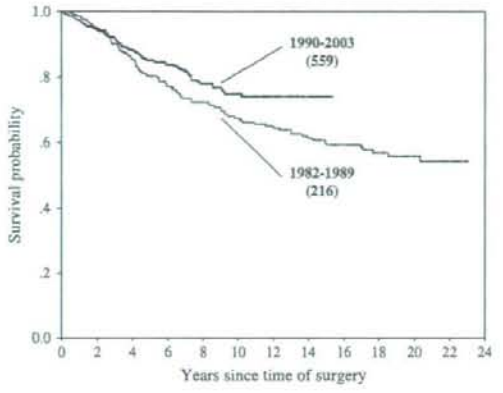


Fig. 1. Chronological changes in the cumulative survival curve with breast cancer after the time of surgery by two periods: 1982–1989 and 1990–2003. (Number) = number of patients. 1982–1989 versus 1990–2003, $P = 0.037$ (log-rank test).

factor.[12] It is also well known that stage is the most significant independent prognostic factor for determining survival in breast cancer.[13] The present study suggests that survival curves significantly improved between period I (1982–1989) and period II (1990–2003) (Fig. 1), although the population of <35-year-old patients and stage IV patients in period II increased compared to that in period I (Table 2). After 1990, breast-conserving surgery was included in our institute. All patients with breast-conversing surgery received radiation therapy. The patients also received adjuvant chemotherapy with LH–RH agonist after 2001, and CEF or CMF regimen after 2002. Therefore, there is a possibility that this improvement was due to the rational development and the exhaustive employment of innovative, targeted anti-cancer agents as adjuvant chemotherapy. Yoshimoto et al.[1] recently also reported that the survival curves in the 1990s significantly improved compared with the 1980s, and that the 10-year overall survival rate in the 1990s (83%) was better than that in the 1980s (77%). The present study also suggested the possibility that stages II and III patients, 35–54-years-old patients, or ER positive patients were received benefits by the above-mentioned rational treatments.

Table 2
Chronological changes of 10-year survival probability between period I (1982–1989) and period II (1990–2003)

| | Period I (1982–1989) % | Period II (1990–2003) % | *P*-value |
|---|---|---|---|
| Age | | | |
| <35 | 100 | 59.8 | 0.33 |
| 35–54 | 65.2 | 79.0 | 0.002 |
| ≥55 | 69.2 | 72.0 | 0.92 |
| pTNM stage | | | |
| Stage I | 90.8 | 89.2 | 0.87 |
| Stage II | 75.9 | 85.8 | 0.036 |
| Stage III | 36.8 | 50.8 | 0.028 |
| Stage IV | 22.2 | 10.2 | 0.43 |
| ER status | | | |
| Positive | 68.1 | 79.4 | 0.029 |
| Negative | 61.2 | 67.4 | 0.29 |
| Unknown | 70.1 | 82.4 | 0.12 |

This present study was based on the results performed at a single institution: Kyoto University Hospital. The merit of the long-term survival study in a single institution is the convenient and efficient way to investigate the characteristics of the patients' categories or backgrounds. Brinkley and Haybittle[14] previously reported that the benefit of analyzing data from a single institution is that after about 20 years, the survival curve of the breast cancer patients runs parallel to the expected survival curve of a similar normal population, and suggested that 18% of all the breast cancer patients might be regarded as cured of their disease in the sense defined by Easson and Russell.[15] This fact confirmed the benefit of analyzing data from a single institution, as mentioned above. On the while, the demerit of study in a single institution is the shift in patient outcome based on the alternating of main surgeons or pathologists for the treatment of breast cancer.

In conclusion, recently, breast cancer is commonly treated by various combinations of surgery, radiation therapy, chemotherapy, and hormone therapy. The present study presented that survival curves have significantly improved between 1982–1989 and 1990–2003 of female patients with breast cancers at our institution, and the recent advance of the survival rates might be due to the rational development of breast cancer treatment.

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# The reversal of recurrence hazard rate between ER positive and negative breast cancer patients with axillary lymph node dissection (pathological stage I-III) 3 years after surgery

Takayoshi Kiba[1,2], Takashi Inamoto*[3], Tsutomu Nishimura[4], Masaya Ueno[1], Kazuhiro Yanagihara[1], Satoshi Teramukai[2,4], Hironori Kato[5], Masakazu Toi[5] and Masanori Fukushima[1,2,4]

Address: [1]Outpatient Oncology Unit, Kyoto University Hospital, Kyoto, Japan, [2]Translational Research Informatics Center, Kobe, Japan, [3]Department of Breast Surgery, Kitano Hospital, The Tazuke Kofukai Medical Research Institute, Osaka, Japan, [4]Translational Research Center, Kyoto University Hospital, Kyoto, Japan and [5]Department of Breast Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Email: Takayoshi Kiba - kiba@tri-kobe.org; Takashi Inamoto* - t-inamoto@kitano-hp.or.jp; Tsutomu Nishimura - t246ra@kuhp.kyoto-u.ac.jp; Masaya Ueno - Umasaya@aol.com; Kazuhiro Yanagihara - kazuhiro@kuhp.kyoto-u.ac.jp; Satoshi Teramukai - steramu@kuhp.kyoto-u.ac.jp; Hironori Kato - hkato@kuhp.kyoto-u.ac.jp; Masakazu Toi - toi@kuhp.kyoto-u.ac.jp; Masanori Fukushima - mfukushi@kuhp.kyoto-u.ac.jp

* Corresponding author

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## Abstract

**Backgrounds:** Prognostic factors are defined as biological or clinical measurement associated with overall survival and/or disease-free survival. Previous studies have shown that patients with estrogen receptor (ER) positive cancers have a better prognosis than patients whose cancers do not have these receptors.

**Methods:** This study investigated the assessment of variables in defining prognosis of 742 breast cancer women with pathological stage (pTNM) I-III diagnosed between 1980 and 2005 at the Kyoto University Hospital in Japan, by age, clinical stage (cTNM), pTNM, the numbers of positive lymph nodes (pN), and ER status.

**Results:** Multivariate analysis demonstrated that pTNM and ER status were the independent prognostic factors for overall survival, and that pTNM and pN were the independent prognostic factors for disease-free survival. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients.

**Conclusion:** The present study confirmed the previous reports which showed favorable prognosis of the patients with lesser pTNM or positive ER status. A reversal of recurrence hazard rate between ER positive and negative breast cancer patients beyond 3 years after operation was detected. The fact may indicate the importance of long term adjuvant hormone therapy for ER positive cancer patients.

## Background

A prognostic factor is defined as a biological or clinical measurement that is associated with overall survival and/or disease-free survival [1]. The knowledge of prognosis forms an integral part of the decision-making process in medicine [2]. Moreover, prognostic factors are important in the treatment of cancer to help identify subgroups of patients who may need more aggressive approach to therapy [3]. Further, prognostic factors also play a critical role in designing clinical trial as stratification and allocation factors [4]. Prognostic factors, i.e., those that predict the risk of recurrence or death from breast cancer, include stage, number of positive axillary nodes, tumor size, lymphatic and vascular invasion, the estrogen-receptor (ER) and progesterone-receptor (PR) positivity, and HER2/neu gene amplification [3,5]. We previously reported that the recent advance of the survival rates in breast cancer patients may be due to the rational development of treatment [6]. In order to assess the independent value of variables in defining prognosis, in the present study, we have investigated the survival of 742 breast cancer patients with pathological stage (pTNM) I-III, by the age, clinical stage (cTNM), pTNM, the numbers of positive lymph nodes (pN) and ER status.

## Methods

### Patients

742 female beast cancer patients aged between 21 and 80 with stage I-III of pTNM were selected from the patients treated at Kyoto University Hospital in Japan from 1980 to 2005. Based on the section 2 in chapter 1 of Japanese ethical guidelines for epidemiological research http:www.niph.go.jp/english2/english ver/ethical-gl/guide lines.htm, this study was exempt from ethical approval under Japanese law and guidelines. Moreover, all treatments for breast cancer were undertaken with informed consent and consents were also taken to confirm cancer diagnosis. These patients underwent surgery with axillary lymph node dissection. The operation methods were classified into three groups: breast conserving surgery, modified radical mastectomy, and standard radical mastectomy. All the patients with breast conserving surgery received radiation therapy. Staging of cTNM and pTNM was evaluated according to UICC stage [7]. Number of lymph node metastasis and ER status of the primary tumors were analyzed by staff members of the Department of Pathology at Kyoto University Hospital. Using immunohistochemistry on the whole series of tumors, they assessed estrogen receptor (ER) status in a standardized way. In our institute, the pathologists routinelyhave examined the ER status of tumors by using the immunohistochemistry since the 1980s. The contents of treatments for breast cancer patients were previously described [6]. According to the years of surgery the patients were grouped into two cohorts: period I (1980–1989) and period II (1990–2005). In period I, modified radical mastectomy with lymph node dissection was included. In this period, breast-conserving surgery was not performed, because it was not recognized to be the prevailing method in Japan. In period II, breast-conserving surgery was the treatment of choice for women with relatively small breast cancers during this past decade in Japan. In our institute, all patients with breast-conversing surgery received radiation therapy. In the treatment stage I, II, IIIA, and operable stage IIIC breast cancer, breast-conserving surgery or modified radical mastectomy with lymph node dissection and with or without breast reconstruction surgery was included. In the treatment of stage IIIB and inoperable stage IIIC breast cancer, systemic chemotherapy, or systemic chemotherapy followed by surgery, with lymph node dissection followed by radiation therapy were included. If necessary, additional systemic therapy such as chemotherapy, hormone therapy, or both were given. Moreover, if necessary, adjuvant therapy such as systemic chemotherapy (*per os* only) with or without hormone therapy (tamoxifen or tremifene) was included. The patients received adjuvant chemotherapy with LH-RH agonist after 2001, cyclophosphamide, epirubicin and 5-fluorouracil (CEF) or Cyclophosphamide, methotrexate and 5-fluouracil (CMF) regimen after 2002, and rational developers such as taxane, trastuzumab, or aromatase inhibitor therapy after 2004.

