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# Transcriptome Analysis of Mouse Stem Cells and Early Embryos

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**Understanding and harnessing cellular potency are fundamental in biology and are also critical to the future therapeutic use of stem cells. Transcriptome analysis of these pluripotent cells is a first step towards such goals. Starting with sources that include oocytes, blastocysts, and embryonic and adult stem cells, we obtained 249,200 high-quality EST sequences and clustered them with public sequences to produce an index of approximately 30,000 total mouse genes that includes 977 previously unidentified genes. Analysis of gene expression levels by EST frequency identifies genes that characterize preimplantation embryos, embryonic stem cells, and adult stem cells, thus providing potential markers as well as clues to the functional features of these cells. Principal component analysis identified a set of 88 genes whose average expression levels decrease from oocytes to blastocysts, stem cells, postimplantation embryos, and finally to newborn tissues. This can be a first step towards a possible definition of a molecular scale of cellular potency. The sequences and cDNA clones recovered in this work provide a comprehensive resource for genes functioning in early mouse embryos and stem cells. The nonrestricted community access to the resource can accelerate a wide range of research, particularly in reproductive and regenerative medicine.**

## Introduction

With the derivation of pluripotent human embryonic stem (ES) (Thomson et al. 1998) and embryonic germ (EG) (Shamblott et al. 1998) cells that can differentiate into many different cell types, excitement has increased for the prospect of replacing dysfunctional or failing cells and organs. Very little is known, however, about critical molecular mechanisms that can harness or manipulate the potential of cells to foster therapeutic applications targeted to specific tissues.

A related fundamental problem is the molecular definition of developmental potential. Traditionally, potential has been operationally defined as "the total of all fates of a cell or tissue region which can be achieved by any environmental manipulation" (Slack 1991). Developmental potential has thus been likened to potential energy, represented by Waddington's epigenetic landscape (Waddington 1957), as development naturally progresses from "totipotent" fertilized eggs with unlimited differentiation potential to terminally differentiated cells, analogous to a ball moving from high to low points on a slope. Converting differentiated cells to pluripotent cells, a key problem for the future of any stem cell-based therapy, would thus be an "up-hill battle," opposite the usual direction of cell differentiation. The only current way to do this is by nuclear transplantation into enucleated oocytes, but the success rate gradually decreases according to developmental stages of donor cells, providing yet another operational definition of developmental potential (Hochedlinger and Jaenisch 2002; Yanagimachi 2002).

What molecular determinants underlie or accompany the potential of cells? Can the differential activities of genes provide the distinction between totipotent cells, pluripotent cells, and terminally differentiated cells? Systematic genomic methodologies (Ko 2001) provide a powerful approach to these questions. One of these methods, cDNA microarray/chip technology, is providing useful information (Ivanova et al. 2002; Ramalho-Santos et al. 2002; Tanaka et al. 2002),

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Abbreviations: ATCC, American Type Culture Collection; 2D, two dimensional; 3D, three dimensional; EG, embryonic germ (cell); ES, embryonic stem (cell); EST, expressed sequence tag; FDR, false discovery rate; GAP-DH, glyceraldegyde-3-phosphate dehydrogenase; HS, hematopoietic stem/progenitor (cell); LIF, leukemia inhibitory factor; MS, mesenchymal stem (cell); NCBI, National Center for Biotechnology Information; NIA, National Institute on Aging; NS, neural stem/progenitor (cell); ORF, open reading frame; PC1, first principal component; PCA, principal component analysis; PGC, primordial germ cell; TS, trophoblast stem (cell); VRML, virtual reality modeling language

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although analyses have been restricted to a limited number of genes and cell types. To obtain a broader understanding of these problems, it is important to analyze all transcripts/genes in a wide selection of cell types, including totipotent fertilized eggs, pluripotent embryonic cells, a variety of ES and adult stem cells, and terminally differentiated cells. Despite the collection of a large number of expressed sequence tags (ESTs) (Adams et al. 1991; Marra et al. 1999) and full-insert cDNA sequences (Okazaki et al. 2002), systematic collection of ESTs on these hard-to-obtain cells and tissues has been done previously only on a limited scale (Sasaki et al. 1998; Ko et al. 2000; Solter et al. 2002).

Accordingly, we have attempted to (i) complement other public collections of mouse gene catalogs and cDNA clones by obtaining and indexing the transcriptome of mouse early embryos and stem cells and (ii) search for molecular differences among these cell types and infer features of the nature of developmental potential by analyzing their repertoire and frequency of ESTs. Here we report the collection of approximately 250,000 ESTs, enriched for long-insert cDNAs, and signature genes associated with the potential of cells, various types of stem cells, and preimplantation embryos.

