Ⅳ．研究成果の刊行（平成24年度）に関する一覧表

書籍

出版	書籍名 (出版社)	タイトル	ページ	出版地	著者氏名
2013 年	幹細胞医療の 実用化技術と 産業展望	第 7 章　品質評価 2.新しい評価法としての アレイ CGH 法	256-261	JPN	伊東紀子, 佐藤正人, 的場 亮, 梅澤明弘

雑誌

出版	発表誌名	論文タイトル	ページ・巻号	著者氏名
2012 年	Journal of Orthopaedic Research	MicroRNA-199a-3p, microRNA-193b, and microRNA-320c are correlated to aging and regulate human cartilage metabolism	1915-1922 ・30(12)	Ukai T, Sato M, Akutsu H, Umezawa A, Mochida J
2012 年	Biomaterials	Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells	5278-5286 ・33(21)	Ito S, Sato M, Yamato M, Mitani G, Kutsuna T, Nagai T, Ukai T, Kobayashi M, Kokubo M, Okano T, Mochida J
2012 年	BMC Musculoskeletal Disorders	Human telomerase reverse transcriptase and glucose-regulated protein 78 increase the life span of articular chondrocytes and their repair potential	13(1):51	Sato M, Shin-ya K, Lee JI, Ishihara M, Nagai T, Kaneshiro N, Mitani G, Tahara H, Mochida J
2012 年	整形外科	【総説】ナノ秒パルスレーザー	980・63(9)	佐藤正人

雑誌（その他）

出版	発表誌名	タイトル	ページ・巻号	著者氏名
2012 年	Medical Tribune 医学情報新聞	［変形性膝関節症］ 3 週間以内で関節軟骨細胞シート作製に成功	26・45(49)	佐藤正人

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			
2013 年 3 月	第 12 回日本再生医療学会総会	【The Johnson & Johnson Innovation Award 受賞講演】 Realization of cartilage regeneration by cell sheet accelerating joint treatment	佐藤正人

2013年 3月	第12回日本再生医療学会総会	【パネルディスカッション】細胞シートによる関節軟骨の再生医療と体内環境の構築	佐藤正人, 持田譲治
2013年 3月	第12回日本再生医療学会総会	Array Comparative Genomic Hybridization およびGバンド分染法を用いた軟骨細胞の安全性評価	小林美由希, 佐藤正人, 河毛知子, 横山宗昂, 小久保舞美, 三谷玄弥, 高垣智紀, 的場 亮, 伊東紀子, 持田譲治
2013年 2月	総合医学研究所第4回シンポジウム&私立大学戦略的研究基盤形成支援事業進捗報告会	家兎変形性関節症モデルを用いた抗VEGF抗体剤による軟骨修復再生効果	佐藤正人
2013年 2月	第4回スーパー特区シンポジウム「細胞シートによる再生医療実現プロジェクト」	【シンポジウム】細胞シートによる関節軟骨再生医療の実現へ向けた取り組み	佐藤正人
2013年 1月	Annual Meeting of the Orthopaedic Research Society	Anti VEGF antibody contributes to better repair of articular cartilage	Nagai T, Sato M, Ukai T, Kobayashi M, Mochida J
2013年 1月	Annual Meeting of the Orthopaedic Research Society	Analysis of the humoral factors produced by layered chondrocyte sheets	Hamahashi K, Sato M, Mitani G, Ito S, Nagai T, Ebihara G, Kutsuna T, Mochida J
2012年 11月	第50回日本人工臓器学会大会	積層化軟骨細胞シートの同種T細胞におよぼす影響	加藤玲子, 佐藤正人, 小久保舞美, 河毛知子, 宮島敦子, 持田譲治, 松岡厚子
2012年 11月	栃木県臨床整形外科医会	【教育研修講演】細胞シートによるヒト幹細胞臨床研究	佐藤正人
2012年 11月	The 7th International Congress of Chinese Orthopaedic Association (COA2012)	【講演】Biological articular cartilage repair using cell sheet	Sato M
2012年 11月	文部科学省イノベーションシステム整備事業「再生医療本格化のための最先端技術融合拠点」第5回シンポジウム	【招待講演】軟骨細胞シートによるヒト幹細胞臨床研究	佐藤正人
2012年 10月	第27回日本整形外科学会基礎学術集会	抗VEGF抗体ヒト化モノクローナル抗体による関節軟骨予防効果	長井敏洋, 佐藤正人, 鵜養 拓, 小林美由希, 持田譲治
2012年 10月	第27回日本整形外科学会基礎学術集会	積層化軟骨細胞シートによって分泌された液性因子の検討	浜橋恒介, 佐藤正人, 三谷玄弥, 伊藤 聡, 長井敏洋, 海老原吾郎, 沓名寿治, 持田譲治

2012年 9月	3rd TERMIS World Congress 2012, Tissue Engineering and regenerative Medicine	【Symposium】Cartilage repair in transplanted scaffold-free chondrocyte sheets using a minipig model	Sato M, Ebihara G, Nagai T, Mitani G, Kutsuna T, Kokubo M, Kobayashi M, Yamato M, Okano T, Mochida J
2012年 9月	3rd TERMIS World Congress 2012, Tissue Engineering and regenerative Medicine	Safety evaluation of cultured chondrocytes by comparative genomic hybridization analysis	Kobayashi M, Sato M, Kawake T, Yokoyama M, Kokubo M, Ukai T, Ito S, Nagai T, Hayashi K, Ito N, Mochida J
2012年 9月	第2回レギュラトリーサイエンス学会学術大会	【シンポジウム】軟骨細胞シートの事例からみたヒト幹細胞臨床研究の取り組み	佐藤正人
2012年 6月	第11回日本再生医療学会総会	【シンポジウム】細胞シートによる関節治療を目指した臨床研究	佐藤正人, 加藤俊一, 加藤玲子, 阿久津英憲, 長嶋比呂志, 村井邦彦, 石原美弥, 中村嘉彦, 三谷玄弥, 高垣智紀, 海老原吾郎, 伊藤 聡, 小林美由希, 小久保舞美, 持田譲治
2012年 6月	第11回日本再生医療学会総会	積層化軟骨細胞シートと培養滑膜細胞移植による関節軟骨治療効果の検討	伊藤 聡, 佐藤正人, 小久保舞美, 小林美由希, 鵜養 拓, 長井敏洋, 沓名寿治, 三谷玄弥, 持田譲治
2012年 6月	第11回日本再生医療学会総会	培養軟骨細胞の安全性評価 －CGH(Comparative Genomic Hybridization)解析による検討－	小林美由希, 佐藤正人, 小久保舞美, 河毛知子, 横山宗昂, 鵜養 拓, 伊藤聡, 長井敏洋, 林 克之, 伊東紀子, 持田譲治
2012年 6月	第11回日本再生医療学会総会	*in vitro*における同種軟骨細胞（シート）の免疫応答におよぼす影響	加藤玲子, 佐藤正人, 小久保舞美, 持田譲治, 松岡厚子
2012年 6月	第11回日本再生医療学会総会	ウサギ軟骨細胞シートの新規ガラス化保存法の開発	前原美樹, 松成ひとみ, 金井貴博, 小久保舞美, 松村和明, 玄丞烋, 佐藤正人, 長嶋比呂志
2012年 5月	第51回日本生体医工学会大会	【シンポジウム】医工融合による臨床応用－関節鏡視下光音響プローブの開発	佐藤正人, 石原美弥, 菊地 眞, 持田譲治
2012年 5月	再生医療を推進する議員の会	【講演】関節軟骨の再生医療と軟骨修復に適した体内環境の構築	佐藤正人
2012年 4月	2012 World Congress on Osteoarthritis Research Society International	Early intervention to prevent cartilage degeneration by administration of anti-VEGF antibody in rabbit model	Nagai T, Sato M, Kutsuna T, Ukai T, Kobayashi M, Mochida J