### Statistical analysis

Disease-free survival was defined from the operation day to the identification date of recurrence of cancer or death from any cause, and overall survival was defined from the operation day to death from any cause. Survival curves were estimated with the Kaplan-Meier method. To identify prognostic factors independently associated with the overall survival or disease-free survival and to estimate the hazard ratios, the Cox proportional hazard model was applied. Two-sided $p < 0.05$ was regarded as statistically significant. The statistical analysis was conducted with SPSS version 11.0 statistical software.

## Results

### Patient Characteristics

Patient characteristics are summarized in Table 1. The medianfollow-uptime of the investigated period in this study was as same as the median follow-up time forsurvivingpatients (5.7 years).

### 10-year overall survival

The 10-year overall survival rates classified by age, cTNM, pTNM, pN, ER status and types of breast surgery are shown in Table 2. Figure 1 shows the overall survival curves in ER-positive and ER-negative patients.

**Table 1: Patient characteristics (n = 742)**

| | number | % |
|---|---|---|
| Gender | | |
| female | 742 | 100 |
| male | 0 | 0 |
| Age | | |
| <35 (21–34) | 35 | 4.7 |
| 35–54 | 337 | 45.4 |
| ≥ 55 (55–91) | 370 | 49.9 |
| cTNM stage | | |
| Stage I | 197 | 26.6 |
| Stage II | 452 | 60.9 |
| Stage III | 93 | 12.5 |
| pTNM stage | | |
| Stage I | 189 | 25.5 |
| Stage II | 397 | 53.5 |
| Stage III | 156 | 21.0 |
| pN | | |
| pN0 | 422 | 56.9 |
| pN1 | 189 | 25.5 |
| pN2 | 88 | 11.9 |
| pN3 | 43 | 5.8 |
| ER status | | |
| negative | 290 | 39.1 |
| positive | 452 | 60.9 |
| Breast surgery | | |
| Breast conserving surgery | 305 | 41.1 |
| Modified radical mastectomy | 429 | 57.8 |
| Standard radical mastectomy | 8 | 1.1 |

### *10-year disease-free survival*

The 10-year disease-free survival rates classified by age, cTNM, pTNM, pN, ER status and types of breast surgery are shown in Table 3. The approximate 10-year disease-free survival between ER positive and negative patients was reversed (Figure 2). According to age, cTNM, pTNM and pN, the reversal of disease-free survival was not detected in the present study (Table 3).

### *Estrogen receptor status*

Because beyond 10 years hazard had increased statistical errors, we investigated the annual hazard of recurrence until 10 years after operation. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients (Figure 3). Figure 4 shows that the overall survival of ER-positive cancer patients was increased by adjuvant hormone therapy ($p = 0.009$). Moreover, among 452 ER-positive cases, at 1 year after surgery, the hazard of recurrence was higher for the patients with adjuvant hormone therapy than the patients without adjuvant hormone therapy, but between 2 and 4 years, the hazard was higher for the patients without adjuvant hormone therapy (Figure 5).

### *Prognostic factor analysis*

Age (<35; 35–54; ≥ 55), cTNM (stage I-III), pTNM (stage I-III), pN (pN0, pN1, pN2, pN3), ER status (negative, positive, unknown), and types of breast surgery (breast conserving surgery, modified radical mastectomy, radical mastectomy) were analyzed as potential prognostic factors by the Cox proportional hazard model. Both univariate analyses to determine prognostic factors associated with overall survival and disease-free survival that the features with $p < 0.05$ were 5 features: cTNM, pTNM, pN, ER status, and type of surgery (Table 2 &3). The important prognostic factor associated with overall survival determined by multivariate analyses with backward variables selection were 2 features: pTNM and ER status (Table 4). The important prognostic factor associated with disease-free survival determined by multivariate analyses with backward variables selection were two features: pTNM and pN (Table 4).

## Discussion

Tumor staging systems provide information about extent of disease that can be used to guide treatment recommendations and provide estimates of patient prognosis. It is well known that pathological stage is the most significant independent prognostic factor for determining survival in breast cancer [8]. Our study documents the fact that pathological stage is the independent prognostic factor for both overall survival and disease-free survival.

Many studies have shown that women with ER positive cancers have a better prognosis than patients whose cancers do not have this receptor [9,10]. In this study cohort, ER status were the independent prognostic factors for overall survival by the multivariate Cox regression analysis, but ER status did not affect disease-free survival (Table 3 &4). Nomura et al. [11] previously reported that in a retrospective multicenter study to investigate the ER status in primary breast cancer with patient prognosis, 3,118 patients with operable breast cancer (stages I-III) were investigated from ten hospitals in Japan who underwent surgery from October 1972 to December 1982, and that Cox's multivariate analysis showed that overall survival, but not disease-free survival was affected by ER status. They speculated the possibility that this was due to the longer postrelapse survival in patients with ER-positive cancer based on the effectiveness of endocrine treatment. Preceding paper has reported that the patients of positive ER status enjoyed benefits from the recent development of breast cancer treatments [6]. In fact, the present study showed that the overall survival of ER-positive cancer patients was increased by adjuvant hormone therapy (Figure 4).

Hortobagyi et al. [12] previously reported that the disease-free survival in estrogen receptor (ER) positive and/or pro-

**Table 2: The 10-year overall survival rates and univariate Cox regression analysis**

| Factors | overall survival rates | | Hazard ratio | | Log-rank test p-value |
|---|---|---|---|---|---|
| | 10-year (%) | 95% CI[a] | | 95% CI [a] | |
| Age | | | | | |
| < 35 | 69.6 | 57.7–81.5 | 1.00 | - | 0.30 |
| 35–54 | 78.1 | 75.2–81.0 | 0.69 | 0.30–1.59 | |
| ≥ 55 | 73.4 | 69.9–77.0 | 0.90 | 0.39–2.08 | |
| cTNM | | | | | |
| Stage I | 85.7 | 81.3–90.0 | 1.00 | - | <0.001 |
| Stage II | 75.8 | 73.1–78.5 | 2.32 | 1.31–4.09 | |
| Stage III | 54.5 | 46.9–62.0 | 4.85 | 2.55–9.22 | |
| pTNM | | | | | |
| Stage I | 89.4 | 85.8–93.1 | 1.00 | - | <0.001 |
| Stage II | 81.7 | 79.1–84.4 | 2.10 | 1.10–4.03 | |
| Stage III | 46.4 | 40.8–51.9 | 7.77 | 4.08–14.81 | |
| pN | | | | | |
| pN0 | 86.7 | 84.2–89.1 | 1.00 | - | <0.001 |
| pN1 | 76.4 | 72.1–80.7 | 1.74 | 1.08–2.82 | |
| pN2 | 46.6 | 39.7–53.4 | 5.25 | 3.34–8.27 | |
| pN3 | 38.2 | 26.9–49.5 | 5.34 | 3.01–9.47 | |
| ER status | | | | | |
| Negative | 71.0 | 67.7–74.3 | 1.00 | - | 0.012 |
| Positive | 79.5 | 76.5–82.5 | 0.63 | 0.44–0.91 | |
| Breast surgery | | | | | |
| Breast conserving surgery | 76.1 | 71.2–81.0 | 1.00 | - | 0.093 |
| Modified radical mastectomy | 76.2 | 73.7–78.7 | 1.31 | 0.84–2.04 | |
| Standard radical mastectomy | 19.1 | 2.29–35.8 | 4.09 | 1.57–10.64 | |

[a] CI: Confidence Interval.