## Results and Discussion

### Novel Genes Derived from Early Mouse Embryos and Stem Cells

Twenty-one long-insert-enriched cDNA libraries with insert ranges from 2–8 kb (Piao et al. 2001) were generated from preimplantation embryos (unfertilized egg, fertilized egg, two-cell embryo, four-cell embryo, eight-cell embryo, morula, and blastocyst), ES cells (Anisimov et al. 2002) and EG cells (Matsui et al. 1992), trophoblast stem (TS) cells (Tanaka et al. 1998), adult stem cells (e.g., neural stem/progenitor [NS] cells) (Galli et al. 2002), mesenchymal stem (MS) cells (Makino et al. 1999), osteoblasts (Ochi et al. 2003), and hematopoietic stem/progenitor (HS) cells (Ortiz et al. 1999), their differentiated cells, and newborn organs (e.g., brain and heart) (see Protocol S1 and Dataset S1 for methods, full list of libraries, and references). In total, 249,200 ESTs (170,059 cDNA clones: 114,437 5′ ESTs and 134,763 3′ ESTs) were generated and assembled together with public data into a gene index (see Materials and Methods; Protocol S1).

Of 29,810 mouse genes identified in our gene index (Figure 1; Dataset S2; Dataset S3), 977 were not present as either known or predicted transcripts in other major transcriptome databases, such as RefSeq (Pruitt and Maglott 2001), Ensembl (Hubbard et al. 2002), and RIKEN (Okazaki et al. 2002) (see Dataset S3 for details and Dataset S4 for sequences). These genes represent possible novel mouse genes, as they either encode open reading frames (ORFs) greater than 100 amino acids or have multiple exons. In particular, 554 of the 977 genes remained novel with high confidence even after more thorough searches against GenBank and other databases. Comparisons of these 977 genes against all National Center for Biotechnology Information (NCBI) UniGene representative sequences showed that 377 genes did not match even fragmentary ESTs and are therefore unique to the National Institute on Aging (NIA) cDNA collection (see Dataset S3). A random subset of 19 cDNA clones representing these genes was sequenced completely to confirm their novelty (Figure 2). Protein domain searches using InterPro (Mulder et al. 2003)

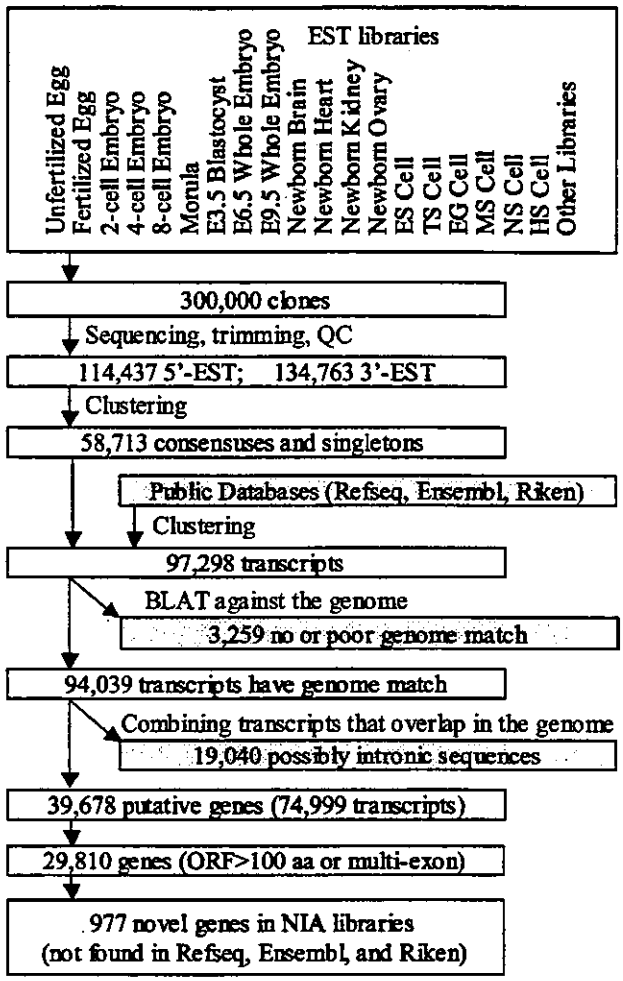


**Figure 1.** Flow Chart of Sequence Data Analysis

Using TIGR gene indices clustering tools (Pertea et al. 2003), 249,200 ESTs were clustered, generating 58,713 consensuses and singletons. NIA consensuses and singletons were further clustered with Ensembl transcripts (Hubbard et al. 2002), RIKEN transcripts (Okazaki et al. 2002), and RefSeq transcripts and transcript predictions (Pruitt and Maglott 2001). Alignments of these sequences to the mouse genome (UCSC February 2002 freeze data, available from ftp://genome.cse.ucsc.edu/goldenPath/mmFeb2002) (Waterston et al. 2002) using BLAT (Kent 2002) helped to avoid false clustering of similar sequences at nonmatching genome locations. Erroneous clusters were reassembled based on the analysis of genome alignment. A total 94,039 putative transcripts were thus generated and then grouped into 39,678 putative genes based on their overlap in the genome on the same chromosome strand and on clone-linking information. Using criteria of an ORF greater than 100 amino acids or of multiple exons (excluding sequences that are potentially located in a wrong strand), 29,810 mouse genes were identified. Finally, 977 genes unique to the NIA database were identified.