研究分担者　阿久津英憲			
2012年 8月	4th Congress of the Asia Pacific Initiative on Reproduction	（シンポジウム）“Human ES cells: acquired pluripotency from blastocysts”	Akutsu H
2012年 7月	第48回大会日本小児循環器学会総会・学術集会	（シンポジウム）「ヒトES細胞～小児難治疾患へ挑む～」	阿久津英憲
2012年 5月	International Conference on "Stem Cell and Regenerative Medicine: Research to Business"	（招待講演）“iPSCs for regenerative medicine”	Akutsu H
2012年 5月	日本組織培養学会第85回大会	（シンポジウム）「ゼノフリーヒトES/iPS細胞の培養システムの確立」	阿久津英憲
研究分担者　長嶋比呂志			
2012年 11月	第39回日本低温医学会総会	【招待講演】再生医学における凍結保存の役割：ブタ胎仔臓器原基のガラス化保存について	長嶋比呂志
2012年 11月	第1回川島腎カンファレンス	【招待講演】クローンブタ・遺伝子改変ブタのトランスレーショナルリサーチへの利用	長嶋比呂志
2012年 7月	17th International Congress on Animal Reproduction	【招待講演】Advancing pig cloning technologies towards application in regenerative medicine	Nagashima H, Matsunari H, Nakano K, Watanabe M, Umeyama K, Nagaya M
2012年 6月	第12回日本抗加齢医学会総会	【招待講演】再生医療研究のための大型動物モデルの開発	長嶋比呂志, 松成ひとみ, 中野和明, 渡邊将人, 梅山一大, 長屋昌樹
2012年 6月	自治医大ピッグシンポジウム	【招待講演】Yamaton　K-2計画：完全ヒト化腎臓作製に向けて	長嶋比呂志, 松成ひとみ, 横尾 隆, 松本 啓, 横手伸也, 岩井聡美, J.A.Medin, 渡邊將人, 梅山一大, 中野和明, 金井貴博, 小林英司
2012年 6月	第55回日本腎臓学会	【招待講演】臓器再生研究へのクローンブタの利用	長嶋比呂志, 松成ひとみ, 横尾隆, 岩井聡美, 小林英司, 中内啓光
2012年 6月	第12回日本抗加齢医学会総会	再生医療研究のための大型動物モデルの開発	長嶋比呂志, 松成ひとみ, 中野和明, 渡邊將人, 梅山一大, 長屋昌樹
2012年 6月	第55回日本腎臓学会学術総会	臓器再生研究へのクローンブタの利用	長嶋比呂志, 松成ひとみ, 横尾隆, 岩井聡美, 小林英司, 中内啓光
2012年 5月	JALAM（日本実験動物医学会）	遺伝子改変ブタ・クローンブタのトランスレーショナルリサーチへの応用	長嶋比呂志, 松成ひとみ, 渡邊將人, 梅山一大, 長屋昌樹

研究分担者　加藤玲子			
2012年 11月	第50回日本人工臓器学会大会	積層化軟骨細胞シートの同種T細胞におよぼす影響	加藤玲子, 佐藤正人, 小久保舞美, 河毛知子, 宮島敦子, 持田譲治, 松岡厚子
2012年 6月	第11回日本再生医療学会	in vitroにおける同種軟骨細胞（シート）の免疫応答におよぼす影響	加藤玲子, 佐藤正人, 小久保舞美, 持田譲治, 松岡厚子

Ⅴ．研究成果の刊行物・別刷

バイオテクノロジーシリーズ

幹細胞医療の実用化技術と産業展望

Implementation Technologies and Industry Outlook of Stem Cell Therapy

監修：江上美芽，水谷　学
Supervisor : Mime Egami, Manabu Mizutani

HIGH TECHNOLOGY INFORMATION

シーエムシー出版

2　新しい評価法としてのアレイ CGH 法

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的場　亮[*3]，梅澤明弘[*4]

2.1　はじめに

近年，培養技術・分子細胞学・生体組織工学・遺伝子工学の発達とともに幹細胞の研究が盛んになり，幹細胞を応用した再生医療は実現化に向け動き始めている。

米国ではヒト ES 細胞製剤を用いた臨床試験が開始され，2010 年 10 月 11 日，Geron 社が ES 細胞由来のオリゴデンドロサイト前駆細胞製剤「GRNOPC1」の臨床試験を脊髄損傷の患者を対象に開始した（翌年経営上の理由で撤退）。2012 年 1 月 23 日，Advanced Cell Technology 社は，2011 年に始めた ES 細胞により作製した網膜細胞を用いて加齢黄斑変性症およびスタッガード病の視力を回復させることに成功したという研究成果を，イギリスの医学誌 Lancet に発表した[1)]。我が国でも現在臨床研究計画が進められている。

ヒト幹細胞を臨床応用する上で安全性の確保は，重要な課題の一つである。特に長期間培養を継続する際，それに伴う細胞の形質変化，ゲノム構造変化などの有無を調べることは非常に重要である。我々は投与する細胞が形質転換を起こしていないか，腫瘍化していないかを調べるツールとして，従来の核型分析試験よりも少ない日数・低コストで実施可能なアレイ CGH 解析が有用であると考え検証試験を開始した。

2.2　アレイ CGH 法とは

CGH 法（Comparative Genomic Hybridization：比較ゲノムハイブリダイゼーション）は，1992 年に Kallioniemi らが，Science 誌で発表した方法であり[2)]，FISH 法（Flourescence In Situ Hybridization)[3)] を応用し，全染色体を対象として，ゲノム DNA が増幅（gain），欠失（loss）した領域を調べる方法である。アレイ CGH 法は，マイクロアレイと CGH 法を組み合わせることで，ハイスループットに目的遺伝子，ゲノム DNA 領域のコピー数変化の検出を可能にした。特に，高密度マイクロアレイの登場により，染色体上の約 2 kb ごとの位置にプローブが配置できるようになり，解像度が格段に向上した。

アレイ CGH 法は，微細ゲノム異常の探索を可能にする技術であり，ゲノムコピー数異常に起因する疾患の病態解明の糸口となる微細ゲノム異常の発見に威力を発揮している。また，既知の染色体異常症やがんの遺伝子・染色体診断にも応用されている。FISH 法や核型分析では発見できない微細な染色体異常を検出することができ，また定量 PCR では 1 回の実験でカバーしきれ

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＊3　Ryo Matoba　㈱DNA チップ研究所　代表取締役社長
＊4　Akihiro Umezawa　㈱国立成育医療研究センター　再生医療センター長

ない網羅性で，全ゲノム領域を解析することができる点がアレイ CGH 最大のメリットである。

2.3　アレイ CGH 法手順

ここでは，Agilent Technologies 社 Human Genome CGH マイクロアレイを用いた標準的な手順について述べる[4]。

解析対象サンプル，比較したい正常コントロールサンプルをそれぞれ用意し，制限酵素 *Alu I*, *Rsa I* を用いて短いサイズに断片化する。その後ランダムプライマーと Exo-Klenow を用いた反応により，解析対象サンプルを Cyanine-5（Cy5）という赤い蛍光色素でラベル化し，比較したい正常コントロールサンプルを Cyanine-3（Cy3）という緑の蛍光色素でラベル化する。遺伝子領域を中心としたゲノム配列のプローブが網羅的に搭載されているスライドグラス上で，両サンプルの競合的なハイブリダイゼーションを行う。各プローブの蛍光強度を専用スキャナーを用いてスキャンし，Feature Extraction ソフトウエアを用いて蛍光強度を数値に変換し，正規化を行い，対数表示させ染色体の位置ごとに並べ直す。その後，Genomic Workbench ソフトウエアを用いてゲノムコピー数の異常の有無を検出する。実験概要とフローを（図 1）に示す。