gesterone receptor (PgR) positive patients was higher than that in ER/PgR negative patients until 5 years after administration of the state-of-the-art adjuvant therapy, however, the disease-free survivals between these groups was reversed after 5 years. Saphner et al. [13] reported that compared with ER negative patients, ER positive patients had lower annual hazard of recurrence until around 3.5 years after surgery, but thereafter higher. In the present study, Figure 3 shows that a positive ER status was associated with a lower hazard of recurrence in the first 2 years after surgery, but a higher hazard of recurrence from years 3 to 10. [14]. Results from the EBCTCG meta-analysis of systemic treatment of early breast cancer by hormone, cytotoxic, or biologic therapy methods in randomized trials involving 144,939 women show a highly significant advantage of 5 years versus 1 to 2 years of tamoxifen with respect to the risk of recurrence [14]. In the present study, in ER-positive cases, between 2 and 4 years after surgery, the hazard of recurrence of patients without adjuvant hormone therapy was higher than the patients with adjuvant hormone therapy (Figure 5). It is noteworthy that this observation emphasizes the importance of adjuvant hormone therapy for ER positive cancer patients beyond 3 years after operation. Moreover, comparing with the 10-year survival rate between ER-positive patients with or without hormone therapy and ER-negative patients (Figure 1 &4), the survival rate between ER-positive patients without hormone therapy and ER-negative patients was similar, but the adjuvant hormone therapy led about 13%

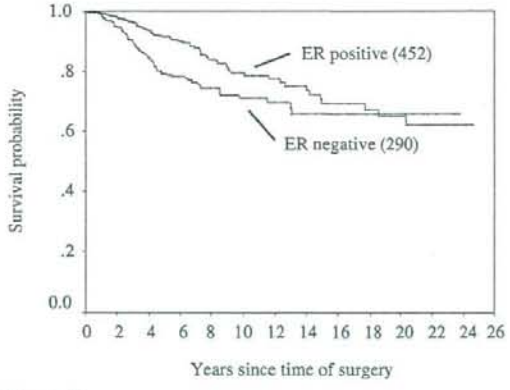


**Figure 1**
**Overall survival curves in ER-positive and ER-negative patients.** (Number) = number of patients. $p = 0.012$.

**Table 3: The 10-year disease-free survival rates and univariate Cox regression analysis**

| Factors | disease-free survival rates | | Hazard ratio | | Log-rank test p-value |
|---|---|---|---|---|---|
| | 10-year (%) | 95% CI [a] | | 95% CI [a] | |
| Age | | | | | |
| < 35 | 47.0 | 33.3–60.7 | 1.00 | - | 0.49 |
| 35–54 | 59.0 | 55.7–62.4 | 0.91 | 0.50–1.64 | |
| ≥ 55 | 63.1 | 59.4–66.7 | 0.90 | 0.50–1.63 | |
| cTNM | | | | | |
| Stage I | 72.4 | 67.3–77.6 | 1.00 | - | <0.001 |
| Stage II | 60.9 | 58.0–63.9 | 2.05 | 1.45–2.91 | |
| Stage III | 31.5 | 24.8–38.2 | 5.03 | 3.36–7.52 | |
| pTNM | | | | | |
| Stage I | 81.7 | 77.4–85.9 | 1.00 | - | <0.001 |
| Stage II | 67.5 | 64.4–70.5 | 2.18 | 1.47–3.24 | |
| Stage III | 17.7 | 13.1–22.4 | 7.66 | 5.13–11.43 | |
| pN | | | | | |
| pN0 | 76.6 | 73.8–79.5 | 1.00 | - | <0.001 |
| pN1 | 56.4 | 51.6–61.2 | 1.85 | 1.36–2.53 | |
| pN2 | 15.5 | 10.3–20.7 | 5.75 | 4.22–7.83 | |
| pN3 | 26.8 | 16.6–37.1 | 4.88 | 3.25–7.32 | |
| ER status | | | | | |
| Negative | 59.8 | 56.4–63.2 | 1.00 | - | 0.183 |
| Positive | 60.0 | 56.6–63.4 | 0.83 | 0.63–1.09 | |
| Breast surgery | | | | | |
| Breast conserving surgery | 59.1 | 54.1–64.0 | 1.00 | - | 0.007 |
| Modified radical mastectomy | 61.5 | 58.7–64.3 | 1.14 | 0.86–1.52 | |
| Standard radical mastectomy | ND[b] | - | 3.34 | 1.76–6.33 | |

[a] CI: Confidence interval, [b] ND: not determined.

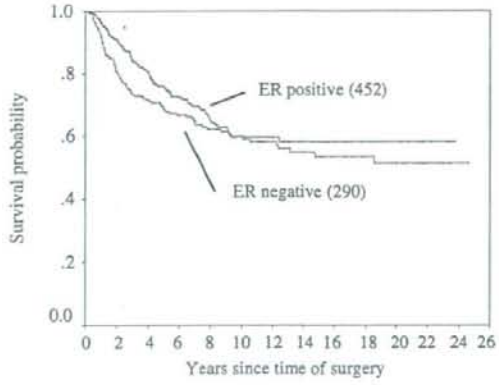


**Figure 2**
**Disease-free survival curves in ER-positive and ER-negative patients.** (Number) = number of patients. *p = 0.18.*

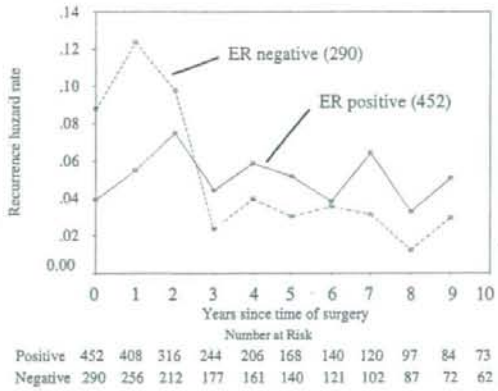


**Figure 3**
**Annual hazard of recurrence of patients separated by ERstatus.** (Number) = number of patients.

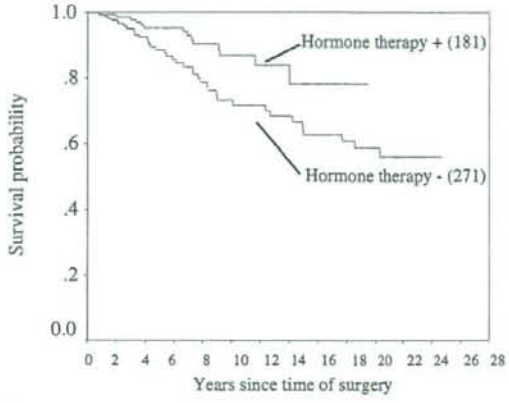


**Figure 4**
**Overall survival curves in ER-positive patients with and without adjuvant hormone therapy.** (Number) = number of patients. *p = 0.009*.

survival gains. Therefore, this fact also suggests adjuvant hormone therapy may have more important roles in the treatment. In addition, the disease-free survival at 10 years after surgery between ER positive and negative patients was reversed (Figure 2). This may be related to the fact that the percentage of number of patients who received adjuvant hormone therapy in ER positive patients between 1980 and 1991 (11/84: 13%) was smaller to that between 1991 and 2005 (170/368: 46%), because of reasons including poor understanding of modern treatment for adjuvant chemotherapy, the cost for drugs, and so on. On

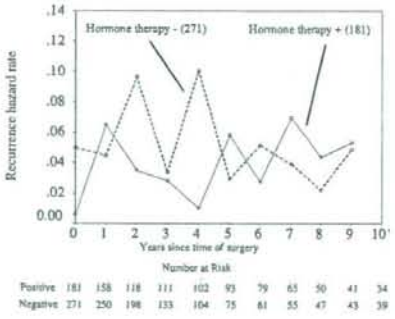


**Figure 5**
**Annual hazard of recurrence of ER-positive patients separated by adjuvant hormone therapy.** (Number) = number of patients.

**Table 4: Multivariate Cox regression analysis for overall survival and disease-free survival.**

| Factors | Overall survival | | |
|---|---|---|---|
| | Hazard ratio | 95% CI [a] | *p*-value |
| pTNM | | | |
| Stage I | 1.00 | - | |
| Stage II | 2.05 | 1.07–3.94 | 0.03 |
| Stage III | 8.09 | 4.24–15.43 | <0.001 |
| ER status | | | |
| Negative | 1.00 | - | |
| Positive | 0.57 | 0.40–0.82 | 0.002 |
| **Factors** | **Disease-free survival** | | |
| | Hazard ratio | 95% CI [a] | *p*-value |
| pTNM | | | |
| Stage I | 1.00 | - | |
| Stage II | 1.87 | 1.12–3.11 | 0.017 |
| Stage III | 3.72 | 1.59–8.70 | 0.002 |
| pN | | | |
| pN0 | 1.00 | | |
| pN1 | 1.49 | 1.01–2.20 | 0.044 |
| pN2 | 2.47 | 1.14–5.34 | 0.022 |
| pN3 | 1.91 | 0.83–4.39 | 0.129 |

[a] CI: Confidence interval

the other hand, the current recommendation is that adjuvant tamoxifen be discontinued after 5 years in all patients as current standard therapy, because there was a trend toward a worse outcome associated with a longer duration of treatment [15]. Further analyses may be needed to clarify the optimal duration of adjuvant hormone therapy in operated breast cancer patients.

Traditional prognostic factors, i.e., those that predict the risk of recurrence or death from breast cancer, include number of positive axillary nodes [3]. It has been reported that the pN is the most important prognostic factor affecting disease-free survival and overall survival in operable breast cancer patients [2]. However, our study suggested that pN is the independent prognostic factor for disease-free survival, but not for overall survival. The patients with axillary lymph node metastasis have received chemotherapy, hormonal therapy or both. Over the past 20 years, various systemic adjuvant therapies have been studied to improve survival [6]. Therefore, there may be a possibility that the other factors such as these therapies may affect the overall survival more stronger than pN, although further investigations are needed to clarify this matter.