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revealed that one of them, *U004160*, is an orthologue of human gene Midasin (*MDN1*), but the remaining 18 genes do not encode any known protein motifs. However, they were split into multiple exons in the alignment to the mouse genome sequences, and we therefore considered them genes. As these sequences are mainly derived from early embryos and stem cells, they most likely represent new candidates for genes specific to particular types of stem cells. RT-PCR analysis revealed that they are expressed in specific cell types

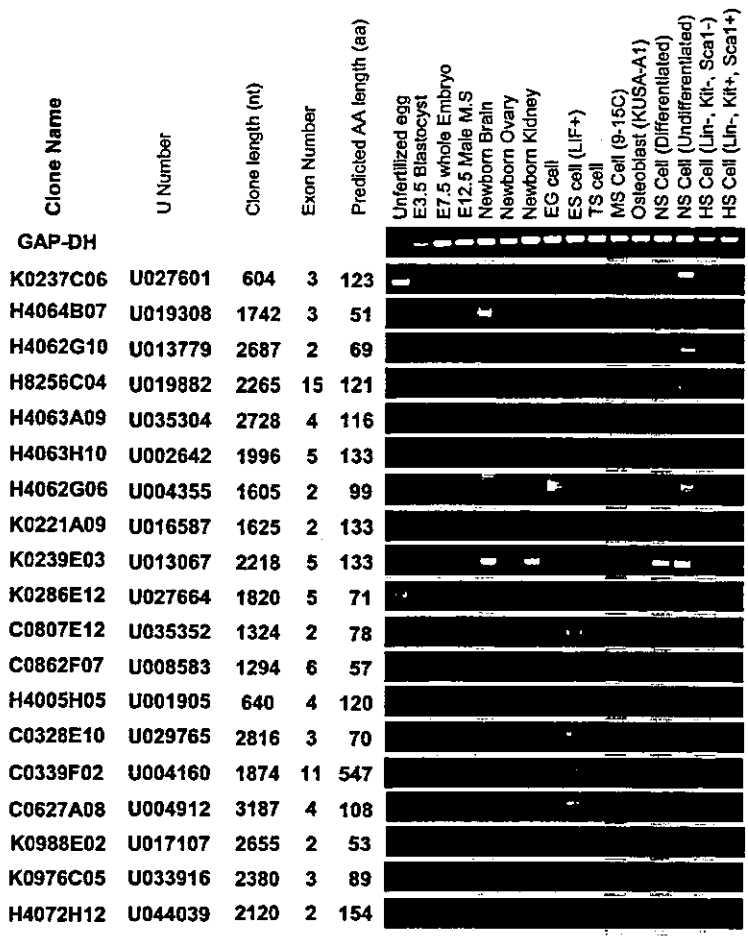


**Figure 2.** Examples of NIA-Only cDNA Clones and RT–PCR Results

Expression pattern of 19 novel cDNA clones in 16 different cell lines or tissues: unfertilized egg, E3.5 blastocyst, E7.5 whole embryo (embryo plus placenta), E12.5 male mesonephros (gonad plus mesonephros), newborn brain, newborn ovary, newborn kidney, embryonic germ (EG) cell, embryonic stem (ES) cell (maintained as undifferentiated in the presence of LIF), trophoblast stem (TS) cell, mesenchymal stem (MS) cell, osteoblast, neural stem/progenitor (NS) cell, NS differentiated (differentiated neural stem/progenitor cells), and hematopoietic stem/progenitor (HS) cells. Glyceraldegyde-3-phosphate dehydrogenase (GAP-DH) was used as a control. A U number is assigned to each gene in the gene index (see Dataset S2). The exon number was predicted from alignment with the mouse genome sequence, and the amino acid sequence was predicted with the ORF finder from NCBI.

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(Figure 2; Dataset S5). For example, the expression of gene *U035352* was unique to ES cells, expression of *U004912* unique to ES and TS cells, and expression of *U001905* unique to ES and EG cells. In addition, one gene showed apparent specific expression in several stem cells and is thus a potential pan-stem cell marker (*U029765*). Taken together, these data suggest that most of the putative genes represented only in the NIA cDNA collection are bona fide genes that have not been previously identified.

## Signature Genes That Characterize Preimplantation Embryos and Stem Cells

To identify genes that were consistently overrepresented in a given set of cDNA libraries when compared with other libraries, we performed the correlation analysis of log-transformed EST frequency combined with the false discovery rate (FDR) method (Benjamini and Hochberg 1995) (FDR = 0.1) (Figure 3; Dataset S6; Dataset S7).