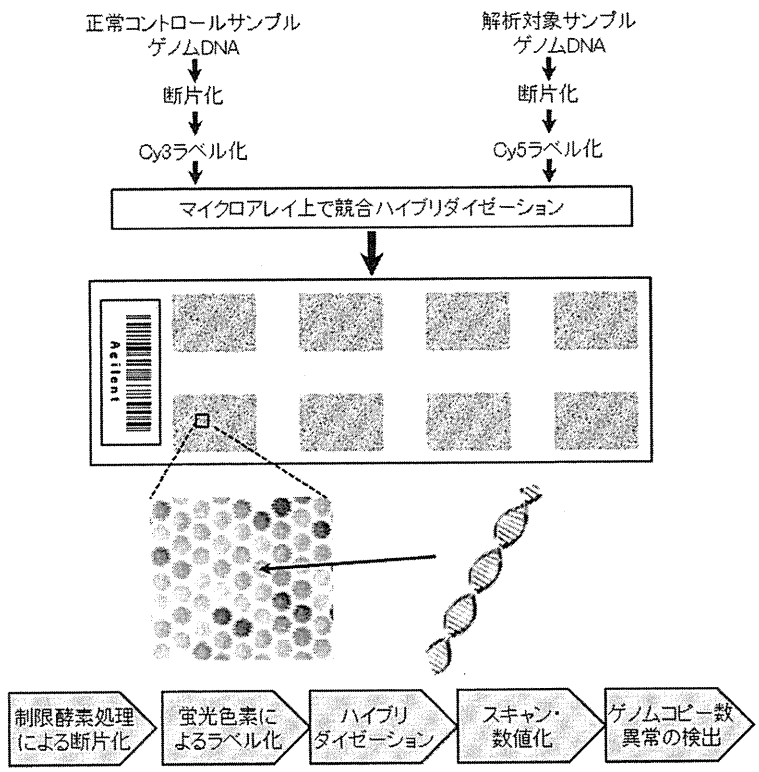

図 1　アレイ CGH 実験概要とフロー

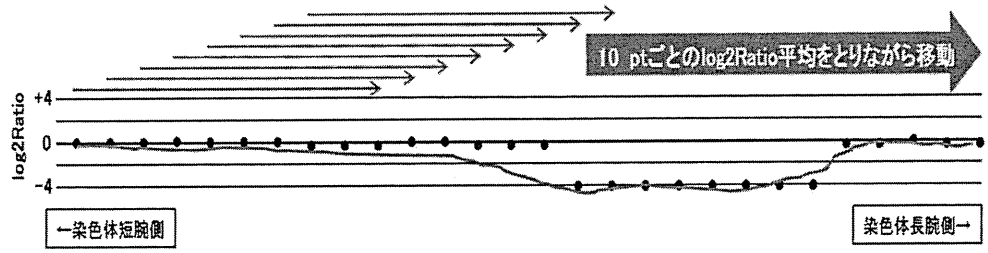

図2　移動平均（Moving Average）10pt

2.4　ゲノムコピー数の異常の検出

Genomic Workbench ソフトウエアでは，ADM-2（Aberration Detection Method-2）アルゴリズム[5] を用いた解析が推奨されている。ADM-2 は Window 幅が可変で Log_2Ratio の変化が大きい領域を検出できるという特徴を持っている。これは，Homozygous Deletion のように大きな Log_2Ratio の変化が起きていれば 1 プローブでも検出できる高感度アルゴリズムである。Agilent 社は閾値（Threshold）6.0 以上の設定を推奨しているが，ADM-2 は変異の幅を大きく検出する傾向があり，使用するアレイフォーマットやサンプルによって最適値の検討が必要である。

ゲノムコピー数の重複・欠失をわかりやすく表示させるために，移動平均の可視化が重要である。例えば移動平均 10pt の場合は，染色体短腕側から 10 プローブの平均値を計算，表示し，次に一つずらした 10 プローブの平均値（2 プローブ目から 11 プローブ目の平均値），その次に一つずらした 10 プローブの平均値（3 プローブ目から 12 プローブ目の平均値）というように 1 プローブずつ移動した平均値を直線で結ぶ（図 2）。このように可視化することによりゲノム全体のコピー数異常を一目で表現することができる。より客観的な数値データは Aberration Calling として一覧表としてアウトプットされ，遺伝子の情報，コピー数異常の度合，領域の長さなどの情報が付加される。

尚，移動平均の値についても ADM-2 アルゴリズム同様に使用するアレイフォーマットやサンプルによって最適値の検討が必要である。

2.5　正常 DNA（HAPMAP DNA 日本人男性）対骨肉腫 DNA の比較

まず最初に，解析対象サンプルとして骨肉腫のゲノム DNA と，比較したい正常コントロールサンプルとして HAPMAP DNA 日本人男性のゲノム DNA をそれぞれ 500 マイクログラム用い，Agilent 社 SurePrint G3 Human CGH マイクロアレイ 8×60K（6 万プローブ搭載）を使用し，既存プロトコルに従い実験を行った。

ハイブリダイゼーション後のシグナル強度データの正規化を行った後，ADM-2 アルゴリズム Threshold＝10 を用いて，ゲノムコピー数の異常を検出したところ，ゲノム上の 19 領域でコピー数異常が見られた（図 3，移動平均 10pt の図を示す）。また，それぞれのサンプルについて色素

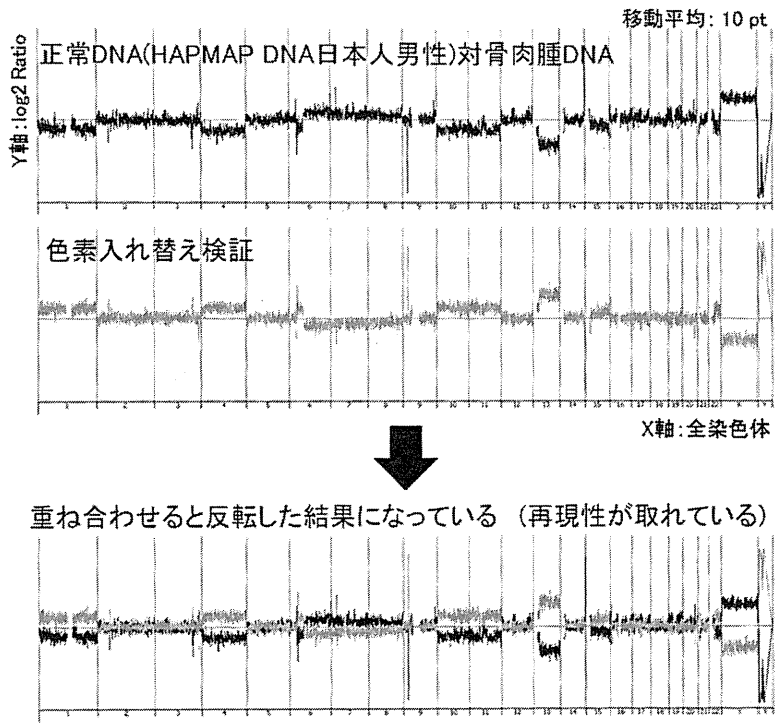

図3　正常 DNA（HAPMAP DNA 日本人男性）対骨肉腫 DNA の比較

入れ替え検証実験を行った。これはラベル化に用いる蛍光色素を逆転させる実験で，逆転したデータが得られた場合再現性が得られたと判断する。その結果，前述した結果と再現性があるデータが得られた（図3)。両者の図を重ね合わせると再現性について，よりわかりやすく確認ができる。以上のように，アレイ CGH 法を用いて網羅的にかつ微細なゲノムコピー数異常が検出できることが確認された。

