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# Cryopreserved human adipogenic-differentiated pre-adipocytes: a potential new source for adipose tissue regeneration

**MH Kim[1], I Kim[1], S-H Kim[1], MK Jung[1], S Han[1], JE Lee[1], J-S Nam[1], S-K Lee[1] and SI Bang[2]**

*[1]Anterogen Co. Ltd and [2]Department of Plastic Surgery, Samsung Medical Center, Sungkyunkwan University, School of Medicine, Seoul, Korea*

**Background**

*Previously, we have shown that* in vitro *adipogenic differentiation of pre-adipocytes before implantation can enhance* in vivo *adipose tissue formation. For large-scale adipose tissue engineering or repeat procedures, cryopreservation of fat grafts has been commonly used in recent years. However, the feasibility of cryopreservation of adipogenic differentiated pre-adipocytes has not been investigated.*

**Methods**

*To examine the impact of cryopreservation on the adipogenic functions of adipogenic-differentiated pre-adipocytes, freeze–thawed adipocytes were compared with fresh differentiated adipocytes* in vitro *and* in vivo. *Adipogenic function was assessed by Oil red O staining, ELISA analysis of leptin secretion and RT-PCR of adipogenic-related genes. After transplantation, adipose tissue formation was assessed by histomorphologic and volumetric analysis.*

**Results**

*Freeze–thawed adipocytes constantly showed typical adipogenic functions in terms of lipid content, leptin secretion and adipogenic gene expression, as well as good viability. Importantly, implants derived from freeze–thawed adipocytes were successfully developed to adipose tissue and newly formed adipose tissues were similar to those developed from fresh differentiated adipocytes, based on histomorphologic and volumetric analysis. In addition, CD34-positive endothelial cells were detected in implants. These results demonstrate that the specific characters of adipogenic-differentiated pre-adipocytes are successfully conserved after cryopreservation without any significant alteration.*

**Discussion**

*Cryopreservation of adipogenic-differentiated pre-adipocytes is a feasible method and extends their clinical use in adipose tissue-engineering applications and transplantation.*

**Keywords**

*adipogenic-differentiated pre-adipocyte, adipose tissue engineering, adipose tissue formation, cryopreservation, human adipose tissue-derived stem cell.*

## *Introduction*

Adipose tissue is a source of adult multipotent stem cells that can differentiate not only along the mesenchymal lineage to produce adipocytes, chondrocytes, myocytes and osteoblasts [1,2] but also along the neuronal lineage [3]. There are many advantages to cell-based therapies using adipose tissue-derived adult stem (ADAS) cells, as adipose tissue can be obtained in liter quantities by liposuction surgery, which requires only a minimally invasive procedure. Thus this abundant and accessible cell population has potential clinical utility for tissue engineering and tissue regeneration [4,5]. In particular, ADAS cells, previously known as pre-adipocytes, are considered useful for adipose tissue engineering because they can be readily made to differentiate into adipocytes *in vitro* given an appropriate adipogenic differentiation-inducing medium. However, the enhancement of the adipogenic conversion of pre-adipocytes *in vivo* after transplantation remains a major task in adipose tissue engineering. It has been reported that the adipogenic differentiation of

*Correspondence to:* **Sa Ik Bang,** MD, Department of Plastic Surgery, Samsung Medical Center, SungKyunkwan University School of Medicine, Seoul, Korea. E-mail: si55.bang@samsung.com



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pre-adipocytes after subcutaneous implantation hardly occurs and that it requires the co-administration of growth factors such as basic fibroblast growth factor to induce adipogenic differentiation [6–8].

In a previous study, we have shown that the implantation of adipogenic-differentiated pre-adipocytes, which are immature adipocytes containing typical lipid droplets in their cytoplasm, enhanced *in vivo* adipose tissue formation [6]. This means that adipogenic-differentiated pre-adipocytes can be utilized as an improved cell source for adipose tissue engineering.