First, we analyzed various combinations of preimplantation stages and identified the following genes: (i) 196 genes specific to unfertilized eggs (oocytes) and fertilized eggs (Group A in Figure 3), (ii) 122 genes specific to two- to four-cell embryos (Group B in Figure 3), (iii) 119 genes specific to eight-cell embryos, morula, and blastocyst (Group C in Figure 3), (iv) 81 genes specific to all preimplantation embryos (Group D in Figure 3), and (v) 143 genes specific to all preimplantation embryos except for blastocysts (Group E in Figure 3) (see also Dataset S7). Blastocyst EST frequencies are unique even among preimplantation embryos, most likely reflecting the switch of the transcriptome from the maternal genetic program to the zygotic genetic program (Latham and Schultz 2001; Solter et al. 2002) or to the differentiation of the trophectoderm. At least 35 out of 196 genes in the egg signature gene list (Group A in Figure 3) have ATP-related protein domains. Genes in the following categories were also enriched in this gene list: the ubiquitin–proteasome pathway, the energy pathway, cell signaling (kinase and membrane) proteins, ribosomal proteins, and zinc finger proteins. Two *SWI/SNF*-related genes (*5930405J04Rik*, the homologue of human *SMARCC2*, and *Smarcf1*) and two *Polycomb* genes (*Scmh1* and *Sfmbt*) overrepresented in eggs may be candidate genes for strong chromatin remodeling activity of eggs during nuclear transplantation of somatic cell nuclei.

Addition of ES and EG cells to preimplantation embryos (143 genes; Group E in Figure 3) yielded only 54 signature genes (Group F in Figure 3). Addition of adult stem cells, MS and NS, or MS, NS, and HS ($Lin^-$, $Kit^+$, $Sca1^+$ and $Lin^-$, $Kit^-$, $Sca1^+$) cells further reduced the number of signature genes to five and one, in Groups G and H, respectively (Dataset S7). Taken together, these results seem to indicate that preim-