The univariate Cox regression analysis for overall survival and disease-free survival demonstrated that the hazard ratio of patients with breast conserving surgery was lower

than that of patients with standard radical mastectomy (Table 2 &3). This fact suggests that breast conserving surgery with radiation therapy may provide not only cosmetic benefit but also better prognosis, although chronological change of breast cancer treatments may affect the survival rates.

In conclusion, the present study presented the data of the long term survival of pathological stage I-III patients with breast cancers at our institution. For the 0- to 2-year interval, the hazard of recurrence was higher for the ER-negative patients than the ER-positive patients, and beyond 3 years the hazard was higher for ER-positive patients. Additionally, disease free survival 10 years after operation was reversed between ER-positive and negative patients. Therefore, the fact may indicate the importance of long term adjuvant hormone therapy for ER positive cancer patients.

## Abbreviations

cTNM: clinical stage; ER: estrogen receptor; pTNM: pathological stage; pN: positive lymph nodes.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

TI and MF designed this study. MU, KY, HK, TI collected and assembled the data. TI organized the data. TK, TN, ST, TI and MF contributed to the statistical analyses and interpretations. TK, TI, MT and MF contributed to writing and finalizing of the manuscript. All authors read and approved the final manuscript.

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# Adipogenesis Induced by Human Adipose Tissue–Derived Stem Cells

Wakako Tsuji, M.D.,[1] Takashi Inamoto, M.D., Ph.D.,[2] Hiroyasu Yamashiro, M.D., Ph.D.,[1]
Takayuki Ueno, M.D., Ph.D.,[1] Hironori Kato, M.D., Ph.D.,[1] Yu Kimura, M.Eng.,[3]
Yasuhiko Tabata, Ph.D., D.Med.Sci., D.Pharm.,[3] and Masakazu Toi, M.D., Ph.D.[1]

Adipose tissue–derived stem cells (ASCs), including preadipocytes, may play an important role in *de novo* adipogenesis and are expected to be a useful external source of cells for adipose tissue engineering. In this study, we examined *in vivo* adipogenesis up to 24 weeks after implantation, induced by human ASCs that were isolated from adipose tissues and expanded *in vitro*. ASCs proliferated *in vitro* in the presence of basic fibroblast growth factor (bFGF), and the number of cells increased by more than 1000-fold at the fourth passage. The ability to differentiate into mature adipocytes was maintained up to the third passage. We incorporated designated numbers of third-passage–expanded cells into a type I collagen scaffold and implanted them into the back of nude mice with or without controlled-release bFGF. After the implantation of $2\times10^6$ ASCs with controlled-release bFGF, the greatest cross-sectional surface area of adipose tissue in the scaffold was 1.19 $mm^2$ at 12 weeks and 2.14 $mm^2$ at 24 weeks. About $2\times10^5$ ASCs with controlled-release bFGF was the best condition for total adipogenesis. Immunohistochemical analysis with antihuman vimentin antibody showed that the area of human-origin adipose tissue was maximum in the group with $8\times10^6$ ASCs incorporated in a scaffold at both 12 and 24 weeks. The amount of human-origin adipose tissue increased in all groups with implanted ASCs from 12 to 24 weeks. Only trace of human-origin adipose tissue was observed in other groups implanted ASCs. Our results show that human ASCs not only function as progenitor cells for *in vivo* adipogenesis, but also induce *de novo* adipogenesis for long period.

## Introduction

BREAST CANCER IS THE MOST COMMON CANCER in women, and surgery remains one of the main treatments. Breast surgery results in deformity of the breast and negatively affects patients' quality of life. Perforator flaps or silicone implants have been used for breast reconstruction, but each has advantages and disadvantages. Several trials of autologous adipose tissue transplantation for breast reconstruction resulted in a 40–60% reduction in adipose tissue volume because of insufficient vascularization.[1–6]

Recent studies in tissue engineering indicate that cell proliferation requires an appropriate cell source, scaffold, and microenvironment, including growth factors.[7,8] The ideal cell source for tissue engineering must have self-renewal capability and immunocompatibility.[9] Mesenchymal stem cells (MSCs) isolated from bone marrow stroma can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages. However, the procurement of cells from bone marrow that are suitable for clinical use has several drawbacks, including severe pain, morbidity, and a low yield.[10] Adipose tissue–derived stem cells (ASCs) can be isolated from collagenase digests of adipose tissue. Various kinds of term have been used for this cell population, for example, adipose-derived stem/stromal cells, adipose-derived adult stem cells, preadipocytes, processed lipoaspirate cells, and adipose mesenchymal stem cells. The International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" to identify the isolated, plastic-adherent, multipotent cell population.[11] ASCs also can differentiate into adipogenic, osteogenic, myogenic, and chondrogenic lineages similar to MSCs.[12] Moreover, a comparison of MSCs and ASCs from the same patient showed no significant differences in the yield of adherent stromal cells, growth kinetics, cell senescence, multilineage differentiation capacity, or gene transduction efficiency.[13] Gene array analysis revealed that less than 1% of genes were differentially expressed between ASCs and MSCs. ASCs were superior to MSCs with respect to maintenance of proliferating ability.[14] The fraction of preadipocytes contributing to adipogenesis

[1]Department of Breast Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
[2]Department of Breast Surgery, Kitano Hospital, The Tazuke Kofukai Medical Research Institute, Osaka, Japan.
[3]Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.

differs among species and ages. In humans the fraction is 1% in children and less than 0.1% in adults.[15] Human adipose tissue is abundant and can be obtained more safely and easily under local anesthesia or at breast surgery than bone marrow cells.

During differentiation into mature adipocytes, preadipocytes express several types of extracellular matrix (ECM) proteins, including fibronectin, laminin, and types I, III, IV, V, and VI collagen. A fibronectin network develops initially, and a type I collagen network is formed last. These ECMs allow preadipocytes to differentiate into mature adipocytes.[16] In response to adipogenic stimulation, ASCs form tissue sheets harboring lipid-filled adipocytes embedded into an abundant human ECM.[17] *In vivo* adipogenesis depends on the type of ECM.[18] Type I collagen has been widely used as a scaffold for adipose tissue engineering[19–22] because of its porous structure. Preadipocytes readily adhere to and grow in type I collagen scaffolds.[23] An *in vitro* study reported a higher rate of adipogenic differentiation with type I collagen than with fibronectin.[24]

*In vitro* proliferation of ASCs is enhanced by basic fibroblast growth factor (bFGF).[25] Although it remains controversial whether bFGF has direct adipogenic activity,[26–29] bFGF has been shown to promote adipogenesis *in vitro* and *in vivo*.[30–32] We found that the controlled-release bFGF more effectively promoted adipose tissue regeneration than aqueous bFGF.[20] We have reported that controlled-release 1 μg of bFGF/site was the most effective concentration on adipogenesis 6 weeks after implantation into nude mice, and a high dose of bFGF caused the inflammatory response in the collagen scaffold.[20]

In a previous study, we implanted up to $5\times10^5$ human ASCs into nude mice and obtained newly formed adipose tissue 6 weeks after implantation. However, quantitative and qualitative differences in the implanted ASCs were not examined, and not examined for long time. The present study was therefore investigated the optimal passage number *in vitro* and the effects of the number of implanted ASCs on adipogenesis *in vivo* over the period up to 24 weeks.

## Materials and Methods

### *Human ASCs*

This study was approved by the Kyoto University ethics committee. Informed consent was obtained from all patients. All patients were women 29–76 years of age. Samples of human adipose tissues were obtained as surgical waste tissue at breast surgery in Kyoto University Hospital (Kyoto, Japan). Donor samples for *in vitro* study were obtained from patients 30–76 years of age with a mean age of 58.6 years ($n=14$). We allocated seven donors individually to with or without bFGF treatment group. There was no significance of age between with and without bFGF treatment groups. Donor samples for *in vivo* study were obtained from patients 29–70 years of age with a mean age of 50.8 years ($n=8$). All donor samples were divided equally. ASCs were isolated from the adipose tissue samples as soon as possible after resection by a modification of the procedure described by Bjorntorp *et al.*[33] Briefly, the adipose tissue samples were washed with phosphate-buffered saline (PBS, pH 7.4) to remove blood cells, minced, and digested with collagenase (2 mg/mL; Wako Pure Chemical, Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) and 20 mg/mL bovine serum albumin at 37°C for 40 min while shaking. The digested tissue was suspended in DMEM:Nutrient Mixture F-12(Ham) (1:1) (DMEM/F-12) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (basal medium). The suspension was filtered through a 250-μm nylon mesh and centrifuged at 400 *g* for 10 min at 20°C. The sediment was suspended in basal medium, placed in 10-cm tissue culture dishes (Falcon; Falcon, New York, NY), and cultured in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C for 1 day. The dishes were gently washed with PBS to remove nonadherent cells and filled with the basal medium and cultured until the adherent ASCs became confluent (P0:Passage0). Then ASCs were detached with 1% trypsin-EDTA solution (Sigma, St. Louis, MO).

