書籍

出版	書籍名 (出版社)	タイトル	ページ	出版地	著者氏名 (編集者名)
2010年	Regenerative medicine and biomaterials for the repair of connective tissues (WOODHEAD PUBLISHING, CRC Press LLC)	Cell sheet technologies for cartilage repair	251-265	UK	Sato M (Archer C, Ralphs J)
2010年	Bioengineering: Principles, Methodlogies and Applications (Nova Science Publishers,Inc)	Chapter 7 : Development of a Diagnostic System for Osteoarthrit is Using a Photoacoustic Measurement Method and Tome-resolved Autofluorescence	179-190	USA	Sato M, Ishihara M, Mitani G, Kutsuna T, Lee J Ik, Kikuchi M, Mochida J (Audric G, Ciel D)

雑誌

出版	発表誌名	タイトル	ページ	巻号	著者氏名
2010年	European Cells and Materials	Primary immune system responders to *nucleus pulposus* cells : evidence for immune response in disc herniation	13-21	19	Murai K, Sakai D, Nakamura Y, Nakai T, Igarashi T, Seo N, Murakami T, Kobayashi E, Mochida J
2010年	SPIE Proceedings	Development of the hyperspectral cellular imaging system to apply to regenerative medicine			Ishihara M, Sato M, Matsumura K, Toguchida J, Mochida J, Kikuchi M

2009年	BMC Biotechnology	The properties of bioengineered chondrocyte sheets for cartilage regeneration		2009, 9:17	Mitani G, Sato M, Lee J Ik, Kaneshiro N, Ishihara M, Kokubo M, Ota N, Sakai H, Kikuchi T, Mochida J
2009年	Tissue Eng Part C Methods	Noninvasive Evaluation of Tissue Engineered Cartilage with Time-Resolved Laser-Induced Fluorescence Spectroscopy			Kutsuna T,Sato M, Ishihara M, Furukawa K, Nagai T, Kikuchi M, Ushida T, Mochida J
2009年	整形・災害外科	【解説・総説】 軟骨再生医療に有効な光技術	1533-1537	52(12)	石原美弥, 菊地眞, 佐藤正人, 沓名寿治, 三谷玄弥, 持田譲治

学会発表

発表	発表学会名	タイトル	著者氏名
研究代表者　佐藤正人			
2010年3月	56th Annual Meeting of the Orthopaedic Research Society	Evaluation of characteristics of chondrocyte sheet constructed of cultured chondrocytes using co-culture method with synovial cells	Kokubo M, Sato M, Mitani G, KutsunaT, Ohta N, Ebihara G, Sakai H, Mochida J
2010年3月	56th Annual Meeting of the Orthopaedic Research Society	Jellyfish mucin may have potential disease modifying effects of osteoarthritis of the knee.	Ohta N, Sato M, Ushida K, Kokubo M, Baba T, Taniguchi K, Urai M, Kihira K, Mochida J
2010年3月	56rd Annual Meeting of the Orthopaedic Research Society	Noninvasive Evaluation of Tissue Engineered Cartilage with Time-Resolved Laser-Induced Fluorescence Spectroscopy	Kutsuna T, Sato M, Ishihara M, Furukawa K, Nagai T, Ushida T, Mochida J

2010年3月	第9回再生医療学会	同種異関節由来細胞間共培養法を用いた軟骨細胞シートの作製とその特性	小久保舞美, 佐藤正人, 三谷玄弥, 内山善康, 繁田明義, 沓名寿治, 太田直司, 海老原吾郎, 持田譲治
2009年11月	第30回日本レーザー医学会総会	ナノ秒パルスレーザーによるScaffold Free組織工学的軟骨の非侵襲的性状評価	沓名寿治, 佐藤正人, 石原美弥, 古川克子, 長井敏洋, 牛田多加志, 菊地眞, 持田譲治
2009年11月	第30回日本レーザー医学会総会	光音響原理に基づく軟骨変性診断法の開発:原理実証から臨床研究まで	石原美弥, 佐藤正人, 番作勲, 三谷玄弥, 沓名寿治, 持田譲治, 菊地眞
2009年11月	第30回日本レーザー医学会総会	高分子圧電フィルムを用いた光音響プローブの改良	番作勲, 石原美弥, 大森努, 佐藤正人, 持田譲治, 菊地眞
2009年11月	第24回日本整形外科学会基礎学術集会	クラゲムチンの関節軟骨に対する影響の検討	太田直司, 佐藤正人, 小久保舞美, 馬場崇行, 谷口佳代子, 浦井誠, 丑田公規, 持田譲治
2009年11月	第24回日本整形外科学会基礎学術集会	抗VEGFヒトモノクローナル抗体(Bevacizumab)による軟骨修復効果	長井敏洋, 佐藤正人, 沓名寿治, 太田直司, 海老原吾郎, 小久保舞美, 持田譲治
2009年11月	第24回日本整形外科学会基礎学術集会	光技術を用いた軟骨変性・再生の評価法の開発	石原美弥, 佐藤正人, 三谷玄弥, 沓名寿治, 持田譲治, 菊地眞
2009年11月	第24回日本整形外科学会基礎学術集会	滑膜細胞と軟骨細胞からなる細胞移植体の作製と評価	李禎翼, 佐藤正人, 三谷弦弥, 持田譲治
2009年11月	第24回日本整形外科学会基礎学術集会	同種異関節における細胞相互作用の検討-共培養法を用いた軟骨細胞シートの特性	小久保舞美, 佐藤正人, 三谷玄弥, 内山善康, 繁田明義, 沓名寿治, 太田直司, 海老原吾郎, 持田譲治
2009年11月	第24回日本整形外科学会基礎学術集会	時間分解自家蛍光スペクトル分析によるscaffold free組織工学的軟骨の非侵襲的性状評価	沓名寿治, 佐藤正人, 石原美弥, 古川克子, 長井敏洋, 牛田多加志, 菊地眞, 持田譲治
2009年11月	第24回日本整形外科学会基礎学術集会	【シンポジウム】 関節軟骨の修復・再生における組織工学的軟骨（軟骨細胞シート/プレート）の役割	佐藤正人, 三谷玄弥, 沓名寿治, 長井敏洋, 海老原吾郎, 太田直司, 小久保舞美, 石原美弥, 古川克子, 牛田多加志, 持田譲治
2009年10月	第36回日本臨床バイオメカニクス学会	【シンポジウム】 光を用いた関節軟骨の機能評価法	佐藤正人, 石原美弥, 三谷弦弥, 沓名寿治, 菊地眞