| Group | Unfertilized Egg | Fertilized Egg | 2-cell Embryo | 4-cell | 8-cell | Morula | E3.5 Blastocyst | E6.5 Whole Embryo | E7.5 Whole Embryo | E7.5 Embryonic Part | E7.5 Extraembryonic Part | E8.5 Whole embryo | E9.5 Whole Embryo | E12.5 Male Gonad/Mesonephros | E12.5 Female Gonad/Mesonephros | E13.5 VMB Dopamine Cell | Newborn Brain | Newborn Heart | Newborn Kidney | Newborn Ovary | ES Cell (LIF+) | ES Cell (LIF-) | TS Cell | EG Cell | MS Cell (9-15C) | Osteoblast (KUSA-A1) | NS Cell (Undifferentiated) | NS Cell (Differentiated) | HS Cell (Lin-, Kit+, Sca1+) | HS Cell (Lin-, Kit+, Sca1-) | HS Cell (Lin-, Kit-, Sca1+) | HS Cell (Lin-, Kit-, Sca1-) | gene number | Gene symbols |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 196 | *Akap1; Alox12e; Apg5l; Arl6; Arl6ip2; Banp; Bcl2l10; Birc2; Bmp15; Bpgm; Btg4; Bub1b; Ccnb1rs1; Cdc25a; Cdc45l; Cryl1; Cyp11a; Dtx2; Eed; Epb4.1l2; Fbxw4; Fmn2; Folr4; Gdf9; Gtr2; H1fo; Hsd3b1; Ing1; Irf1; Itga9; Kcnh1; Kdel1; Krt2_16; Map2k6; Map4k5; Mapkbp1; Mater; Mdm4; Mitc1; Mmp23; Mrg1; Npc1; Oas1d; Oas1e; Obox3; Orc5l; Orc6l; P37nb; Pabpc4; Pcsk6; Phf1; Plat; Pld1; Pole3; Prkab1; Rbbp7; Rdx; Rfpl4; Rgs2; Rnf35; Rnpc1; Sh3d5; Sip1; Slc21a11; Smarcf1; Snrpb2; Tbn; Tcl1; Tcl1b1; Tcl1b3; Tes3; Tex14; Thrsp; Top1; Wbscr21; Xbp1; Zfp296; and 119 unknown genes.* |
| D | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 81 | *Akp5; Bcl2l10; Bmp15; Bpgm; Btg4; Cdkap1; Ctsc; Dusp14; Fbxo15; Fxyd4; Gdf9; Hspa8; Klf5; Obox3; Ovgp1; Prkab1; Rfpl4; Rnf35; Rnpc1; Spin; Tcl1; Tcl1b3; Timd2; Ulk1; and 57 unknown genes.* |
| E | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 143 | *Akp5; Arpc1b; Bcl2l10; Birc2; Bmp15; Bpgm; Btg4; Cd160; Cdkap1; Cry1; Daf1; Degs; Dusp14; E2f1; Fbxo15; Fkbp5; Fkbp6; Fmn2; Folr4; Fxyd4; Gdf9; Gja4; Gstm2; H1fo; Hspa8; Krt2_16; Lcn2; Magoh; Mater; Mbtd1; Mdm4; Mil1; Oas1d; Oas1e; Obox3; Ovgp1; P37nb; Prkab1; Rfpl4; Rgs2; Rnf33; Rnf35; Rnpc1; Rps8; Sat; Slc34a2; Spin; Tcl1; Tcl1b1; Tcl1b3; Timd2; Ybx3; and 91 unknown genes.* |
| F | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | - | - | - | - | 54 | *Bcl2l10; Bmp15; Btg4; Cdkap1; Degs; Dusp14; Fxyd4; Gdf9; Mitc1; Obox3; Ovgp1; Prkab1; Rfpl4; Rnf35; Rnpc1; Spin; Tcl1; Tcl1b3; Timd2; and 35 unknown genes.* |
| I | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | + | - | + | - | - | - | - | - | 140 | *Akap10; Akap12; Amt; Ash2l; Atm; Birc6; Cask; Cbx5; Cdh11; CHD6; Crkol; Dnchc1; Ect2; Edr1; Enah; ENPP3; Fshprh1; Gabrg1; Galnt1; GPCR; Hells; Hmgcr; Hmmr; Impact; Kars_ps1; Kif10; Kif15; Knsl1; Lamc1; Lbr; Lox; Mad5; Mki67; Np95; Oazi; Odf2; Opa1; Pald; Plrg1; Pola1; Ppp4r1; Ptch; Ptch2; Rasa2; Rb1; Rex2; Rpl31; Sh3bp3; Shcbp1; Ski; Slc27a1; Sms; Spna2; Synj2; Tnc; TRB_2; Trif; Trps1; Zfp148; Zfp191; and 80 unknown genes.* |
| J | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | - | - | - | - | 93 | *Abce1; Akap10; Ccnf; Cdh11; Col12a1; Crkol; Dda3; Dmd; Enh; Fanca; GPCR; Hmmr; Img; Lrig1; Iqgap1; Kars_ps1; Kif1b; Lox; Morf; Nedd4; Opa1; Pald; Pola1; Ppp4r1; Ptprf; Rpl31; Sec23a; Sh3bp3; Slc27a1; Slc7a3; Snrp116; Tardbp; Thbs1; TRB-2; Trps1; Zfhx1a; Zfhx1b; Zfp191; and 55 unknown genes.* |
| K | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - | - | - | 75 | *Abhd2; Ccnf; Cldn4; Dda3; Dnmt3b; ENPP3; F11r; Fkbp4; Foxh1; Grb7; Grcc8; Helb; Hmga1; Jmj; Jub; Lsm10; Map4k1; Mif; Morf; Mta3; Mybl2; Ncl; Nek4; Nfyb; Nol5; Pabpn1; Pcnxl3; Pdcd4; Ppp4r1; Prkar2a; Prss8; Rbbp6; Rfng; Rpl13; Rps2; Rps6ka1; Slc29a1; Slc7a3; Sntb2; Sox13; Tdh; Tsbp; Ubb; Unc13h1; Wee1; Xrcc2; Zfp42; and 28 unknown genes.* |
| L | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - | - | 39 | *Akap10; Cldn4; Dnmt3b; ENPP3; Foxh1; Galk1; Grb7; Kars-ps1; Lcn7; Nfyb; Ngfrap1; Pcbp1; Rgds; Ubb; Unc13h1; Wee1; Xrcc2; Zfp278; Zfp42; and 20 unknown genes.* |
| M | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + | - | + | + | - | - | 44 | *Atf2; Cbl; Ccne2; Cd44; Elf1; Fshprh1; Fyn; G7e; Herc3; IFI 203; Itga4; Jak1; MALT-1; Mbnl; Nab1; Nfat5; Phc3; SENP6; Sbxbp4; Tde1l; Tex2; Tm6sf1; Wwp4; and 34 unknown genes.* |
| N | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | + | - | - | - | - | - | - | - | - | 108 | *Abce1; Abhd2; Akap10; Arl6ip2; Ccnf; Col12a1; Dda3; Dstn; Edr1; Enah; ENPP3; Fkbp4; Foxh1; Gfpt2; Helb; Impact; Ing5; Jmj; Jub; Mad5; Map4k1; Mkm1; Morf; Mov10; Mta3; Mtf2; Mybl2; Pask; Pcnxl3; Pola1; Pola2; Ppp2r5e; Ppp4r1; Ptch2; Ranbp17; Rbbp6; Rest; Rex2; Rw1; Slc29a1; Slc7a3; Smarca4; Sntb2; Sox13; Tacc3; Tcof1; Tdgf1; Tdh; Zfp42; and 59 unknown genes.* |
| O | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | - | - | - | - | 113 | *Akap10; Akap12; Ash2l; Atm; Cipp; Col18a1; Dda3; Ect2; Edr1; Enah; ENPP3; Ermelin; Etl1; Gab1; Gfpt2; Hic2; Hmmr; Impact; Ing5; Itga3; Jmj; Lamc1; Lyar; Mad5; Mkm1; Mtf2; Mybl2; Ncoa3; Np95; Opa1; Pald; Pola1; Ppp2r5e; Ptch2; Ranbp17; Rex2; Rnf17; Rw1; Slc29a1; Slc7a3; Smcx; Sms; Taf7; Tdh; Tex20; Trif; Wee1; Zfp110; and 65 unknown genes.* |