### *Proliferation ability of ASCs*

ASCs were suspended in the basal medium, placed in 10-cm tissue culture dishes or 96-well microtiter plate (Falcon) at the density of $1.0\times10^4$ cells/cm$^2$, and cultured with 100 ng/mL bFGF or without bFGF for 1 week.[34] To evaluate proliferative activity, viable cells in each dish were counted by the trypan blue dye exclusion method at the end of culture. These numbers represent the proliferation of undifferentiated ASCs. The proliferation of ASCs was also evaluated by MTT assay.[35] Using a commercially available kit for MTT assay (Chemicon International, Temecula, CA), we spectrophotometrically measured the absorbance of the solution mixture in each well at 570–630 nm.

### *Differentiation ability of ASCs*

ASCs suspended in basal medium were placed in 24-well plates (Falcon) at a density of $1.0\times10^5$ cells/cm$^2$ and cultured for 1 day. The medium was then changed to DMEM/F-12 medium (1000 μL/well) containing 0.05 μM insulin, 0.2 nM 3,5,3-triiodothyronine, 100 nM transferrin, 17 μM calcium pantotenate, 33 μM biotin, and 100 nM dexamethasone (ITT medium)[36] and cultured for 21 days. To evaluate adipogenic differentiation of ASCs, glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using a commercially available kit (GPDH activity measurement kit, JFL003; Hokudo, Hokkaido, Japan).[37] ASCs were washed twice with PBS and homogenized in the buffer solution included with the kit, using a handy sonic homogenizer (UR-20; Tomy Seiko, Tokyo, Japan) on ice. After mixing, the absorbance of the solution mixture was spectrophotometrically measured at 340 nm.

### *Materials*

We prepared a disc form of type I collagen scaffold (diameter, 20 mm; height, 2.5 mm) and gelatin microspheres (isoelectric point, 5.0), as described in our previous report.[20,38] An aqueous solution of human recombinant bFGF was kindly supplied by Kaken Pharmaceutical, Tokyo, Japan. Other chemicals were purchased from Wako Pure Chemical Industries, Kyoto, Japan, and used without further purification. To prepare controlled-release bFGF, 2 mg of gelatin microspheres was swollen with an aqueous solution of bFGF (20 μL, containing 1 μg of bFGF) and allowed to stand at 37°C for 1 h.

### *Implantation of ASCs*

Animal experiments were reviewed by the Committee on the Ethics of Animal Experiments (Faculty of Medicine,

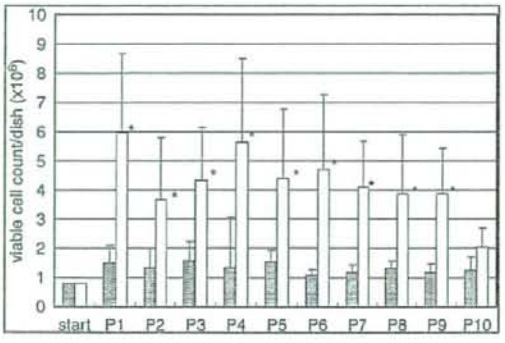



**FIG. 1.** Proliferation of ASCs *in vitro* assessed on the basis of the viable cell count. The number of viable ASCs per 10 cm of the tissue culture dish after culture with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by trypan blue dye exclusion assay. *: $p < 0.05$ versus without bFGF group.

Kyoto University, Kyoto, Japan) and were carried out in accordance with the Guidelines for Animal Experiments of the Faculty of Medicine, Kyoto University.

The designated number of third-passage ASCs (0: A group and B group; $5\times10^5$: C group and D group; $2\times10^6$: E group and F group; $8\times10^6$: G group and H group) were incorporated into the collagen scaffolds with (B, D, F, and H groups) or without controlled-release bFGF (A, C, E, and G groups), and implanted subcutaneously into the back of 6-week-old female BALB/c nude mice (Shimizu Laboratory Supply, Kyoto, Japan) under general anesthesia. Twelve-week group consisted of five mice. Twenty-four-week group consisted of three mice. Twelve and 24 weeks after implantation, the mice were euthanized with an overdose of anesthesia, and the implanted sites including the skin (approximately $2\times2\,cm^2$) were carefully removed for subsequent histological examinations.

### De novo *adipogenesis and human ASC–derived adipogenesis*

One half of each tissue specimen was fixed in 10% neutralized formalin solution and embedded in paraffin. Sections (thickness, 2 μm) of the specimens were stained with hematoxylin and eosin (H-E). The other half of the specimen was frozen, and sections were stained with oil red O to confirm the presence of mature adipose tissue. Paraffin sections were stained with a monoclonal antibody against human vimentin (mahv, clone V9, Code Nr. M 0725 Lot 057; DAKO, Glostrup, Denmark). The antibody was used at a dilution of 1:25. The positive control was human adipose tissue, and the negative control was murine adipose tissue. Adipose tissue area of the scaffolds and the human vimentin–positive area were measured and analyzed with the computer program Image-Pro Plus (Media-Cybernetics, Bethesda, MD). We took pictures of H-E sections with Axio Vision (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) software, and opened these pictures with Image-Pro Plus. We measured the area of scaffolds and newly formed adipose tissue with the manual measurement function. Scaffolds were stained with H-E, but lipid droplets were not stained. We excluded fibroblastic capsules around the scaffolds. Morphologically, collagen scaffolds have a net-like structure, adipose tissue has a granulated structure, and fibroblastic capsules have a layered structure. The average area of the scaffolds was 2.14 $mm^2$ and did not differ significantly among the groups.

### *Statistical analysis*

The Mann–Whitney U-test (Microsoft Exel, Statcel2) was employed for statistical analysis, and $p < 0.05$ was considered to indicate statistical significance.

## Results

### *Proliferative activity of ASCs*

The proliferative activity of the ASCs *in vitro* was retained through the 10th passage as assessed by the viable cell count (Fig. 1) and MTT assay (Fig. 2). The proliferative activity of the ASCs was 1.6–4.0-fold higher in the presence of bFGF than in the absence of bFGF (Fig. 1). The number of ASCs increased by more than a 1000-fold at the fourth passage of ASCs cultured with bFGF. Statistically significance was seen in the presence of bFGF (Figs. 1 and 2). There was no correlation between the proliferative activity of the ASCs and age of donors.

### *Differentiation of ASCs to mature adipocytes*

Differentiation of ASCs to mature adipocytes as assessed by GPDH activity assay was observed at all passages (Fig. 3). The extent of differentiation of ASCs in the presence of bFGF was greater than that in the absence of bFGF from the first to third passages, and decreased from the fourth passage onward. From first to third passage, differentiation of ASCs in the presence of bFGF was significantly greater than any other groups. Open and closed bars at start show intrinsic GPDH. There was no correlation between the extent of differentiation and age of donors.

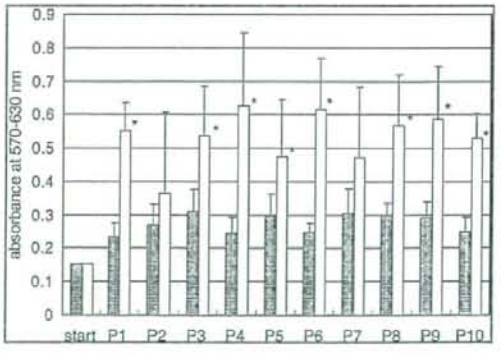



**FIG. 2.** Proliferation of ASCs *in vitro* as assessed by MTT assay. Proliferation of ASCs in 96-well microtiter plates cultured with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week was measured by MTT assay. *: $p < 0.05$ versus without bFGF group.

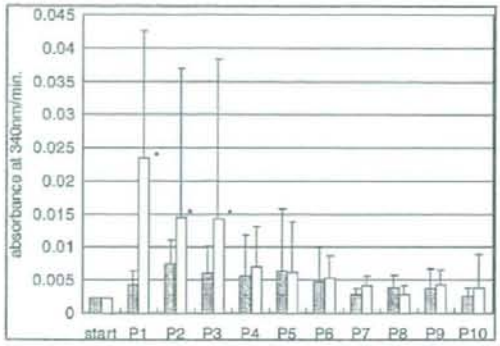



FIG. 3. Adipogenic differentiation of ASCs. GPDH activity of ASCs at each passage after culture with 100 ng/mL bFGF (open bars) or without bFGF (closed bars) for 1 week and incubation in DMEM/F-12 medium containing insulin, 3,5,3-triiodothyronine, transferrin, calcium pantotenate, biotin, and dexamethasone for 21 days was measured using a commercially available GPDH activity measurement kit and a spectrophotometer (absorbance of the solution mixture at 340 nm per minute). *: $p < 0.05$ versus without bFGF group and the group passages 4–10.