Most forms of adipose tissue engineering involving augmentation of soft tissue defects require large-scale volumes [9]. Therefore, repeated injections associated with repeated liposuction surgery and cell harvesting procedures might be necessary. Moreover, autologous fat transplantation requires occasional retouching because of unpredictable and irregular absorption of transplanted fat grafts in the recipient area. Recently, to extend the clinical applications of autologous fat transplantation, cryopreservation of the harvested adipose tissue has been advocated and become a commonly used technique in the field of plastic and reconstructive surgery [10,11].

In this context, if adipogenic-differentiated pre-adipocytes can be stored safely, it becomes possible to avoid repeat liposuction and to produce adipogenic-differentiated pre-adipocytes on a large scale. Consequently, cells can be thawed and injected as needed. Despite recent progress in adipogenic differentiation using ADAS cells, the possibility of cryopreservation of adipogenic-differentiated pre-adipocytes has not been explored. In this study, we investigated, using Oil red O staining, leptin secretion assays and RT-PCR for adipogenic gene expression, whether the adipogenic functions of adipogenic-differentiated pre-adipocytes are maintained after cryopreservation. In addition, adipose tissue regeneration and neovascularization were examined after cell implantation into athymic nude mice.

## *Methods*

### Isolation, differentiation and cryopreservation of human pre-adipocytes

Subcutaneous adipose tissues were obtained from the abdomens of patients undergoing elective liposuction after gaining the patients' informed consent; tissues were washed at least three times with Krebs' ringers buffer (KRB; Sigma Chemical Co., St Louis, MO, USA) to remove contaminating blood. Liposuction tissue samples were digested for 80 min at 37°C with intermittent shaking in KRB containing 1% BSA (Sigma) and 0.025% collagenase (Type I) (Gibco BRL, Gaithersburg, MD, USA). Floating adipocytes were then separated from the stromal-vascular fraction by centrifugation (300 *g*) for 5 min. The stromal-vascular fraction was cultured in DMEM-Ham's F-12 medium (v/v, 1:1) supplemented with 10% (v/v) FBS (Hyclone, Logan, UT, USA) and maintained for 24–72 h. After this period, the flask was washed with PBS to remove any non-adherent cells and cells were fed with expansion medium composed of DMEM-Ham's F-12 medium supplemented with 10% FBS, human-transforming growth factor-1(TGF-β1; 0.25 ng/mL), human epidermal growth factor (EGF; 5 ng/mL) and human basic fibroblastic growth factor (bFGF; 0.25 ng/mL). Adherent cells were expanded for 7–11 days until they achieved confluence. Confluent pre-adipocytes were then induced to undergo adipogenesis using the following differentiation medium: DMEM-Ham's F-12 medium supplemented with 3% FBS, biotin (33 μmol/L), pantothenate (17 μmol/L), human recombinant insulin (1 μmol/L), dexamethasone (1 μmol/L), isobutylmethylxanthine (IBMX; 0.1875 mmol/L) and indomethacin (0.2 mmol/L). After a 3-day induction period, the cells were fed with the same medium without IBMX and indomethacin every 2–3 days for the remaining period in culture. Differentiated pre-adipocytes were identified by light microscopic observations of morphologic changes from a fibroblast-like structure to a round cell structure containing lipid vacuoles. Harvested adipocytes that were differentiated from pre-adipocytes according to the procedure mentioned above were resuspended in cryopreservation medium (90% FBS, 10% DMSO) at $2.5 \times 10^7$ cells/mL and immediately placed into a freezing container (Nalgene 5199–0001, VWR, Belgium), with the temperature at −80°C, overnight, and then transferred to a liquid nitrogen tank (−196°C). After 6 days in liquid nitrogen, the cells were thawed quickly in a 37°C water bath, washed three times in DMEM, counted to determine viability and yield, and used as freeze–thawed adipocytes in this study. Harvested adipocytes that did not undergo the freeze–thaw process were used as fresh adipocytes.

### Oil red O staining and the quantification of lipid accumulation

To observe cell morphologies and Oil red O staining, monolayer cultures of cells (fresh adipocytes or