**Figure 3.** Signature Genes for Specific Groups of Early Embryos and Stem Cells
DOI: 10.1371/journal/pbio.0000074.g003

plantation embryos, particularly totipotent fertilized eggs and highly pluripotent cells (ES and EG cells), have quite distinct genetic programs, but that less pluripotent adult stem cells (MS, NS, and HS) have even more specialized genetic programs. This supports the notion of a gradual decrease of developmental potential from preimplantation embryos to stem cells to differentiated cells.

Additional analysis was done to determine genes that are enriched in stem cells, but not in preimplantation embryos and other tissues (see Figure 3; Dataset S6; Dataset S7). In this

analysis, 140 genes were identified as signature genes for pluripotent stem cells (ES, EG, NS, and MS in Group I in Figure 3), whereas 93 genes were identified as signature genes for these stem cells and their differentiated forms (cultured cells in Group J in Figure 3). Similarly, 75 and 39 genes, respectively, were identified as ES- and TS-specific (Group K in Figure 3), whereas 44 genes were identified as signature genes for adult stem cells (NS, MS, and HS in Group M in Figure 3). Lists of these genes showed that distinctive sets of genes are responsible for cell specificity (Figure 3).

FDR analysis revealed that 113 genes were specifically expressed in ES and EG cells in Group O (the most pluripotent stem cells), but not in all other cell types examined (Figure 3; Dataset S7). The most abundant group of these genes was transcription regulatory factors (about 30% of all specific genes), most of which were members of the zinc finger family, including *Mtf2*, *Ing5*, *Mkrn1*, *Hic2*, and the KRAB box zinc finger. Other abundant genes specifically expressed in ES and EG cells included matrix/cytoskeleton/membrane structural proteins such as *Itga3*, *Dstn*, *Smtn*, *Dctn1*, and *Col18a1* and the DNA remodeling proteins such as *Rcc1*, *Kars-ps1*, *Pola2*, *Mov10*, and *Rad54l*. These two groups of genes may be associated with the unique feature of ES/EG cell cycle structure, where greater than 70% of the cell population are in S phase (Savatier et al. 1996).

Previous studies have identified genes specific to particular stem cells or genes common to a group of stem cells, although there was little agreement about which transcripts are commonly enriched in these studies (e.g., Anisimov et al. 2002; Ivanova et al. 2002; Ramalho-Santos et al. 2002; Tanaka et al. 2002). The difference in the method and platform used could be a major reason for the difficulty in identifying a common gene set. The analysis of limited number of cell types could also contribute to differences in the resulting gene lists, because genes that appeared specific to certain cell types may also be expressed in other cells that were not included in the analysis. In contrast, the current study has analyzed a large number of different stem cells, preimplantation embryos, and newborn organs from our own EST collections as well as all publicly available ESTs that were derived from a few hundred cell types. Combined with stringent FDR statistics (see Materials and Methods), the analysis of this large number of cell types may provide broader perspectives on this issue. Comparison between the gene lists of the present study and the gene lists from the previously published studies identified areas of agreement (common genes), but also revealed that many genes previously reported as specifically expressed in one cell type or group of cells are actually expressed in other cell types and thus are not specific (see the details in Dataset S8). The signature genes identified in this study distinguish different stem cells, and this gene list may provide a way to recognize or purify specific stem cell types and provide insights into stem cell–specific functions.