2009年9月	第58回東日本整形災害外科学会	【ASIA NOW】 関節軟骨修復再生をめざした軟骨滑膜混合細胞体の開発	李禎翼, 佐藤正人, 三谷弦弥, 持田譲治
2009年9月	第7回並木整形外科セミナー	【教育研修講演】 関節軟骨再生 up to date	佐藤正人
2009年9月	World Congress on Medical Physics and Biomedical Engineering	Multifunctional characterization of engineered cartilage using nano-pulsed laser	Ishihara M, Bansaku I, Sato M, Mochida J, Kikuchi M
2009年9月	IFMBE Proceedings	Multifunctional characterization of engineered cartilage using nano-pulsed laser	IshiharaM, Bansaku I, Sato M, Mochida J, Kikuchi M
2009年5月	第82回日本整形外科学会学術総会	【シンポジウム】 光による関節軟骨の力学特性と性状評価	佐藤正人, 石原美弥, 三谷弦弥, 沓名寿治, 菊地眞
2009年5月	第84回日本医療機器学会大会	変形性関節症診断用の光音響プローブの改良	番作勲, 石原美弥, 菊地眞, 佐藤正人, 持田譲治
2009年4月	第53回日本リウマチ学会	クラゲ由来ムチンの関節軟骨に対する影響の検討	太田直司, 佐藤正人, 小久保舞美, 丑田公規, 持田譲治
2009年4月	第53回日本リウマチ学会	滑膜細胞との供培養法を用いた短期間での軟骨細胞シートの作製とその特性評価	小久保舞美, 佐藤正人, 太田直司, 坂井秀明, 持田譲治
2009年4月	第48回日本生体医工学会大会	レーザーによる軟骨の多角的評価システムの開発	石原美弥, 佐藤正人, 持田譲治, 菊地眞
研究分担者　三谷玄弥			
2009年	Annual Meeting of JOSKAS 2009	Evaluation of muscle strength after ACL reconstruction with hamstring tendons focusd on gender and graft selection	Mitani G, Takagaki T, Nakamura Y, Oshima M, Uehara J,Hamahashi K, Mochida J
研究分担者　加藤俊一			
2009年11月	第51回日本小児血液学会総会	多様化する造血細胞移植	加藤俊一
2009年9月	The 3rd International Symposium of Lysosomal Storage Diseases	Hematopoietic Stem Cell Transplantation for Inborn Errors in Metabolism (IEM) - A single institute experiences -	Takakura H, Shimizu T, Moromoto T, Koike T, Yanagimachi N, Yabe M, Yabe H, Tanaka A, Kato S
研究分担者　阿久津英憲			
2009年11月	第36回日本低温医学会総会・学術集会シンポジウム	Human Embryonic stem cells and iPS Cells: Potential tool for Low temperature medical experiments	阿久津英憲

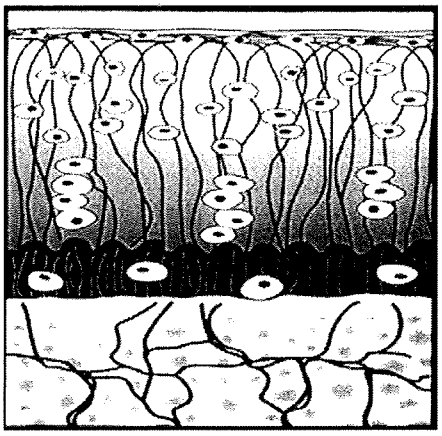
2009年	日本人類遺伝学会第54回大会　ワークショップ	難治性疾患克服に向けたヒトiPS細胞の可能性	阿久津英憲
2009年	第8回日本再生医療学会総会シンポジウム	ヒトiPS細胞遺伝子発現動態の多様性	阿久津英憲
研究分担者　長嶋比呂志			
2010年3月	日本畜産学会第112回大会	糖尿病発症遺伝子改変ブタの長期飼育に関する研究	日高龍路，梅山一大，望月寛徳，関口渓人，松成ひとみ，中野和明，藤原主，渡邊將人，長嶋比呂志
2010年3月	日本畜産学会第112回大会	自動冷却装置を用いた遺伝子改変ブタ精子の凍結保存	藤原主，松成ひとみ，梅山一大，渡邊將人，中野和明，竹内靖浩，中野貞雄，長嶋比呂志
2010年3月	日本畜産学会第112回大会	Kusabira-Orange遺伝子導入トランスジェニッククローン(Tg-C)ブタの繁殖能力および遺伝子伝達	松成ひとみ，竹内靖浩，保谷美恵，関口渓人，望月寛徳，日高龍路，渡邊將人，梅山一大，高柳就子，中野和明，藤原主，長嶋比呂志
2010年3月	日本畜産学会第112回大会	ブタにおける連続核移植の可能性：第6世代クローンの作出	松成ひとみ，中野和明，藤原主，池澤有加，小川武甲，高柳就子，渡邊將人，梅山一大，長嶋比呂志
2010年3月	第9回日本再生医療学会	異種胎仔組織を用いた再生腎臓誘導法の開発	横尾隆，松成ひとみ，岩井聡美，松本啓，長嶋比呂志，小林英司
2010年3月	第9回日本再生医療学会	急性腎不全に陥った腎臓への胎児由来組織の移植効果—バイオイメージング・ラットを用いた検討	岩井聡美，横尾隆，杉本直美，松成ひとみ，長嶋比呂志，小林英司
2010年3月	第9回日本再生医療学会	胎仔腎臓原基の器官培養を利用した幹細胞スクリーニング—手技の仔細と蛍光画像パターンによる分類—	杉本直美，横尾隆，増田茂夫，花園豊，竹内賢吾，松成ひとみ，長嶋比呂志、土居雅子，小林英司
2010年3月	第9回日本再生医療学会	膵臓特異的に緑色蛍光タンパク(Venus)を発現するトランスジェニック(Tg)ブタの作出.	松成ひとみ，小林敏寛，渡邊將人，中野和明，藤原主，長屋昌樹，中内啓光，長嶋比呂志.
2010年3月	第13回日本異種移植研究会	ブタCMP-N-acetylneuraminic acid hydroxylase geneの解析	池田孔佑，山本亜紀，近藤昭宏，松成ひとみ，長嶋比呂志，高間勇一，上野豪久，福澤正洋，宮川周士