2.6　培養軟骨細胞の継代数の差による異常の検出

次に培養細胞安全性評価のモデル実験として，軟骨細胞の継代数とゲノムレベルでの異常蓄積の関係データを取得した。解析対象サンプルとして継代数4（P4)，継代数6（P6）のゲノム DNA と，比較したい正常コントロールサンプルとして継代数2（P2）のゲノム DNA をそれぞれ500マイクログラム用い，Agilent 社 SurePrint G3 Human CGH マイクロアレイ 8×60K（6万プローブ搭載）を使用し，既存プロトコルに従い実験を行った。

また，それぞれのサンプルについて繰り返し再実験，色素入れ替えデータを取得し，データの再現性の検証を行った。解析条件として，ADM-2 アルゴリズム Threshold＝10 を用い，移動平均を 10pt に設定した。その結果，繰り返し再実験，色素入れ替え検証共に，継代数による変化は認められず，培養によるゲノム DNA コピー数異常はないと判断した（図4)。現在合計 20 サンプルについて同様なデータを取得したが，この設定値でのゲノム DNA コピー数異常は検出さ

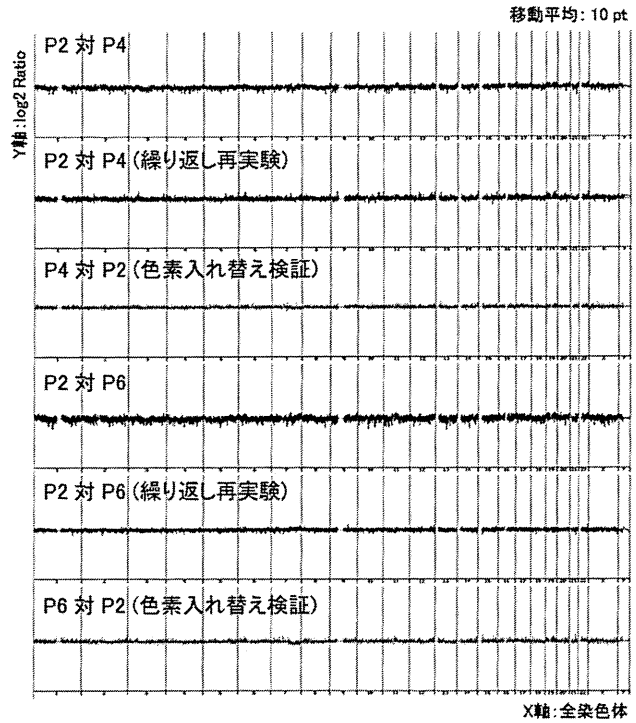

図4　培養軟骨細胞の継代数とゲノムレベルでの異常蓄積の関係

れていない。

2.7　ADM-2（Aberration Detection Method-2）アルゴリズムの Threshold 値検討

前述の，軟骨細胞の継代数とゲノムレベルでの異常蓄積の関係データを用いて，Threshold 値の違いによって，ゲノム上の何箇所がコピー数異常として検出されるか，検討を行った。その結果 Threshold 値を下げるにつれ，コピー数異常が多数検出されるが，繰り返し再実験・色素入れ替え実験ではいずれも再現性がなく，実験データのノイズである可能性が高いことが判明した。つまり，繰り返し再実験や色素入れ替え実験を行うことにより，実験上問題となるノイズを減らすことができると考えられる。今後は，サンプル数や，継代数を増やす等の検討を進め，より安定した閾値を確定したい。

2.8　今後の展開

現在，培養軟骨細胞の継代数の差による異常の検出と同じ手法で，ES 細胞の継代数とゲノムレベルでの異常蓄積の関係データを取得する計画を進めている。最近の研究では，継代数が増えるにつれてゲノム上のある領域にコピー数異常が見られるという報告がある[6]。培養軟骨細胞の実験・解析で得たノウハウから，幹細胞安全性評価に適したプローブを選別し幹細胞安全性評価用カスタムをアレイ作成し，より効率よく高精度にコピー数異常を検出できるシステムを構築し

たい。特にゲノム上の癌関連遺伝子領域については，高精度のコピー数異常を検出できるシステムが必要である。また，現在の実験手法はマニュアル操作の部分も多く，検査として確立するためにはサンプル調製，ハイブリダイゼーションなどの工程について全自動システムを構築する必要がある。

本技術は，幹細胞を応用した再生医療における細胞製剤のゲノム安定性を検証する上で事実上の標準となる。

文　　献

1) Schwartz SD *et al.*, *Lancet*, **379** (**9817**), 713-20 (2012)
2) Kallioniemi A *et al.*, *Science*, **258** (**5083**), 818-21 (1992)
3) Trask BJ, *Trends Genet.*, **7** (**5**), 149-54 (1991)
4) 山本俊至，臨床遺伝に関わる人のためのマイクロアレイ染色体検査，第5章，診断と治療社 (2012)
5) Lipson D *et al.*, *J. Comput. Biol.*, **13** (**2**), 215-28 (2006)
6) Närvä E *et al.*, *Nat. Biotechnol.*, **28** (**4**), 371-7 (2010)

MicroRNA-199a-3p, microRNA-193b, and microRNA-320c Are Correlated to Aging and Regulate Human Cartilage Metabolism

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ABSTRACT: MicroRNAs (miRNAs) are small RNAs of ~22 base pairs that regulate gene expression. We harvested cartilage tissue from patients with polydactylism, anterior cruciate ligament injury, and osteoarthritis undergoing total knee arthroplasty and used microarrays to identify miRNAs whose expression is upregulated or downregulated with age. The results were assessed by real-time PCR and MTT assay in a mimic group, in which synthetic double-stranded RNA from the isolated miRNA was transfected to upregulate expression, and in an inhibitor group, in which the miRNA was bound specifically to downregulate expression. The expression of two miRNAs (miR-199a-3p and miR-193b) was upregulated with age and that of one miRNA (miR-320c) was downregulated with age. A real-time PCR assay showed that type 2 collagen, aggrecan, and SOX9 expression were downregulated in the miR-199a-3p mimic group but was upregulated in the inhibitor group. Similar results were observed for miR-193b. By contrast, ADAMTS5 expression was downregulated in the miR-320c mimic group and upregulated in the inhibitor group. Cell proliferative activity was upregulated significantly in the miR-193b inhibitor group compared with the control group. We believe that miR-199a-3p and miR-193b are involved in the senescence of chondrocytes, and miR-320c is involved in the juvenile properties of chondrocytes. J Orthop Res 30:1915–1922, 2012