## Principal Component Analysis Identified Clusters of Cells/Tissues with Similar EST Frequency

The global expression patterns of 2,812 relatively abundant genes (see Materials and Methods; Dataset S9) were further analyzed by principal component analysis (PCA), which reduces high-dimensionality data into a limited number of principal components. The first principal component (PC1) captures the largest contributing factor of variation, which in this case corresponds to the average EST frequency in all tissues, and subsequent principal components correspond to other factors with smaller effects, which characterize the differential expression of genes. As we were interested in the differential gene expression component, we plotted the position of each cell type against the PC2, PC3, and PC4 axis in three-dimensional (3D) space by using virtual reality modeling language (VRML) (Figure 4A; Video S1; a full interactive view is available on http://lgsun.grc.nia.nih.gov/Supplemental-Information). Genes were also plotted in the same 3D space (a version of PCA called a biplot) (Chapman et al. 2002) to see their association with cell/tissue types. Close examination of the 3D model identified PC2 and PC3 as the most representative views of the 3D model (Figure 4B). A two-dimensional (2D) plot of PC2 and PC3 is therefore used for the following discussion, with references to the 3D model. It is important to keep in mind that the distance between cell types along principal components has a substantial error associated with randomness of clone counts in EST libraries. The estimated error range (2*SE) in the PC3 scale is about 7%–9% based on Poisson distribution (Figure 4B). Nonetheless, PCA identifies major trends and clusters in gene expression among these cell types.

The most conspicuous trend was that cells that differ in their developmental potential appeared well separated along the PC3 axis. In Figure 4A and 4B, preimplantation embryos (unfertilized egg, fertilized egg, two-cell, four-cell, eight-cell, morula, and blastocyst) are positioned at the top of the PC3 axis; embryos and extraembryonic tissues from early- to mid-gestation stage, such as E6.5, E7.5, E8.5, and E9.5, are positioned at the middle; and cells and tissues mostly from terminally differentiated cells (newborn ovary, newborn heart, and newborn brain) are positioned at the bottom. PCA is unsupervised (performed without using knowledge of developmental stages of each cell types), and so this ordering along the PC3 axis seems to reflect the structures of global gene expression patterns among the cells. The PC2 axis provided an additional dimension to separate cells into developmental stages, functional groups, or both. The correlation of the PC2 axis to known biological stages, functions, or both, however, remains unclear.

Interestingly, both ES cells and adult stem cells are positioned at the middle of the PC3 axis together with whole-embryo libraries from early- to mid-gestation stages (Figure 4B). ES and EG cells were derived from embryos, and thus their positions matched with their developmental timing. Although NS, MS, and HS cells were all derived from adult organs (brain, bone marrow, and bone marrow, respectively), their position along the PC3 axis corresponded to early embryonic tissues and embryo-derived stem cells (ES and EG). The results are consistent with the notion that adult stem cells acquire or retain the pluripotency with characters of less-differentiated cell types. This also suggests that the PC3 axis does not represent just developmental timing, but also indicates the developmental potential of cells, with totipotent eggs at the top, pluripotent embryonic cells and stem cells at the middle, and terminally differentiated cells at the bottom.

This hypothesis seems to be consistent with another interesting observation that the differentiated forms of stem cells were always positioned lower than their stem cell