2010年3月	第13回日本異種移植研究会	α1,3ガラクトース転移酵素遺伝子ダブルノックアウトブタの体細胞クローニングにおけるScriptaidの効果	松成ひとみ，池澤有加，渡邊将人，梅山一大，中野和明，藤原　主，竹内靖浩，本田香澄，前原美樹，高柳就子，山田和彦，宮川周士，長嶋比呂志
2009年11月	第54回日本生殖医学会	妊孕性保存およびQOL向上を目的とした卵巣ガラス化保存・移植技術の有効性	香川則子，桑山正成，池田有希，落合恵子，長嶋比呂志，加藤修
2009年10月	65th Annual Meeting of the American Society for Reproductive Medicine	Function of vitrified human ovarian grafts after xeno-transplantation	Kagawa N, Kuwayama M, Ikeda Y, Silber S, Nagashima H, Kato O
2009年10月	The Transplantation Society, IPITA-IXA 2009	Xeno-metanephros as a biocompetent scaffold for kidney regeneration	Yokoo T, Nagashima H, Matsunari H, Iwai S, Hosoya T, Kobayashi E
2009年10月	The Transplantation Society, IPITA-IXA 2009	Lectin microarray analyses of endothelial cells and fibroblasts from the α1,3 galactosyltransferase knockout pig	Miyagawa S, Yamamoto A, Ikeda K, Matsunari H, Nagashima H, Takeishi S, Yamada M, Fukuzawa M
2009年9月	第45回日本移植学会総会	レクチンブロット法によるαGal-knockoutブタの糖鎖抗原の解析	山本亜紀，徐 恒傑，武石俊作，山田正雄，三善英知，池田孔佑，松成ひとみ，長嶋比呂志，福澤正洋，宮川周士
2009年9月	第45回日本移植学会総会	ブタCMP-N-acetylneuraminic acid hydroxylase geneの解析	池田孔佑，山本亜紀，松成ひとみ，中野和明，藤原主，長嶋比呂志，福澤正洋，宮川周士
2009年9月	第102回日本繁殖生物学会大会	抗酸化機能強化培地がブタ体外生産胚の凍結生存性に及ぼす影響	中野和明，中山順樹，小川武甲，松成ひとみ，藤原主，斉藤紗恵子，池澤有加，吉岡耕治，星 宏良，長嶋比呂志
2009年9月	第102回日本繁殖生物学会	若齢時凍結保存卵巣の自家移植による老齢不妊マウスの繁殖能力の回復および寿命の延長	香川則子，桑山正成，池田有希，落合恵子，長嶋比呂志，加藤修
2009年9月	第102回日本繁殖生物学会大会	糖尿病モデルトランスジェニッククローンブタの作出 III. 変異型ヒトHNF-1α遺伝子を導入したDominant-negative変異体の病態の詳細解析	梅山一大，渡邊將人，松成ひとみ，黒目麻由子，小川武甲，中野和明，藤原主，三木敬三郎，長嶋比呂志

2009年9月	第102回日本繁殖生物学会	肝臓特異的赤色蛍光(Kusabira-Orange)発現を示す遺伝子改変ブタの作出	松成ひとみ，渡邊將人，梅山一大，中野和明，藤原主，小川武甲，池田有希，春山エリカ，塩田明，長嶋比呂志
2009年8月	第36回豚の繁殖衛生セミナー	糖尿病モデル遺伝子改変ブタの生産と病態の特徴について	梅山一大，渡邊將人，松成ひとみ，黒目麻由子，小川武甲，中野和明，藤原主，長嶋比呂志
2009年8月	第27回日本受精着床学会	若齢時に凍結保存した卵巣の自家移植による老齢不妊マウスからの正常産子の作出	香川則子，桑山正成，池田有希，落合恵子，長嶋比呂志，加藤修
2009年8月	第27回日本受精着床学会	若齢時に凍結保存した卵巣の老齢マウスへの自家移植による繁殖能力の回復	池田有希，香川則子，落合恵子，桑山正成，加藤修，長嶋比呂志.
2009年6月	25th Annual Meeting of the Europian Society of Human Reproduction and Embryology	Recovery of reproductive function and extension of life expectancy in old infertile mice by ovarian transplantation	Kagawa N, Kuwayama M, Ikeda Y, Nagashima H, Leibo S, Kato O
2009年6月	International Symposium Xenotransplantation	Recent advances in production of genetically modified pigs for xenotransplantation	Nagashima H
2009年5月	第50回日本哺乳動物卵子学会	Cryotop法を用いたマウス4細胞期胚および胚盤胞の再凍結保存後の生存性：凍結・再凍結保存時期の検討	池田有希，松成ひとみ，落合恵子，香川則子，桑山正成，加藤修，長嶋比呂志
研究協力者　村井邦彦			
2009年9月	第24回日本整形外科学会基礎学術集会	椎間板ヘルニアにおける免疫機能の関与	村井邦彦，酒井大輔，中井知子，中村嘉彦，持田譲治
2009年4月	第38回日本脊椎脊髄病学会	椎間板ヘルニアの新治療に向けた基礎的研究 - 椎間板髄核細胞の特異的免疫特性について	村井邦彦，　酒井大輔，中井知子，中村嘉彦，持田譲治
2009年2月	第23回日本ペインクリニック学会東京地方会	椎間板ヘルニアの新治療に向けた基礎的研究—椎間板髄核細胞の特異的免疫特性について	村井邦彦，鈴木英雄，五十嵐孝，瀬尾憲正，酒井大輔，中井知子，中村嘉彦，持田譲治

