cells, is a unique *in vitro* culture system.

Some reports also established a cultured cell line from immortalized MDA-MB453 human breast cancer cells (14) and primary culture of breast cancer (1), which display CSC and EMT characteristics, respectively. These two reports analyzed the expression of only a limited number of genes. In our experiment, comprehensive analysis of gene expression was used to compare the four closely related types of tissues and cells (MCKH cells, CRL-8798 cells, malignant breast tumor, and normal breast tissue). The large amount of data generated in this study will provide accurate information about MCKH cells and will likely make these cells a good experimental model for analyzing the mechanisms of EMT and the maintenance and differentiation of CSCs. Understanding the cellular, molecular, and genetic mechanisms of EMT, and the cellular, molecular, and genetic characteristics of CSCs using a primary culture model system such as MCKH cells may provide potential therapeutic targets for treating the disease.

In this study, using MCKH cells as a model system, we assessed the effects of hyperthermia. MCKH cells were relatively resistant to heat shock, which may be due to overexpression of HSPs, but these data also suggest that prolonged hyperthermia may favorably affect cancer regression. On the other hand, resistance to high temperature supports the idea that MCKH cells have stem cell properties.

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