### *Adipogenesis of implanted human ASCs*

On the basis of the *in vitro* data described above, we implanted the designated number of third-passage ASCs cultured with bFGF into the back of 6-week-old female BALB/c nude mice. The ASCs were incorporated into the collagen scaffolds with or without controlled-release bFGF. In all the groups, adipogenesis was induced in the scaffold and maintained up to 24 weeks after implantation (Figs. 4-1 and 4-2). The greatest cross-sectional surface area of adipose tissue in the scaffold was 1.19 $mm^2$ 12 weeks after implantation, which was equivalent to 68.84% of the scaffold area. In contrast, the adipose tissue area was 0.58 $mm^2$ in the group receiving a scaffold alone (without ASCs) with controlled-release bFGF (B group) (Fig. 5-1). In the group receiving $2\times10^6$ ASCs incorporated in a scaffold without controlled-release bFGF (E group), the adipose tissue area was 0.40 $mm^2$. The area of adipose tissue was 0.08 $mm^2$ in the group receiving a scaffold alone (A group). The extent of adipogenesis in the group receiving $2\times10^6$ ASCs incorporated in a scaffold without controlled-release bFGF (E group) was similar to that in the group receiving a scaffold alone with controlled-release bFGF (B group). In the group receiving $8\times10^6$ ASCs in a scaffold without controlled-release bFGF (G group), adipogenesis in the scaffold decreased to the level of the group receiving a scaffold alone (A group). At 24 weeks, the total adipose tissue area increased in most of the groups (Fig. 5-2). The greatest cross-sectional surface area of adipose tissue in the scaffold was 2.14 $mm^2$ in the F group, which was almost twice as that at 12 weeks. In the group receiving $8\times10^6$ cells without controlled-release bFGF (G group), total adipogenesis was greater than *de novo* adipogenesis (A and B groups). Controlled-release bFGF thus had statistically an additive effect on adipogenesis in most of the groups at both 12 and 24 weeks.

### *Human ASC–derived adipogenesis*

Human vimentin–positive cells were found in all the groups receiving ASCs. Most of these cells were not mature adipocytes. However, at 12 weeks, in the group receiving $8\times10^6$ ASCs in a scaffold with controlled-release bFGF (H group), human ASC–derived mature adipocytes were observed (Fig. 6). The extent of human ASC–derived adipogenesis in the H group 12 weeks after implantation was equivalent to 14.6% of total adipogenesis in the scaffold. In contrast, the extent of human ASC–derived adipogenesis in other groups was less than 1% (Fig. 7-1). At 24 weeks, human ASC–derived adipose tissue area was increased in all the groups receiving ASCs (Fig. 7-2). The greatest cross-sectional surface area of human-derived adipose tissue in the scaffold was 0.35 $mm^2$ in the group receiving $8\times10^6$ ASCs in a scaffold without controlled-release bFGF (G group), and this was 43.8% of total adipose tissue area. In the H group, human-derived adipogenesis was 0.17 $mm^2$, which was equivalent to 44.4% of total adipogenesis in the scaffold.

## Discussion

ASCs are similar to MSCs[13] and proliferate considerably when cultured with bFGF.[39,40] We isolated $5\times10^5$ ASCs from 5 g of fresh adipose tissue obtained during breast cancer surgery. We considered $5\times10^5$ ASCs were scarce as a starting material for adipogenesis *in vivo*, and proliferated with bFGF *ex vivo*. Heimburg *et al.* showed that $8\times10^4$ to $3.5\times10^5$ preadipocytes can be harvested from 1 g of fresh adipose tissue, depending on the donor and the method used for cell retrieval.[41] ASCs are isolated from excised or aspirated adipose tissue. Lipoaspiration from the abdominal wall requires another incision site and is associated with further pain. Moreover, informed consent would have to be additionally received for this procedure. It is therefore not suitable for our study. We used excised adipose tissue obtained during breast surgery in this study. The adipose tissue is usually discarded after operation, and informed consent is readily obtained. Viable cells from adipose tissue decrease with preservation conditions and time periods after resection.[42] We usually preserve resected adipose tissue at 4°C and isolate ASCs as soon as possible after resection to minimize cell damage. We had probatively isolated ASCs 72 h after surgery and obtained about half the number of ASCs from 5 g of adipose tissue.

In this study, we did not exclude patients with axillary lymph node metastasis or advanced stage, and cancer cells might remain in breast or axillary adipose tissue. However, we did not see any cancer cells in ASCs cultured *in vitro* for 10 weeks and in the adipose tissues *in vivo* for 24 weeks after implantation. In the future, the procedure should be clinically performed in carefully selected patients to avoid cancer cell dissemination.

When cultured with 100 ng/mL of bFGF, ASCs proliferated more than 1000-fold at the fourth passage, whereas the ability of ASCs to differentiate into mature adipocytes decreased subsequently. Two reasons may account for this phenomenon. First, the "stem cell" population decreases and other populations of cells, such as fibroblasts, increase after the fourth passage. Stem cells are characterized by self-renewal capacity, long-term viability, and multilineage potential. The stem cell–associated marker CD34 was at peak levels in the stromal vascular fraction cells and early passage ASCs throughout the

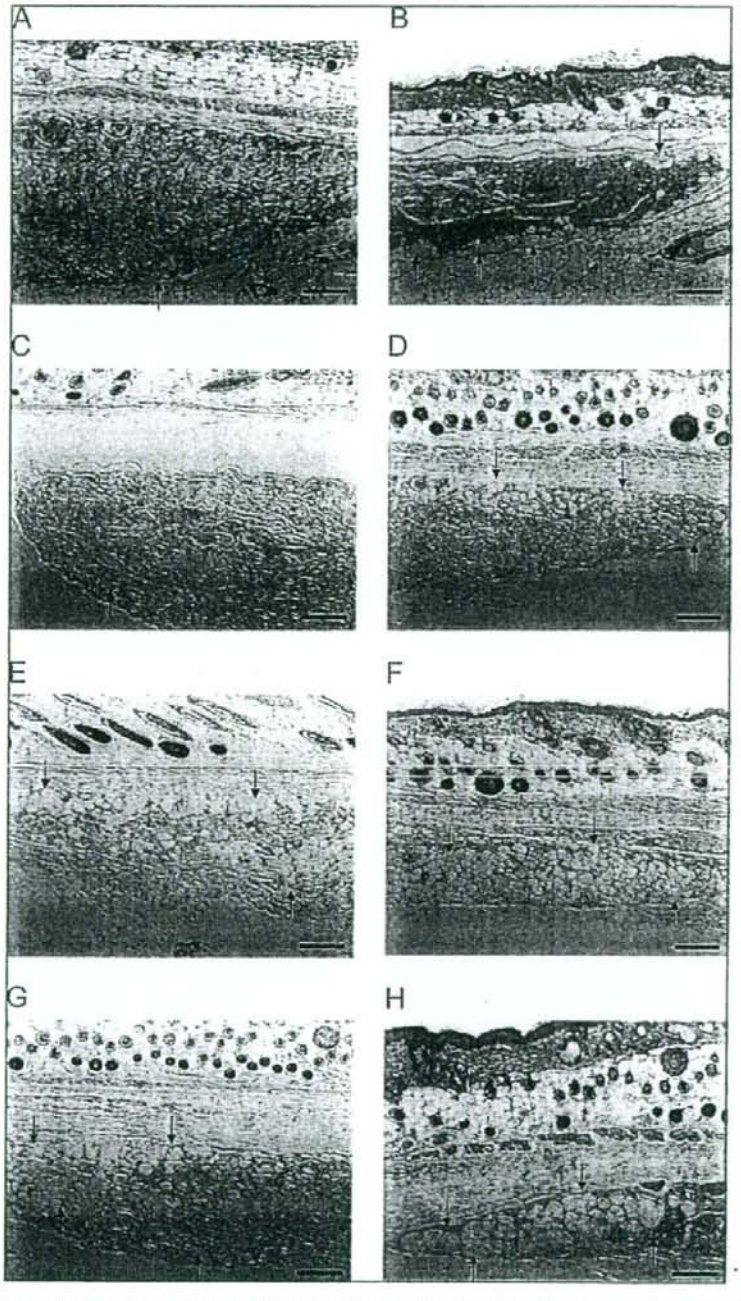



FIG. 4-1. Adipogenesis of implanted human ASCs 12 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF (0: A, B; $5\times10^5$: C, D; $2\times10^6$: E, F; $8\times10^6$: G, H) and incorporated into collagen scaffolds with (B, D, F, H) or without controlled-release bFGF (A, C, E, G) were implanted into the back of 6-week-old female BALB/c nude mice. Formation of adipose tissue in the implanted scaffold 12 weeks after implantation is indicated by the arrows (magnification×100). Scale bar = 100 μm. Color images available online at www.liebertonline.com/ten.