Ⅴ．研究成果の刊行物・別刷

WOODHEAD PUBLISHING IN MATERIALS



Regenerative medicine and biomaterials for the repair of connective tissues

Edited by Charles Archer and Jim Ralphs

CRC

WP

10

Cell sheet technologies for cartilage repair

M. SATO, Tokai University School of Medicine, Japan

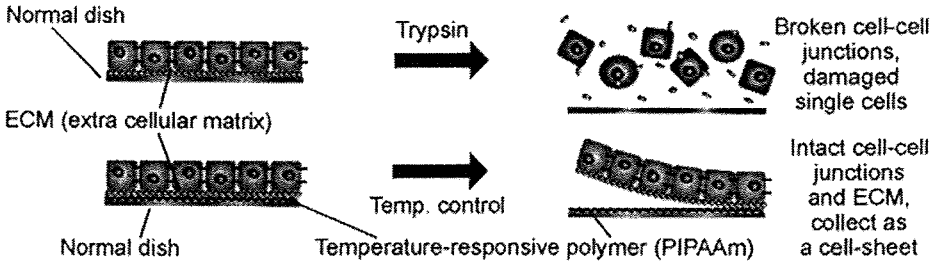
Abstract: This chapter outlines the principles of cell sheet technology, its clinical applications, how to repair cartilaginous defects using layered chondrocyte sheets, the properties of layered chondrocyte sheets, future trends in cartilage repair, and regulation of this area in Japan.

Key words: cell sheet, temperature-responsive culture dish, articular cartilage, cultured chondrocyte, tissue engineering.

10.1 Introduction

This section outlines cell sheet technology, specifically introducing the temperature-responsive culture dish. Cell sheets can be obtained without enzymes. It is also possible to obtain layered constructs. Prof. Okano,[1,2] from Tokyo Women's Medical University, developed this technology involving the building of three-dimensional tissue constructs, consisting of individual units of the cell sheets with tight junctions between the cells and extracellular matrices. A temperature-responsive culture dish is used to obtain the cell sheet. An *N*-isopropylacrylamide monomer solution is spread on commercial tissue culture polystyrene dishes. These dishes are then subjected to electron beam irradiation, thus resulting in polymerization and covalent binding of the isopropylacrylamide to the surface of the dish.

Poly *N*-isopropylacrylamide (PIPAAm) is a unique polymer that exhibits thermally reversible soluble-insoluble changes in an aqueous solution in response to temperature changes across an LCST (lower critical solution temperature) of 32 °C. Polymerized chains of acrylamide hydrate to expand in water below the LCST, while the isopropyl group dehydrates to form compact, insoluble conformations above the LCST. These dishes, therefore, reverse their hydrophobic and hydrophilic properties in response to changing temperature. When the temperature-responsive polymer (PIPAAm) is fixed to a cell-culturing dish using Bionano interface technology, the surface of the plate changes in response to temperature change across an LCST of 32 °C. The surface becomes hydrophobic above 32 °C, which enables cells to attach to the surface and grow. However, when the temperature is reduced to 20 °C, the polymer surface becomes hydrophilic and the hydrated polymer chains allow the cultured cells to



10.1 Cell detachment mechanism of temperature-responsive surface of the dish. Cultured cells by using a temperature-responsive surface could be released from the dish surface only by reducing the temperature without proteolytic enzyme. *N*-isopropylacrylamide monomer solution was spread onto commercial tissue culture polystyrene dishes. The surface of the temperature-responsive culture dish is grafted with a polymer (poly-*N*-isopropylacrylamide) which becomes either hydrophilic or hydrophobic in a reversible manner, depending on the temperature. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophobic surface similar to that of commercially available dishes and it can be used to culture cells in a conventional manner when the temperature is 37 °C or higher. However, the surface of the dish becomes hydrophilic when the temperature falls below the critical solution temperature of 32 °C. Therefore, confluent sheets of cultured cells can be spontaneously released from the hydrophilic dish surface by reducing the temperature to below 32 °C.

detach easily (Fig. 10.1, Movie: http://www.cellseed.com/technology-e/003.html). Cells detach because the hydrophobic surface they are attached to disappears below the LCST of 32 °C. Since cell-damaging enzymes are not required, cells can detach while maintaining the cell–cell junction. This enables the cultured cells to be harvested as a single 'sheet'. The cell sheets are highly effective when transplanted into patients due to the tight connections between the cells. The cultured cell sheet can be easily moved and layered upon other cultured cell sheets to generate a 3-dimensional cell culture. If fibronectin is present, the multiple-layer cell sheets are easily constructed by simply laying one cell sheet on top of another. The technology is ultimately applicable to the construction of organs by layering different types of cell sheet.

10.2 Overview of present clinical applications

This section will discuss the present clinical applications of cell sheet technology (i.e., the regeneration of the cornea, myocardium, esophageal mucosa, etc.). Prof. Okano and his colleagues are working on building three-dimensional tissue constructs. Cell sheet technology has already been used for regenerative medicine in corneal[3,4] and myocardial tissues.[5–8] In Japan, the lack of cornea and heart donors and the immune system's reaction to a transplanted organ are recognized as serious problems. Prof. Nishida, at Tohoku University, has produced cell sheets from the cultured epithelial stem cells of cornea and oral

mucosal cells. He has transplanted these two types of cell sheet into more than 12 patients without sutures for tissue regeneration and has had excellent clinical results. Prof. Sawa, at Osaka University, made cell sheets from cultured myoblasts of the femur of a patient with dilated cardiomyopathy who underwent surgery for the implantation of a ventricular assist device in February 2006. He transplanted 20 cell sheets into the walls of the left ventricle of the heart in May 2007. The patient recovered well and he no longer needed the ventricular assist device, which was removed in September 2007. This was the first report of successful cell sheet therapy for a patient with dilated cardiomyopathy. The cell sheet therapy can provide regenerative treatment as an alternative for patients who need cardiac transplantation. Prof. Sawa plans to treat six patients, using the same type of cell sheet, in Osaka University Hospital. Other preclinical studies using cell sheets are under way. Clinical studies of cell sheet therapy for urothelial tissue[9] (Tokyo Women's Medical University), oesophageal tissue[10–12] (Tokyo Women's Medical University), periodontal tissue[13–15] (Tokyo Women's Medical University and Tokyo Medical and Dental University), hepatic tissue[16–19] (Tokyo Women's Medical University) and articular cartilaginous tissue[20,21] (Tokai University School of Medicine) are currently taking place.