Keywords: miR-199a-3p; miR-193b; miR-320c; cartilage metabolism; osteoarthritis

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of ~22 base pairs that regulate gene expression by repressing translation. In 1993, the first miRNA, lin-4 was discovered in nematodes, and subsequent studies have shown that miRNAs regulate ~30% of all human genes.[1] miRNA is transcribed initially from miRNA genes into a long primary transcript (pri-mRNA), which is then cleaved by Drosha to produce precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm using exportin-5, and there it is cleaved by Dicer to yield miRNA. The miRNA then combines with an argonaute protein to form RISC, which sequence-specifically recognizes and represses the translation of target RNAs.[2] Abnormalities in cartilage development have been observed in Dicer gene dysfunction models, and miRNA is known to play a key role in cartilage differentiation.[3] miRNA plays a key role in differentiation and growth in organs and tissues,[2] and miRNAs expressed specifically in cartilage[4–8] have been reported. Till date, miR21,[4] miR22,[9] miR27a,[6] miR140,[10] and miR146[8] have been reported as miRNAs that are associated with cartilage metabolism. Many studies have reported the use of a microarray analysis for isolating miRNAs that are expressed in a tissue-specific manner. For example, Miyaki et al.[10] performed microarray analysis of MSC and articular chondrocytes, and they observed a high expression of miR140 in chondrocytes. They also observed the expression of miR-140 increased during chondrogenesis. In addition, they reported that miR140 expression was lower in the OA group than in the normal group. Iliopoulos et al.[9] performed microarray analysis using normal chondrocytes and chondrocytes isolated from OA. They reported an increased expression of miR22 and a decreased expression of miR140 in the OA group. They also reported that the expressions of IL-1β and MMP13 were increased, whereas the expressions of aggrecan was decreased in the group that showed increased miR22 expression. In contrast, using microarray analysis, we focused on three types of miRNAs, that is, miR199a-3p, miR193b, and miR320c, and their expressions were observed to vary with age.

The purpose of this study was to identify an miRNA that could be used as a biomarker to evaluate cartilage in OA or regenerative medicine. To identify an miRNA that was involved with cartilage metabolism, we isolated miRNAs with expression levels that increased or decreased with age, by performing microarray analysis using chondrocytes isolated from patients with three different conditions, that is, polydactylism(PD), anterior cruciate ligament (ACL) injury, and OA undergoing total knee arthroplasty (TKA). We evaluated whether the extracted miRNAs were involved with cartilage metabolism by comparing two groups, that is, the overexpression group where miRNA levels increased and the inhibitor group where miRNA levels decreased. Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, while gene expression was evaluated using real-time polymerase chain reaction (PCR). For real-time PCR, we focused on three anabolic factors, that is, aggrecan, type 2 collagen, and SOX9, which affect cartilage synthesis. We also focused on three catabolic factors, that is, MMP3, MMP13, and ADAMTS5, which affect cartilage matrix

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destruction. In addition, we focused on type 1 collagen, which is expressed in high levels in fibrocartilage. We also analyzed miRNA-controlled genes, OA-related genes, and cartilage-related genes by pathway analysis to verify whether miRNAs had direct or indirect effects on cartilage.

MATERIALS AND METHODS

Cartilaginous Tissue

The protocols were performed under the approval and guidance of the Clinical Research Review Committee of the Tokai University School of Medicine. Cartilaginous tissue was obtained from 17 patients (age, 60–79 years; average age, 73.4 years) who had been diagnosed with OA and who had undergone TKA at the Tokai University Hospital from April 2010 to June 2011; three patients (age, 15–31 years; average age, 22.3 years) who had an ACL injury and who had undergone surgery at the same hospital during the same period; and six patients (age, 11–16 months; average age: 13 months) who had been diagnosed with polydactylism and who had undergone plastic surgery at the National Center for Child Health and Development during January and February 2010. We used all layers of the normal articular cartilage from PD, ACL, and TKA. Cartilage tissues were harvested from the joints of the resected fingers in patients with polydactylism, arthroscopically excised non-loading parts of injured ACLs, and femur and tibia from patients with knee OA who underwent total knee arthroplasty.

Isolation and Culture of Chondrocytes

The harvested cartilaginous tissue of each patient was sliced finely with scissors and treated with enzymes in a Petri dish for 4 h using Dulbecco's modified Eagle medium F12 (DMEM/F12; GIBCO, Invitrogen Corporation, Carlsbad, CA) containing 0.05% collagenase type 1 (Worthington, Inc., Lakewood, NJ). The enzyme-treated cartilaginous tissue was passed through a cell strainer (BD Falcon^{TM}; BD Biosciences, Bedford, MA) with a pore size of 100 μm, and the cells were retrieved by centrifugation. The chondrocytes were seeded into a 1×10^4 cells/cm^2 plate and incubated in culture medium of DMEM/F12 supplemented with 20% fetal bovine serum (FBS; Gibco, NY) and 1% antibiotic–antimycotic solution (Gibco). After 4 days, the culture was maintained by adding a further 50 μg/ml ascorbic acid (Wako Junyaku Kougyou Corp, Kanagawa, Japan). All incubations were performed at 37°C in 5% CO_2 and 95% air. The isolated chondrocytes were seeded at passage 0 and subcultured until passage 2.

Microarray

Microarray analysis was performed using a total of 15 specimens, that is, articular cartilage from the resected fingers of patients with polydactylism ($N = 6$), excised non-loading parts of injured ACLs ($N = 3$), and knee articular cartilage from patients with knee OA ($N = 6$). RNA was isolated from the chondrocytes after they were seeded into 1×10^4 cells/cm^2 24-well plates and incubated in 5% CO_2 and 95% air. An SV Total RNA Isolation System (Promega Corp., Madison, WI) was used to perform total RNA extraction, according to the manufacturer's instructions. The quality of RNA in each sample was verified using the A260/280 absorbance ratio. Using miRNA kits (Qiagen, Tokyo, Japan), 10 μl of RNA was isolated according to the manufacturer's instructions. Intensity values of each scanned feature were quantified using Agilent feature extraction software version 10.7.3.1, which performs background subtractions. Normalization was performed using Agilent GeneSpring GX version 11.0.2. (per chip: quantile normalization; per gene: normalization to median of all samples). There are total of 939 probes on Agilent Human miRNA Microarray (Design ID: 021827) without control probes. The probes which were differentially expressed between the three conditions (PD, ACL, and TKA) were extracted.

Target Scan: Target Gene Prediction

We used the GeneSpring GX11 *TargetScan* function (Agilent) to predict the miRNA target genes. The context percentile was set at 90.0, and conserved and nonconserved databases were chosen.

Pathway Analysis

Pathway analysis of the association between the miRNA-regulated genes and the OA- and cartilage-related genes, which were detected using MetaCore, was performed using GenMAPP (http://www.genmapp.org/) pathway analysis software.

cDNA Synthesis

The incubated chondrocytes were rinsed twice in phosphate-buffered saline (PBS), placed in 700 μl of QIAzol Lysis Reagent (Qiagen, Inc., Valencia, CA), and the RNA was isolated using a miRNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocols. RNA at a concentration of 1 μg/μl that was isolated using the miScript Reverse Transcription Kit (Qiagen) was mixed with 4 μl of miScript RT Buffer, 1 μl of miScript Reverse Transcriptase Mix, and RNase-free Water. The solution was incubated for 60 min at 37°C and then 5 min at 95°C, and the cDNA was prepared.

Real-Time PCR

In the PCR used to confirm the miRNA, 25 μl of QuantiTect SYBR Green PCR Master Mix, 5 μl of miScript Universal Primer, 5 μl of miScript Primer Assay (Qiagen), a suitable quantity of RNase-free Water, and template cDNA were mixed for 15 min at 95°C, 15 s at 94°C, 30 s at 55°C, 30 s at 70°C (40 cycles), 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C (dissociation step) using a miScript Primer Assay kit (Qiagen) and a miScript SYBR Green PCR kit (Qiagen). In the calculations, the values were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the expression level was calculated using the $2^{-\Delta\Delta CT}$ values.