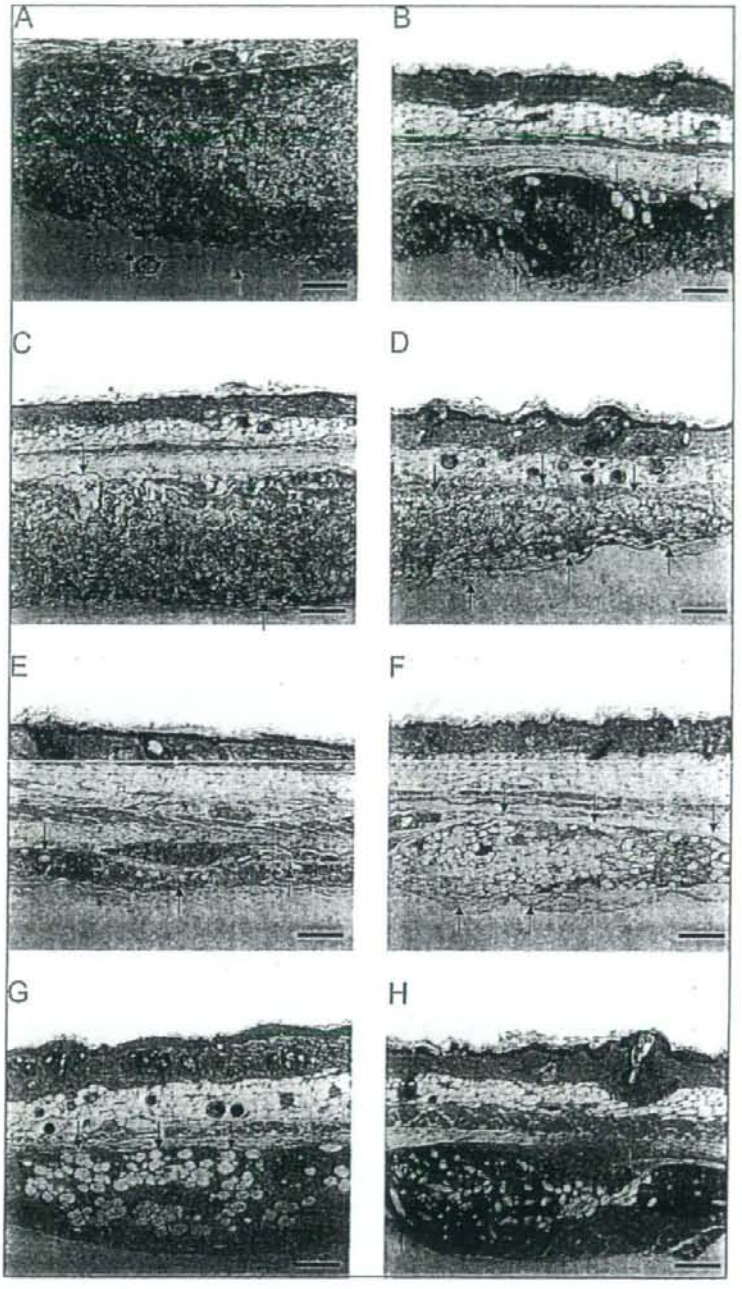



FIG. 4-2. Adipogenesis of implanted human ASCs 24 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF (0: A, B; $5\times10^5$: C, D; $2\times10^6$: E, F; $8\times10^6$: G, H) and incorporated into collagen scaffolds with (B, D, F, H) or without controlled-release bFGF (A, C, E, G) were implanted into the back of 6-week-old female BALB/c nude mice. Formation of adipose tissue in the implanted scaffold 24 weeks after implantation is indicated by the arrows (magnification×100). Scale bar = 100 μm. Color images available online at www.liebertonline.com/ten.

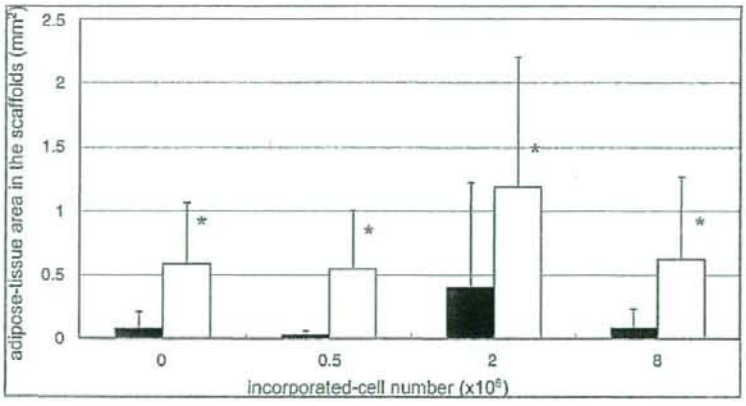



FIG. 5-1. Adipose tissue area in the scaffolds 12 weeks after implantation. The designated numbers of third-passage ASCs incorporated into scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The adipose tissue area in the scaffolds was measured and analyzed with the computer program Image-Pro Plus. The average area of the scaffolds was 2.14 $mm^2$ and did not differ significantly among the groups. *: $p < 0.05$ versus without controlled-release bFGF and group A.

culture period.[43,44] Second, because the ASCs were cultured in medium containing 10% FBS, bFGF expression was progressively lost, leading to impaired self-renewal ability.[45] To obtain ASCs that retain the ability to differentiate into mature adipocytes, ASCs should optimally be cultured with bFGF until the third passage. A previous study has reported that human adipose tissue–derived MSCs retain their capacity to differentiate into mature adipocytes (GPDH activity) for least 15 passages.[46] However, peak GPDH activity is at the fifth passage. These findings are consistent with our results.

*De novo* adipogenesis occurs without implanting ASCs,[19,31,32,47–49] because preadipocytes are recruited from surrounding adipose tissue and differentiate into mature adipocytes. Ideally, adipose tissue engineering techniques would simulate this phenomenon after breast surgery in the future. In women with breast cancer, however, less ASCs would survive at the implantation site of ASCs incorporated in a collagen scaffold with controlled-release bFGF, because the conserved breast usually receives radiation therapy after breast conserving surgery.[50] Because *de novo* adipogenesis is unlikely, we require exogenous progenitor cells. Implanted progenitor cells are expected to differentiate into mature adipocytes. Cell implantation therapy has some benefits, and ASC-assisted lipotransfer has been used for cosmetic breast augmentation.[51] Clinically, the implantation of ASCs including preadipocytes is prerequisite to a successful outcome of adipose tissue engineering.

One of our objectives was to confirm how many mouse-derived cells can be recruited and how many human-derived cells must be implanted. We immunohistochemically distinguished human-origin from mouse-origin adipose tissue with the use of antihuman vimentin antibody. Vimentin is not specific for preadipocytes or adipocytes, but it is expressed on these cells. Franke *et al.* concluded that lipid droplets are

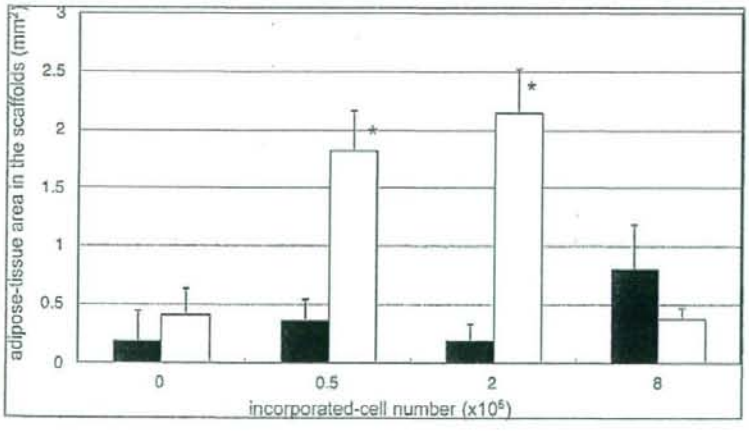
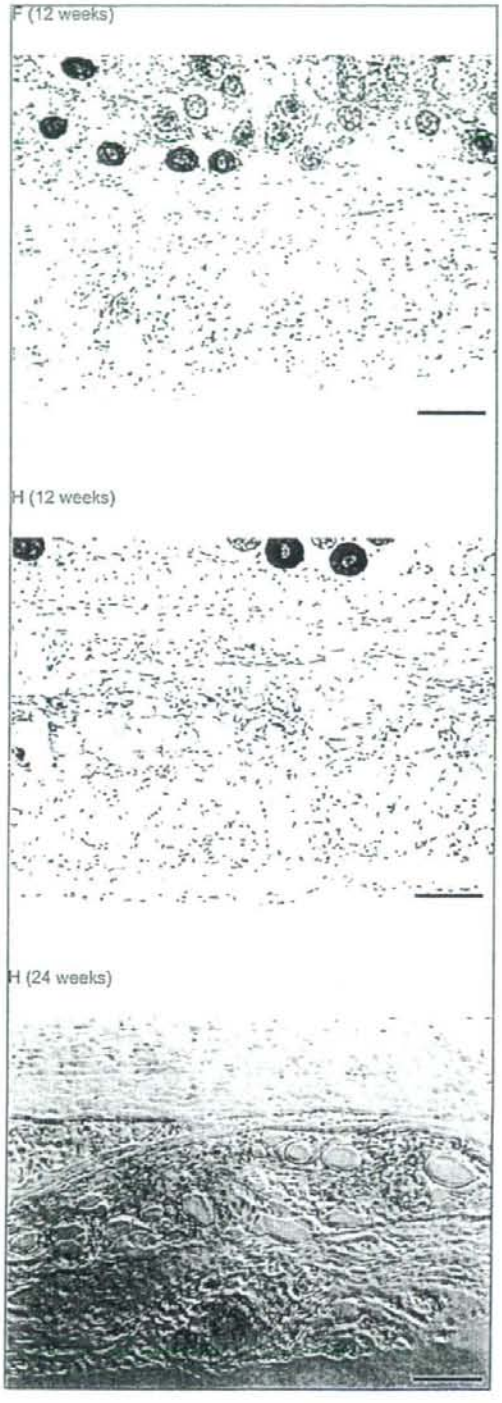