10.3 Challenge for cartilage repair

This section will discuss how to repair cartilaginous defects using layered chondrocyte sheets. Articular cartilage is an avascular tissue that is nourished by synovial fluid. Adult articular cartilage shows poor self-repair after degeneration or injury and it is therefore unlikely to be restored to normal once it has been damaged. The current treatments available for cartilage defects include the application of a periosteal patch to cover the defect[22] and mosaicplasty, in which an osteochondral pillar is grafted from a non-weight-bearing site.[23] However, the use of periosteal patches has limitations owing to problems with ossification and the limited area that can be treated. Although the microfracture technique is widely used, in which drilling is employed to induce bone marrow cells to differentiate into chondrocytes, the cartilage obtained by this technique is fibrocartilage, with different characteristics to those of hyaline cartilage. Since promising results for the transplantation of cultured autologous chondrocytes have been reported,[24] various articular cartilage regeneration techniques have been applied clinically, including the use of scaffolds such as atelocollagen[25] and cell transplantation therapy with bone marrow-derived mesenchymal stem cells.[26] However, current cartilage regeneration techniques are intended for the treatment of full thickness defects and there have been no reports on the clinical application of a technique for partial thickness defects in patients with early osteoarthritis. Defects in articular cartilage are classified as either full or partial thickness defects, according to whether or not they penetrate the marrow spaces of the subchondral bone. Partial thickness defects are analogous to the clefts and

fissures that are seen in the early stages of osteoarthritis in humans. These fibrillated lesions grow larger and deeper during the course of the disease but never repair themselves spontaneously. It has also been suggested that partial thickness defects do not heal because they are walled off from the marrow and thus have no access to the macrophages, endothelial cells and mesenchymal cells that reside therein.[27]

Articular cartilage is composed of scattered chondrocytes embedded in an abundant extracellular matrix (ECM). The matrix is mainly composed of type II collagen and proteoglycans and is responsible for specific joint functions, including smooth movement and shock absorption. When cultured chondrocytes are employed *in vitro*, it is important to harvest the cells without damaging the ECM. However, current methods damage the cultured cells and disrupt the ECM because proteolytic enzymes are used when harvesting the cultured cells. To achieve the repair and regeneration of partial thickness articular cartilage defects, cultured chondrocytes can be harvested without ECM damage by using temperature-responsive culture dishes. Such cell sheets have been reported to have various advantages, including the preservation of the early phenotype and the expression of adhesion proteins on the base.[1] Furthermore, these cell sheets can be layered onto each other to prepare a layered 'tissue' because the ECM is preserved on the base and such three-dimensional manufactured tissues have already been used for transplantation.[5]

In this study, human chondrocyte sheets with the ECM were obtained using the temperature-responsive culture dish method and were then combined in layers. Following this, the 'tissue' was compared with that of a single sheet and the adhesion of the sheets was examined both *in vivo* and *ex vivo*. This demonstrated the first step towards bioengineering cartilaginous tissues for the treatment of partial thickness cartilage defects using cell sheet technology (Fig. 10.2).

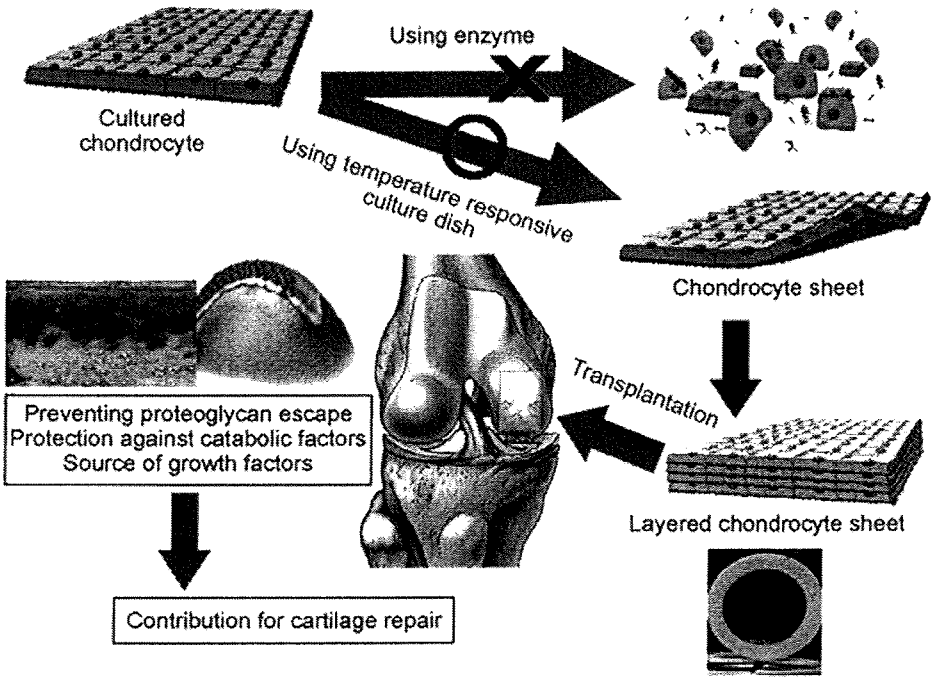
10.3.1 Allograft study

Articular chondrocytes from Japanese white rabbits

Twelve Japanese white rabbits aged 3–4 weeks and weighing between 800 and 1000 g were used as the source of articular cartilage cells. Cartilage samples were collected from the femoral compartment of the knee joint and were subjected to the same enzymatic treatment process as that used for human articular cartilage cells. Thereafter, the isolated cells were seeded and cultured in temperature-responsive culture dishes.

Cell proliferations on a temperature-responsive surface

Chondrocytes were digested for 1 h in Dulbecco's modified Eagle's medium/F12 (D-MEM/F12; GIBCO, NY USA) containing 0.4% Pronase E



10.2 Cell sheet technology for articular cartilage repair. The advantages of using such cell sheets are that they are easy to culture and they proliferate easily, they have good adhesion, and a barrier function which is also very important because it enables the protection of intra-articular catabolic factors, while also preventing proteoglycans from escaping. Furthermore, cell sheets are useful for various types of cartilageous defect in conjunction with the use of scaffold free tissue-engineered cartilage.