In the PCR used to confirm the gene expression, 12.5 μl of SYBR Green Master Mix, 0.5 μl of 20 nmol of both Primer Front and Primer Reverse, 1 μl of cDNA and 10.5 μl of distilled water were mixed for 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, 1 min at 60°C (60 cycles), 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C (dissociation step). In the calculations, the values were standardized to GAPDH, and the expression level was calculated using the $2^{-\Delta\Delta CT}$ values. Real-time PCR was used to confirm the gene expression of aggrecan ($N = 6$), ADAMTS5 ($N = 8$), type 1 collagen ($N = 9$), type 2 collagen ($N = 6$), SOX9 ($N = 7$), MMP3 ($N = 7$), and MMP13 ($N = 7$). The primer sequences used in the real-time PCR are shown in Table 1.

MTT Assay

We used samples of knee articular cartilage tissue that were harvested from a total of 12 patients with knee OA who had

Table 1. List of Primers Used in Real-Time PCR

Primer ID	Accession No.	Sequence	Expect Size (bp)
Aggrecan-F	NM_001135	TCG AGG ACA GCG AGG CC	94
Aggrecan-R		TCG AGG GTG TAG GCG TGT AGA GA	
ADAMTS5-F	NM_007038	GAG CCA AGG GCA CTG GCT ACT A	120
ADAMTS5-R		CGT CAC AGC CAG TTC TCA CAC A	
Type I collagen-F	NM_000088	AAG GGT GAG ACA GGC GAA CAA	170
Type I collagen-R		TTG CCA GGA GAA CCA GCA AGA	
Type II collagen-F	NM_033150	GGA CTT TTC TTC CCT CTC T	113
Type II collagen-R		GAC CCG AAG GGT CTT ACA GGA	
SOX9-F	NM_009428	AAC GCC GAG CTC AGC AAG A	138
SOX9-R		CCG CGG CTG GTA CTT GTA ATC	
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	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPDH-R		ATG GTG GTG AAG ACG CCA GT	

undergone TKA. We prepared a solution by dissolving DMEM High Glucose 1× (Invitrogen) at a concentration of 4 mg/ml. Subsequently, we seeded the cells into 24-well plates at a density of 4.0×10^4 cells/cm^2. Before adding the MTT solution, the DMEM medium was removed completely and the cells were washed with PBS once. To each 24-well plate, 500 μl of MTT solution was added, and the plate was incubated for 2 h at 37°C in the dark. After incubation, the MTT solution was removed, 300 μl of dimethyl sulfoxide was added to each well, 100 μl of the supernatant liquid was transferred to each well of a 96-well plate, and the absorbance was measured at 590 nm.

miRNA Overexpression

1×10^5 chondrocytes were seeded in 24-well plates along with synthetic double-stranded RNA (Syn-rno-miR-199a-3pmi miScript miRNA Mimic, Syn-hsa-miR-193bmi miScript miRNA Mimic, and Syn-hsa-miR-320c miScript miRNA Mimic), a 2 nM solution and 5 μl/well of HiPerFect Transfection Reagent (Qiagen) was added, and the cells were incubated for 6 h at 37°C in 5% CO_2 and 95% air. In the control group, we added only 5 μl/well of the HiPerFect Transfection Reagent (Qiagen) after cell seeding (Table 2).

Repression of miRNA Expression

The chondrocytes were seeded at a density of 1×10^5 cells/cm^2 into 24-well plates, and anti-rno-miR-199a-3pmi miScript miRNA inhibitor, anti-hsa-miR-193bmi miScript miRNA inhibitor, or anti-hsa-miR-320c miScript miRNA inhibitor (Qiagen) and 5 μl/well of HiPerFect Transfection Reagent (Qiagen) was added. A 2 nM solution was made, and the cells were incubated for 6 h at 37°C in 5% CO_2 and 95% air. In the control group, we added only 5 μl/well of the HiPerFect Transfection Reagent (Qiagen) after cell seeding. The chondrocytes used in the miRNA overexpression group and inhibitor group were harvested from the knee joints of 10 patients with knee OA who had undergone TKA (Table 2).

Statistical Analysis

Steel's test, a nonparametric multiple-comparison test, was used to evaluate the real-time PCR and MTT assay data. One-way ANOVA was used to evaluate microarray analysis.

RESULTS

Microarray Analysis

Microarray analysis was performed to isolate the miRNA whose expression differs between PD, ACL, and TKA, and with age. A test microarray to compare the three groups (PD, ACL, and TKA) showed a significant difference ($p < 0.05$) by analysis of variance. Isolation of the miRNAs whose expression varied consistently showed that expression was upregulated incrementally with age in two types (miR-199a-3p and miR-193b) and was downregulated incrementally with age in one type (miR-320c; Fig. 1).

Table 2. List of the RNA Sequences Used for Transfection

Name	Sequence
rno-miR-199a-3p Mimic	ACAGUAGUCUGCACAUUGGUUA
rno-miR-199a-3p Inhibitor	TGTCATCAGACGTGTAACCAAT
hsa-miR-193b Mimic	CGGGGUUUUGAGGGCGAGAUGA
hsa-miR-193b Inhibitor	TTGACCGGGAGTTTCAGGGCGA
hsa-miR-320c Mimic	AAAAGCUGGGUUGAGAGGGU
hsa-miR-320c Inhibitor	TTTTCGACCCAACTCTCCCA

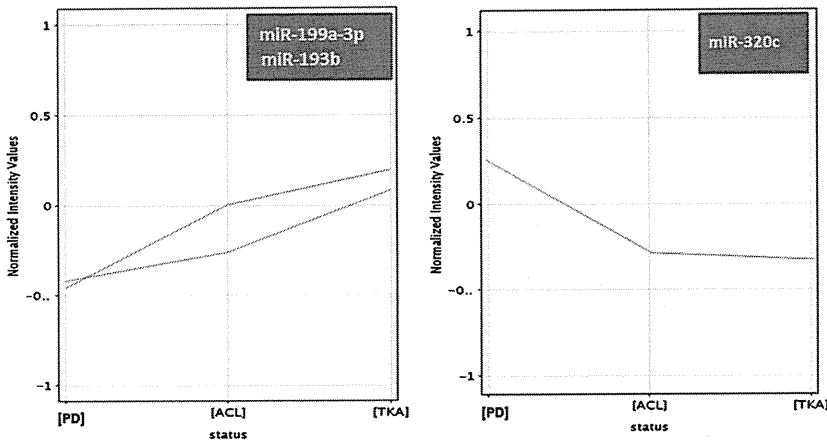

Figure 1. Result of microarray. Microarray analysis was performed to isolate the miRNA whose expression differs between PD, ACL, and TKA, and with age. MiR-199a-3p and miR-193b were incrementally upregulated, and miR-320c was incrementally downregulated with age.

Target Scan

A target scan was performed to investigate the genes regulated by the identified miRNAs: seven genes were regulated by miR-199a-3p, 10 were regulated by miR-193b, and 19 were regulated by miR-320c (Table 3).

Pathway Analysis

Analysis of differences in the pathways between the isolated miRNAs and the OA-related genes and cartilage-related genes revealed that the miRNA-regulated genes were those involved in the metabolism of cartilaginous tissue, such as type 1 collagen, MMP13, stromelysin 1(MMP3), and aggrecanase 2 (ADAMTS5; Fig. 2A,B).

Real-Time PCR

Gene expression analysis was performed using miRNAs that were isolated by microarray (miR-199a-3p, miR-193b, and miR-320c). MiR-199a-3p and miR-193b, for which expression is upregulated with age, regulated anabolic factors such as aggrecan, type 2 collagen, and SOX9. MiR-320c, in which expression is downregulated with age, regulated the catabolic factor ADAMTS5 (Table 4).