FIG. 5-2. Adipose tissue area in the scaffolds 24 weeks after implantation. The designated numbers of third-passage ASCs incorporated into scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The adipose tissue area in the scaffolds was measured and analyzed with the computer program Image-Pro Plus. *: $p < 0.05$ versus without controlled-release bFGF and group A.




encaged in a vimentin-containing structure.[52] The antihuman vimentin antibodies used to confirm newly formed adipose tissue were human derived in many *in vivo* studies.[20,22,53,54] We previously reported that human-derived adipocytes were distinguishable from *de novo* adipocytes with antihuman vimentin staining.[20] In our study, structures around lipid vacuoles were stained with antihuman vimentin antibody, indicating the presence of human-derived mature adipocytes. No human vimentin–positive cells were found in the groups receiving a scaffold alone, with or without controlled-release bFGF (A and B groups). At 12 weeks, in the group receiving $2\times10^6$ ASCs with controlled-release bFGF (F group), the human vimentin–positive area was accounted for less than 1% of newly formed adipose tissue. And in the group receiving $8\times10^6$ ASCs with controlled-release bFGF (H group), the human vimentin–positive area was equivalent to about 15% of newly formed adipose tissue. At 24 weeks, human-derived adipose tissue area increased in every group. In the F group, the human vimentin–posiive area was 2.8% of newly formed adipose tissue. In the H group, the human vimentin–positive area was equivalent to 44.4% of newly formed adipose tissue. Both human adipose tissue area and percentage of the scaffold were increased from 12 to 24 weeks in the H group. Human ASCs take longer time to differentiate into mature adipocytes than mouse ASCs. Implanted human ASCs differentiate into mature adipocytes in the host and continue to differentiate for a long time. At 24 weeks in the G group, human-derived adipogenesis was 0.35 $mm^2$, while *de novo* adipogenesis was 0.45 $mm^2$ and greater than the A group. From this result, implanted human ASCs not only differentiate into mature adipocytes but also promote *de novo* adipogenesis. Implanted human ASCs function for a long time as progenitor cells for *in vivo* adipogenesis and induce *de novo* adipogenesis.

An optimal cell seeding concentration for scaffold formation may exist. Our results suggested that $2\times10^6$ ASCs/site was the best concentration at both 12 and 24 weeks. At 12 weeks, in the group receiving $2\times10^6$ ASCs incorporated in a scaffold without controlled-release bFGF (E group), the area of newly formed adipose tissue was similar to that in the group receiving the scaffold alone with controlled-release bFGF (B group). Although bFGF has an obvious effect on adipogenesis, the number of implanted cells is also an important factor. Heimburg *et al.* seeded $10^6$ preadipocytes cultured in the medium supplemented with epidermal growth factor onto collagen sponges, which were then implanted into mice. They found that implantation of a large number of preadipocytes is important for the promotion of adipogenesis.[22] Torio-Padron *et al.* injected human ASCs in fibrin into nude mice and also concluded that an increased cell concentration enhances the formation of adipose tissue.[55] However,

FIG. 6. Human ASC–derived adipogenesis. Immunohistochemical sections of newly formed adipose tissue 12 and 24 weeks after implantation. Human-origin adipose tissue is stained by antihuman vimentin antibody. Group receiving $2\times10^6$ ASCs with controlled-release bFGF at 12 weeks (F), group receiving $8\times10^6$ ASCs with controlled-release bFGF at 12 weeks (H), and group receiving $8\times10^6$ ASCs with controlled-release bFGF (H) at 24 weeks (magnification× 200). Scale bar = 100 μm. Color images available online at www.liebertonline.com/ten.

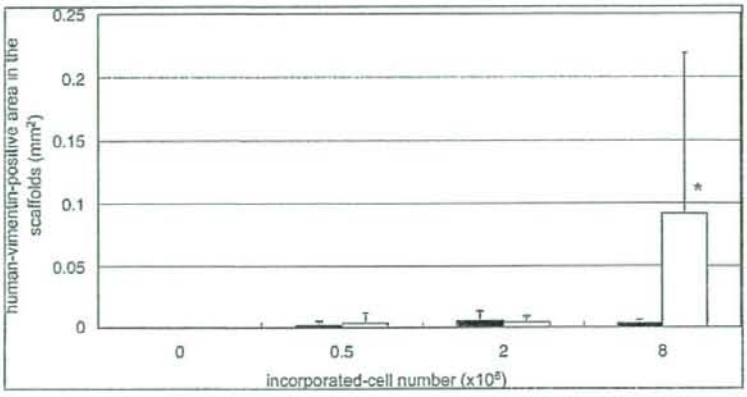



FIG. 7-1. Human vimentin–positive area in the scaffolds 12 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF incorporated into the scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The human vimentin–positive area in the scaffolds was measured and analyzed with Image-Pro Plus. *: $p < 0.05$ versus any other group.

from our data, there might be the best cell concentration for total adipogenesis. Both 12 and 24 weeks after implantation, maximum adipose tissue area was observed in the group receiving $2\times10^6$ ASCs with controlled-release bFGF (F group), not in the group receiving $8\times10^6$ ASCs (G and H groups). Implanted ASCs not only differentiate into mature adipocytes, but also secrete ECM to promote maturation. The amount of ECM or cytokines secreted by ASCs may increase with an increased number of implanted ASCs and produce an appropriate micromilieu for proliferation and differentiation of ASCs themselves. Stillaert *et al.* reported that ASCs secrete additional ECM components and that these ECM components were able to act as inductive factors to further enhance adipogenesis *in vivo*.[5,6] In our study, $2\times10^6$ ASCs with controlled-release bFGF (F group) might be the optimum condition in terms of secreted ECM, cytokines, and succeeding cell survival. Implanting $8\times10^6$ ASCs (G and H groups) was not good condition for total adipogenesis. The number of ASCs that can survive in the scaffold might be limited. Since there are many inflammatory cells in the specimens of the G and H groups, some of $8\times10^6$ ASCs are supposed to be dead. The dead cells might cause inflammation and inhibit differentiation into mature adipocytes. Only in the H group at 24 weeks, bFGF did not have additive effects for both total and human-derived adipogenesis. Total adipogenesis decreased, but human-derived adipogenesis increased in the H group. Too many ASCs might inhibit *de novo* adipogenesis.

In summary, our results indicate that the implantation of optimum number of ASCs with controlled-release bFGF is the key for a successful outcome of functional adipose tissue engineering for long term. Newly formed adipose tissue induced by human ASCs fully matured and functioned for a long time. There are few papers that report *in vivo* human-derived adipogenesis for such a long period as our paper. This study is baseline for future clinical practice. Further studies are needed to discover the most efficient ways of generating adipose tissue for clinical practice.

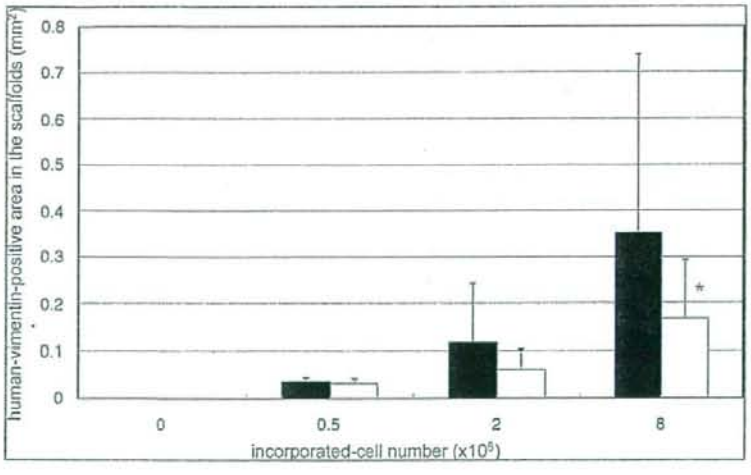



FIG. 7-2. Human vimentin–positive area in the scaffolds 24 weeks after implantation. The designated number of third-passage ASCs cultured with bFGF incorporated into the scaffolds with (open bars) or without controlled-release bFGF (closed bars) were implanted into the back of 6-week-old female BALB/c nude mice. The human vimentin–positive area in the scaffolds was measured and analyzed with Image-Pro Plus. *: $p < 0.05$ versus groups C and D.

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Address reprint requests to:
*Wakako Tsuji, M.D.*
*Department of Breast Surgery*
*Kyoto University, Graduate School of Medicine*
*54 Kawara-cho Shogoin, Sakyo-ku*
*Kyoto 606-8507*
*Japan*

*E-mail:* w-sato@kuhp.kyoto-u.ac.jp

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