(Kakenseiyaku Inc.) and subsequently for 4 h in DMEM/F12 containing 0.016% Collagenase P (Roche, Mannhein, Germany). The digested tissue was passed through a cell strainer (BD Falcon™) with a pore size of 100 μm. The cells were then seeded at a high density (10 000 cells/cm^2) onto the surfaces of temperature-responsive culture dishes (UpCell™, diameter: 35 mm provided by CellSeed, Tokyo, Japan) and cultured in DMEM/F12 supplemented with 20% Fetal Bovine Serum (FBS; GIBCO, NY) and 50 μg/ml ascorbic acid (Wakojunyakukougyou Corp. Japan) and 1% Antibiotics-Antimycotic (GIBCO, NY) at 37 °C in an atmosphere of 5% CO_2 and 95% air for a week. Human articular cartilage cells were also seeded onto commercially available culture dishes (diameter: 35 mm, Iwaki, Japan) and cultured under the same conditions.

Harvesting of cell sheets

Each culture dish was removed from the incubator when the cells reached confluence and was then left to stand at about 25 °C for 30 minutes. After the

culture medium has been removed, the cell sheet was harvested using a polyvinylidene difluoride (PVDF) membrane according to the method reported by Yamato *et al.*[28] In brief, the PVDF membrane was placed on the cell sheet and then the sheet was rolled up with the membrane from one corner. Cultured human chondrocytes were able to be successfully harvested as a single contiguous cell sheet using this method. Then each cell sheet was placed on top of another confluent cell sheet to create multilayered sheets. Since the multilayered sheets floated in the culture medium, a 0.4 μm cell culture insert (Falcon, USA) was placed on top to prevent this and then the culture of the sheets was continued for 1 week.

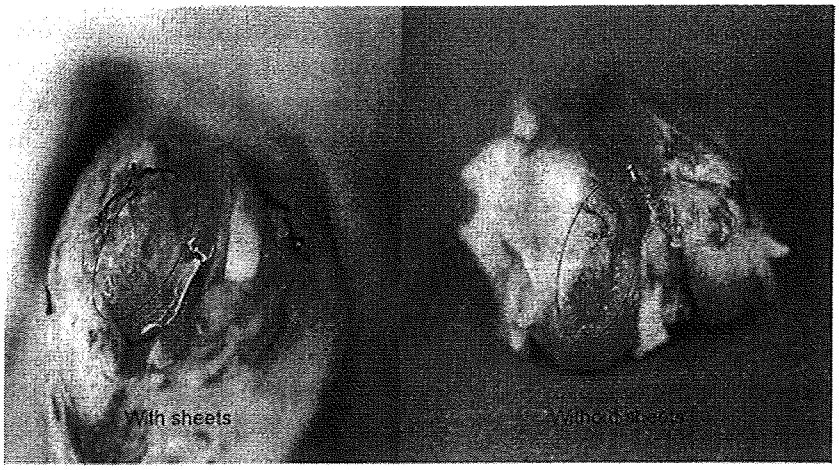
Three-layer sheets of cartilage cells from Japanese White rabbits were also prepared. A 0.4 μm filter was also used to compress these sheets onto the culture dish and sheets were incubated for 3 weeks to prepare the multilayered sheets for transplantation.

Transplantation of chondrocyte sheets

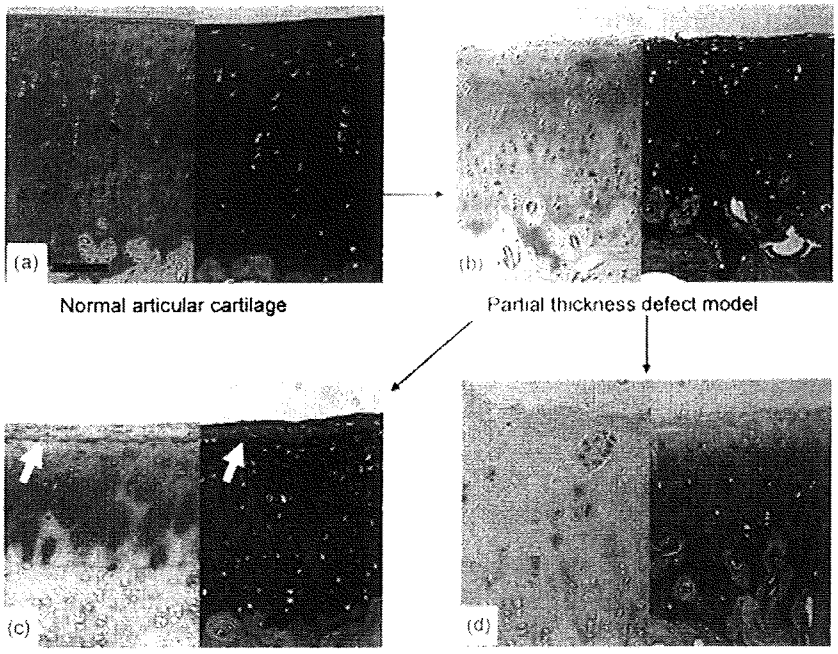
The articular cartilage of the medial femoral condyle of Japanese white rabbits, weighing about 3000 g, was removed to a depth of less than 1 mm using a file to prepare a model of partial thickness cartilage damage. The damaged cartilage was covered with a three-layered chondrocyte sheet, which was stabilized with a nylon suture until the initial fixation was achieved. This was done in four knees of two rabbits as the transplantation group. At the same time, the articular cartilage of the medial femoral condyle was similarly filed, but not covered with a cell sheet, in four knees of two rabbits (the control group). The cartilage was harvested after 4 weeks, fixed in 4% PFA for one week and decalcified with K-CX Decalcifying Solution (Fujisawa Pharmaceutical, Japan) for 1 week. The specimens were then embedded in paraffin, cut into sections and stained with safranin-O and toluidine blue for evaluation.

Histological findings of the allografted chondrocyte sheet and injured sites

The three-layered cell sheet remained well attached to tissue sections 4 weeks after transplantation. The area covered by the sheet was better stained than the area not covered with it, as observed in the previously mentioned *ex vivo* experiment. In the partial damaged cartilage model, the area not covered with the multilayered sheets showed progressive cartilage degeneration with fibrillation and poor staining of the matrix at 4 weeks. In contrast, the area covered with the three-layered sheets showed relatively mild degeneration and a well-stained matrix (Figs 10.3 and 10.4). This study confirmed that chondrocytes could be harvested as sheets and thus be made into multilayered 'tissue' by culturing in temperature-responsive dishes and then collecting them using a temperature recovery system.