MTT Assay

The MTT assay showed no significant difference in the cell proliferation capacity between miR-199a-3p and miR-320c. By contrast, the cell proliferation capacity was upregulated significantly in miR-193b in the inhibitor group on day 7 (Fig. 3).

MiRNA Expressions

Each mimic group increased in the miRNA expression. On the other hand, each inhibited group decreased (Table 5).

DISCUSSION

MiRNAs are noncoding RNAs of ~22 base pairs that are involved in cell proliferation, differentiation, development, and tumorigenesis. miRNAs bind to the 3′-end of the untranslated region of mRNA (the target) where they induce posttranscriptional repression and mRNA degeneration, and modulate gene expression.[11–18] Since Lee et al.[19] reported the first miRNAs, several hundred have been discovered in plants and animals, and miRNAs specific to certain animal species and tissues are also known. Several types of miRNA have been discovered in fetal skeletal tissue, and it is believed that they play a key role in cartilage development.[20]

In terms of the miR-199a, which was the focus of this study, in experimental models using articular chondrocytes from mice, Lin et al.[21] reported that Col2A1, COMP, and SOX9 expression was downregulated in the miR-199a expression-enhanced group and upregulated in the miR199a expression-repressed group compared with the control group. Because the expression of miR-199a after BMP2 induction declines initially and then increases gradually for several days, it is probably essential in the final stages of cartilage development, including cartilage hypertrophy and cartilage maturity.[21] MiR-193b may also target SOX5. In miRNA expression analysis on differentiated and dedifferentiated chondrocytes, miR-193b expression is about 7.6-fold higher in dedifferentiated chondrocytes than in differentiated chondrocytes (Table 5).[22] However, there is no report on the functional analysis of miR-193b. Our study is the first report that miR-193b affects human cartilage metabolism by regulating type 2 collagen, aggrecan, and SOX9 expression. Although an association between miR-320c expression and other abnormalities such as impaired glucose tolerance[23] has been reported, no relationship with cartilage has been reported.

The miRNAs identified by microarray varied with age, and this pattern may affect articular cartilage metabolism. The results of the real-time PCR analysis of the three types of miRNA (Table 4) suggest that miR-199a-3p and miR-193b expression is upregulated with age and may be involved in chondrocyte senescence by downregulating anabolic factors such as type 2 collagen, aggrecan, and SOX9. Conversely, the results also suggest that miR-320c expression is downregulated with age and may be involved in the juvenile properties of chondrocytes by downregulating the

Table 3. Results of Target Scan Analysis

Probe name	miRNA	Gene Symbol
(A)		
A_23_P19987	hsa-miR-199a-3p	IGF2BP3
A_23_P134125	hsa-miR-199a-3p	MAP3K5
A_33_P3377130	hsa-miR-199a-3p	MAP3K5
A_33_P3389188	hsa-miR-199a-3p	TFAM
A_33_P3276475	hsa-miR-199a-3p	CHMP1B
A_23_P30995	hsa-miR-199a-3p	CYBSR4
A_23_P307392	hsa-miR-199a-3p	DPF3
A_32_P160883	hsa-miR-199a-3p	NEDD4
A_33_P3619171	hsa-miR-193b	PMAIP1
A_23_P47614	hsa-miR-193b	PHLDA2
A_33_P3357949	hsa-miR-193b	ETV1
A_32_P78491	hsa-miR-193b	ETV1
A_33_P3401156	hsa-miR-193b	ETV1
A_33_P3357954	hsa-miR-193b	ETV1
A_32_P192376	hsa-miR-193b	ENPP1
A_23_P156880	hsa-miR-193b	ENPP1
A_23_P48339	hsa-miR-193b	IFT88
A_33_P3408320	hsa-miR-193b	LASS1
A_23_P502312	hsa-miR-193b	CD97
A_32_P12610	hsa-miR-193b	E2F6
A_23_P88580	hsa-miR-193b	ARID3B
A_23_P30655	hsa-miR-193b	NFKBIE
(B)		
A_23_P329198	hsa-miR-320C	OBFC2A
A_32_P181638	hsa-miR-320C	BVES
A_23_P68031	hsa-miR-320C	STAT4
A_33_P3312182	hsa-miR-320C	C10orf47
A_23_P82990	hsa-miR-320C	OGN
A_33_P3519683	hsa-miR-320C	ZBTB8OS
A_24_P77904	hsa-miR-320C	HOXA10
A_33_P3231297	hsa-miR-320C	CREG1
A_24_P99838	hsa-miR-320C	ZNF223
A_23_P127140	hsa-miR-320C	RAB11FIP2
A_23_P219084	hsa-miR-320C	ZNF3
A_33_P3289113	hsa-miR-320C	COX11
A_23_P309865	hsa-miR-320C	ZNF449
A_23_P81650	hsa-miR-320C	C5orf15
A_33_P3304372	hsa-miR-320C	TMEM144
A_23_P97700	hsa-miR-320C	TXNIP
A_23_12896	hsa-miR-320C	FANCF
A_23_P343398	hsa-miR-320C	CCR7
A_33_P3392537	hsa-miR-320C	TK2

(A) Of the two types of miRNA whose expression is upregulated with age, miR-199a-3p regulated 7 types of genes and miR-193b regulated 10 types of genes. (B) miR-320c, whose expression is downregulated with age, regulates 19 types of genes.

expression of the catabolic factor ADAMTS5. MiR-199a-3p and miR-193b may be involved in cartilage degeneration, and we found nothing that contradicts previous reports.[21,22] ADAMTS5 is an efficient aggrecanase and induces cartilage degeneration. Although miR-320c has not been reported in relation to cartilage, the expression of ADAMTS5 was downregulated in the miR-320c mimic group and was upregulated in the inhibitor group. This suggests that miR-320c is involved in the metabolism of chondrocytes by regulating ADAMTS5.

We used the TargetScan program to investigate the genes regulated by the three identified types of miRNA. ETV1 (ER81), which is regulated by miR-193b, has also been reported as a transcription factor unique to mesenchymal stem cells (MSCs).[24] In the study by Kubo et al.,[24] ETV1 expression was upregulated by 5–10 times in MSC compared with fibroblasts, chondrocytes, synovial cells, and adipose cells. Kubo et al. also reported that cell differentiation and juvenile properties are affected when ETV1 is knocked down using short interfering RNA; for example, the self-renewal capacity of MSCs was repressed. Another study reported that the expression of STAT4, which is regulated by miR-320c, was upregulated in cells in which chondrocyte differentiation has been induced compared with a control group,[25] suggesting that STAT4 affects cartilage differentiation.

In the analysis of the pathways between the OA-related genes and cartilage-related genes detected by MetaCore and the miRNAs we identified in this study, ER81 (ETV1), which is controlled by miR-193b, produced positive feedback on the OA-related genes MMP1, stromelysin-1(MMP3), and MMP13 (Fig. 2A). Although the pathway of the E2F6 gene, which is regulated by miR-193b, is unclear, the pathway analysis suggested that this gene is involved in the feedback on aggrecanase-2(ADAMTS5) and transforming growth factor β1 (TGFβ1; Fig. 2A). NEDD4, which is regulated by miR-199a-3p, produced negative feedback on connexin 43, another OA-related gene (Fig. 2A). On the basis of the comparison of pathway analysis between genes regulated by miRNAs and cartilage-related genes, we believe that HOXA10, which is regulated by miR-320c, may regulate MMP13 through RUNX2 (Fig. 2B).