10.3 Layered chondrocyte sheets transplantation to the cartilageous defects of the rabbit knee joints. In this rabbit animal model, the allografted cell sheet maintained its cartilageous thickness, but the group without any cell sheets showed an exposure of subchondral bone and severe osteoarthritis.



10.4 Histological findings after transplantation of layered chondrocyte sheets. A histological analysis of an *in vivo* study (left side: Safranin-O staining, right side: Toluidine blue staining): (a) the normal articular cartilage of the Japanese white rabbit femoral chondrocyte; (b) the partial thickness defect model; (c) the partial thickness defect models which covered the three-layered chondrocyte sheets. These showed a better stainability than those not covered by the chondrocyte sheets (d). The partial thickness defect models themselves showed progressive cartilage degeneration with fibrillation (d). The arrows demonstrate the layered chondrocyte sheets (c) (bar: 100 μm).

The importance of treatment and prophylaxes for osteoarthritis is increasing due to a progressively ageing society. However, there are only a few conservative therapies available at this time, such as non-steroidal anti-inflammatory drug (NSAID) administration and the injection of hyaluronic acid. There is still no means of preventing the future exacerbation of cartilage degeneration.

Based on the results of this study, the use of bioengineered chondrocyte sheets may be potentially useful in the treatment of partial thickness defects of articular cartilage. The advantages of such cell sheets are that they are easy to culture and grow and, most importantly, they show good adhesion and barrier functions. This means that they can protect against intra-articular catabolic factors while also preventing the escape of proteoglycan from the injured site. They have a promising growth factor supply and furthermore, such cell sheets could be useful as an alternative to periosteum grafting, which is the most commonly used treatment.

Good adhesion and the inhibition of cartilage degeneration at the injured sites were also confirmed even after the experimental study of allografts of layered chondrocyte sheets had been running for 2 months. The sites where the cell sheets showed adhesion were well stained with safranin-O. Therefore, it is suggested that multilayered chondrocyte sheets may serve as a barrier for preventing proteoglycan loss from damaged cartilage, while also protecting the injured site from the catabolic factors in synovial fluid. A strategy has been developed for repairing a full thickness defect of articular cartilage using the layered cell sheets because it is necessary to treat the bleeding from the bone marrow.

10.4 Properties of chondrocyte sheets

This section will discuss the properties of chondrocyte sheets. In particular, layered chondrocyte sheets contain few of the destructive factors that cause cartilage to degenerate and they also have good adhesion properties that help to both protect and repair the cartilage surface.

10.4.1 Human articular chondrocytes

The cells used for this *in vitro* experiment included human articular chondrocytes obtained from patients who had undergone anterior cruciate ligament reconstruction and had given their informed consent at the Tokai University Oiso Hospital from December 2004 to August 2005. Chondrocytes were obtained while forming the interfoveolar ligament and they were then isolated by enzymatic treatment. Twenty-five knees of 25 patients aged 14 to 49 years (average 23 years, 19 males and 6 females) were used as the source of these cells. The chondrocytes were enzymatically dissociated and were then seeded and cultured according to the method of Sato *et al.*[29]

10.4.2 RNA isolation and cDNA synthesis

Total RNA was isolated using the *RNeasy* Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The RNA quality in each sample was confirmed by the A260/280 absorbance ratio and by electrophoresis on a 1.2% agarose formaldehyde gel. Approximately 1.0–2.0 μg of total RNA was reverse transcribed into single strand cDNA using Moloney murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems, Foster City, CA). The reverse transcriptase (RT) reaction was carried out for 60 min at 42 °C and then for 5 min at 95 °C in a thermocycler.

10.4.3 Primer design and real-time polymerase chain reaction (PCR)

All oligonucleotide primer sets were designed based upon the published mRNA sequence. The expected amplicon lengths ranged from 70 bp to 200 bp. The oligonucleotide primers used in this study are listed in Table 10.1. Real-time polymerase chain reaction (PCR) was carried out in a SmartCycler II (Cepheid, Sunnyvale, CA) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). For real-time PCR, 2–2.5 μl of cDNA template was used in a final volume of 25 μl. The cDNA was amplified according to the following conditions: 95 °C for 15 s and 60 °C for 60 s for 35 to 45 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A

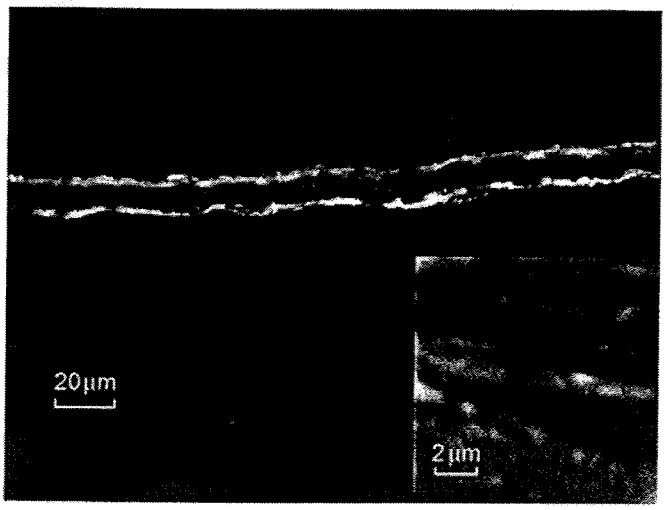
Table 10.1 List of primers used in real-time PCR