Although there is a great deal of literature on miRNAs, there are few reports on the relationship between miRNAs and the cell proliferation capacity of chondrocytes. The MTT assay performed in this study (Fig. 3) suggests that the effects on cell replication capacity did not differ between miR-199a-3p and miR-320c. Although miR-193b was upregulated significantly in the inhibitor group compared with the control group on day 7, mimic group was no significant difference. Thus these three miRNAs(miR-199a-3p, miR-193b, miR-320c) may not affect the cell replication capacity of chondrocytes.

Our findings suggest that miR-199a-3p and miR-193b may be involved in chondrocyte aging by regulating aggrecan, type 2 collagen, and SOX9, and that miRNA-320c may be involved in the juvenile properties of chondrocytes by regulating ADAMTS5. Our findings suggest that miR-199a-3p, miR-193b, and miR-320c may be useful for a marker of cartilage as donor tissue of cartilage graft and tissue culture for cell therapy (Fig. 4).

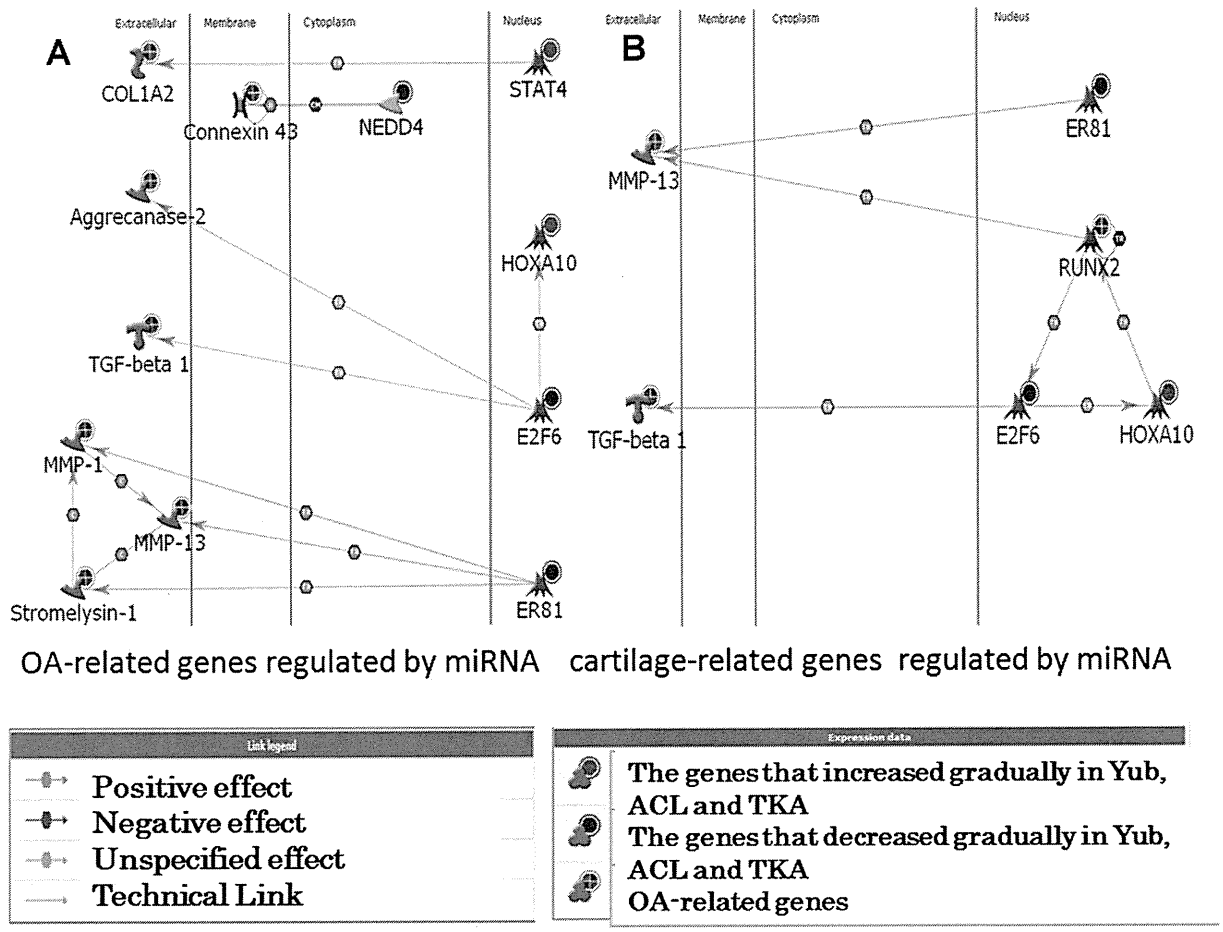

Figure 2. Pathway analysis. (A) Pathway analysis between OA-related genes and the genes regulated by miRNA. ER81 (ETV1) controlled by miR-193b produced positive feedback on MMP-1, stromelysin-1(MMP3), and MMP-13. Similarly, although the pathway of E2F6 gene, which is controlled by miR-193b, is unclear, it is involved in aggrecanase-2(ADAMTS5) and TGFβ1 feedback. NEDD4, which is controlled by miR-199a-3p, produced negative feedback on connexin 43, an OA-related gene. (B) Pathway analysis between cartilage-related genes and the genes regulated by miRNA. In the pathway analysis of genes regulated by miRNA and cartilage-related genes, HOXA10, which is regulated by miR-320c, regulates MMP13 through RUNX2.

Table 4. Relative Expression of mRNA

	miR199a-3p			miR193b			miR320c		
Gene Name	Fold Change	*p* Value	SE	Fold Change	*p* Value	SE	Fold Change	*p* Value	SE
Mimic COL1	0.5	<0.05	0.16	2.34	0.88	0.94	0.80	0.07	0.24
Inhibit COL1	0.79	<0.01	0.33	2.11	0.72	1.38	3.59	<0.01	1.56
Mimic COL2	0.5	<0.05	0.18	0.34	<0.05	0.16	0.80	0.13	0.22
Inhibit COL2	8.5	<0.01	4.21	4.07	<0.05	5.65	1.18	0.42	0.30
Mimic MMP3	1.08	0.85	0.09	1.11	0.06	0.35	0.79	<0.05	0.27
Inhibit MMP3	0.73	0.10	0.20	0.75	<0.05	0.10	1.63	0.57	0.79
Mimic MMP13	0.58	<0.05	0.15	1.03	0.34	0.33	0.47	<0.01	0.19
Inhibit MMP13	0.75	1.00	0.36	0.95	<0.01	0.29	1.35	1.00	0.28
Mimic aggrecan	0.86	<0.01	0.05	0.68	<0.01	0.09	1.59	0.07	0.31
Inhibit aggrecan	6.12	<0.05	5.66	2.66	<0.01	0.90	0.70	0.07	0.38
Mimic ADAMTS5	3.53	0.27	2.21	1.05	0.58	0.40	0.39	<0.05	0.17
Inhibit ADAMTS5	2.61	1.00	1.59	0.80	0.08	0.37	2.81	<0.05	0.70
Mimic SOX9	0.7	<0.05	0.15	0.73	<0.05	0.13	0.95	1.00	0.30
Inhibit SOX9	1.24	<0.05	0.11	2.61	<0.01	0.82	1.05	0.78	0.38

Expression of mRNA in the type 2 collagen, aggrecan, and SOX9 mimic group was downregulated by miR-199a-3p but was upregulated in the inhibitor group. Similarly, expression in the type 2 collagen, aggrecan, and SOX9 mimic group was downregulated by miR-193b but was upregulated in the inhibitor group. Expression of miR-320c was downregulated in the ADAMTS5 mimic group but upregulated in the inhibitor group.