Primer ID	Accession No.	Sequence	Expected size (bp)
Collagen Type I-F	NM_000088	AAG GGT GAG ACA GGC GAA CAA	170
Collagen Type I-R		TTG CCA GGA GAA CCA GCA AGA	
Collagen Type II-F	NM_033150	GGA CTT TTC TTC CCT CTC T	113
Collagen Type II-R		GAC CCG AAG GGT CTT ACA GGA	
Aggrecan1-F	NM_001135	TCG AGG ACA GCG AGG CC	94
Aggrecan1-R		TCG AGG GTG TAG GCG TGT AGAGA	
Fibronectin1-F	NM_001030524	GCA CAG GGG AAG AAA AGG AG	189
Fibronectin1-R		TTG AGT GGA TGG GAG GAG AG	
MMP3-F	NM_002422	ATT CCA TGG AGC CAG GCT TTC	138
MMP3-R		CAT TTG GGT CAA ACT CCA ACT GTG	
MMP13-F	NM_002427	TCA CGA TGG CAT TGC TGA CA	77
MMP13-R		AGG GCC CAT CAA ATG GGT AGA	
TIMP1-F	NM_003254	CAG CGT TAT GAG ATC AAG ATG GAC CA	186
TIMP1-R		AGT GAT GTG CAA GAG TCC ATC CTG	
ADAMTS5-F	NM_007038	GAG CCA AGG GCA CTG GCT ACT A	120
ADAMTS5-R		CGT CAC AGC CAG TTC TCA CACA	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPGH-R		ATG GTG GTG AAG ACG CCA GT	

melting-curve analysis was performed (0.5 °C/s increase from 55 to 95 °C with continuous fluorescence readings) at the end of the cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. All reactions were repeated in six separate PCR runs using RNA isolated from four sets of human samples. The results were evaluated using the SmartCycler II software program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize the samples. To monitor crossover contamination of PCR, RNase-free water (Qiagen Inc., Valencia, CA) was included in the RNA extraction and used as a negative control. To ensure the quality of the data, a negative control was always applied in each run.

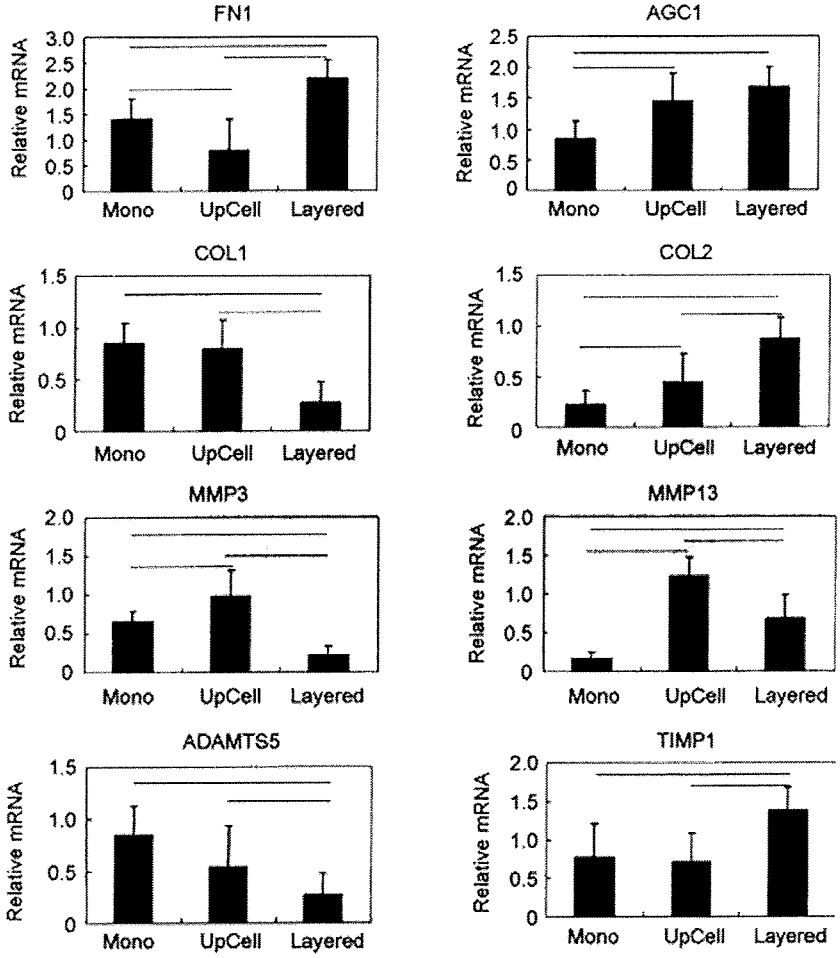
10.4.4 Analysis of gene expression

Layered chondrocyte sheets were shown to adhere to porcine cartilage after a one-day organ culture. It is possible that an increase of fibronectin in the multilayered chondrocyte sheets may have been involved (Fig. 10.5). Good adhesion could thus be obtained because harvesting without enzymatic treatment makes it possible to better preserve the activity of both fibronectin and adhesion proteins such as integrin. Matrix–matrix interactions therefore play an important role in the adhesion between chondrocyte sheets and injured cartilage, and specific enzymes may modify the surface of each matrix and permit interaction between integrin proteins and fibronectin. Although this adhesive phenomenon is currently being studied using a cDNA microarray, some of the results were demonstrated here. The expression of fibronectin1, collagen type II, aggrecan 1 and TIMP1 mRNA



10.5 The expression of fibronectin. The localization of fibronectin was the surface of the layered chondrocyte sheet. The high magnification demonstrated that the expression of fibronectin localized among extracellular matrices and cell-cell junctions.

was observed at significantly high levels, while the expression of collagen type I, MMP3 and ADAMTS5 was at significant levels in the layered chondrocyte sheets in comparison to the monolayer culture. Another interesting aspect of these chondrocyte sheets is the fact that catabolic factors, such as MMP3,[30–33] MMP13[32–35] and ADAMTS5[36,37] were observed to decrease at the time of layering, while the expression of TIMP1 with antagonistic actions against MMP3



10.6 The relative expressions of mRNA of key genes. The mRNA expression of fibronectin 1 of three-layered chondrocyte sheets was higher than monolayer culture. The mRNA expressions of aggrecan and type II collagen of chondrocyte sheets were higher than that of monolayer culture. The mRNA expression of type I collagen demonstrated a low level in the three-layered chondrocyte sheets. The expressions of MMP3 and ADAMTS5, which promote cartilage degeneration were low in the three-layered chondrocyte sheets while the mRNA expression of TIMP1, which is an antagonistic factor of MMP3, showed a significantly high level in the three-layered chondrocyte sheets. Mono: conventional monolayer culture, UpCell: monolayer culture using temperature-responsive culture dish, Layered: layered chondorocyte sheets (—: $P < 0.05$).