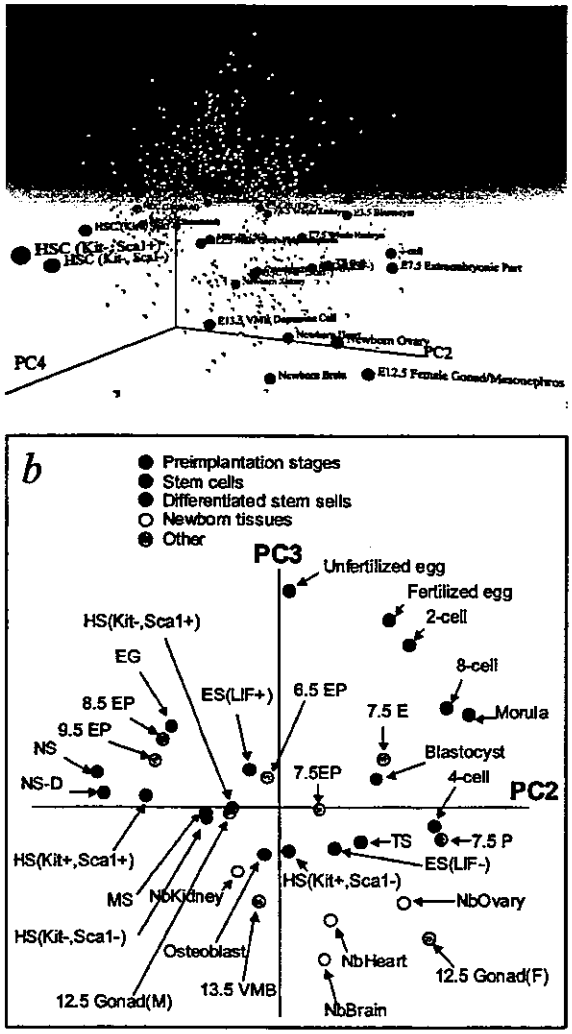


**Figure 4.** PCA Analysis of EST Frequency

The results were obtained by analyzing 2,812 genes that exceeded 0.1% in at least one library. (A) 3D biplot that shows both cell types (red spheres) and genes (yellow boxes). (B) 2D PCA of cell types. EST frequencies were log-transformed before the analysis. Names of some cells and tissues are abbreviated as follows: 6.5 EP, E6.5 whole embryo (embryo plus placenta); 7.5 EP, E7.5 whole embryo (embryo plus placenta); 8.5 EP, E8.5 whole embryo (embryo plus placenta); 9.5 EP, E9.5 whole embryo (embryo plus placenta); 7.5 E, E7.5 embryonic part only; 7.5 P, E7.5 extraembryonic part only; NbOvary, newborn ovary; NbBrain, newborn brain; NbHeart, newborn heart; NbKidney, newborn kidney; 13.5 VMB, E13.5 ventral midbrain dopamine cells; 12.5 Gonad (F), E12.5 female gonad/mesonephros; 12.5 Gonad (M), E12.5 male gonad/mesonephros; HS ($Kit^-$, $Sca1^-$), hematopoietic stem/progenitor cells ($Lin^-$, $Kit^-$, $Sca1^-$); HS ($Kit^-$, $Sca1^+$), hematopoietic stem/progenitor cells ($Lin^-$, $Kit^-$, $Sca1^+$); HS ($Kit^+$, $Sca1^-$), hematopoietic stem/progenitor cells ($Lin^-$, $Kit^+$, $Sca1^-$); HS ($Kit^+$, $Sca1^+$), hematopoietic stem/progenitor cells ($Lin^-$, $Kit^+$, $Sca1^+$); and NS-D, differentiated NS cells.

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counterparts (undifferentiated forms) in the PC3 axis (Figure 4A and 4B). For example, the position of NS (differentiated) cells, a mixture of neuron and glia obtained after culturing NS cells in the differentiation conditions, was lower and nearer to the terminally differentiated cells than were NS cells. Osteoblast cells, which are more differentiated than the MS cells from which they are derived, were again positioned lower than the MS cells. The same holds true for ES ($LIF^-$) cells (lower PC3 position), which were obtained by culturing ES cells in the absence of leukemia inhibitory factor (LIF), allowing ES cells to differentiate into many different cell types, and ES ($LIF^+$) cells (higher PC3 position), which were maintained as highly pluripotent by culturing them in the presence of LIF. For HS cells, all four cell types were selected first as lineage marker-negative cells, and thus they were all relatively undifferentiated cells. These cells were then sorted by c-$Kit^+$ and $Sca1^+$ into four separate fractions. The most pluripotent cells ($Lin^-$, c-$Kit^+$, $Sca1^+$) were again positioned higher than other three cell types in the PC3 axis. Finally, TS cells were positioned at the least-potent place among stem cells, which seemed to fit to their known characteristics. It has previously been shown that TS cells are already committed to the extraembryonic lineage and are less pluripotent than ES and EG cells, because TS cells injected back to mouse blastocysts only differentiate into extraembryonic trophoblast lineages (Tanaka et al. 1998). The microarray analysis of TS cells also shows that they already express many placenta-specific genes, which is a sign of lineage-committed cells (Tanaka et al. 2002).

Finally, it is interesting to note that EG cells were positioned closely to E8.5 whole embryos and E9.5 whole embryos, whereas ES cells were positioned closely to blastocysts, E6.5, and E7.5 whole embryos (Figure 4). Because ES cells are derived from E3.5 blastocysts and EG cells are derived from primordial germ cells (PGCs) of E8.5 (in this particular line), these results indicate that the expression patterns of relatively abundant genes in ES and EG cells reflect their developmental stages of origin. Although ES and EG cells were established from different sources, EG cells are often considered to be ES cells and the distinction of their origin is ignored. However, the result here suggests potentially significant differences between the genetic programs of EG cells and ES cells.

## Genes Correlated with the Developmental Potential of Cells

To identify a group of genes associated with the PC3 axis, we first fixed the coordinate of each cell type on PC3 and searched for genes whose log-transformed frequencies correlated with this coordinate in each cell type. Correlation analysis combined with the FDR method (FDR = 0.1) revealed 88 genes whose expression levels were significantly associated with PC3 (Dataset S10). To test how well these genes represent PC3, we plotted the sum of log-transformed EST frequencies for these 88 genes versus PC3 projections of the same cell types (Figure 5). Most cells were positioned diagonally relative to the original PC3 coordinates, indicating that the average expression levels of these 88 genes can roughly represent cell type position along the PC3 coordinate. Because the PC3 axis does not have a unit and cannot be directly translated to variables measured by molecular biological techniques, the possible use of 88 genes as a surrogate for the PC3 axis will help to test this working hypothesis in the future.

What are the characteristics of these 88 potential correlating genes? Based on the available protein domain information, Gene Ontology (GO) annotation (Ashburner et al. 2000; http://www.geneontology.org/doc/GO.annotation.html), and literature, 58 genes can be classified into putative functional categories (Dataset S10). For example, signature genes in the

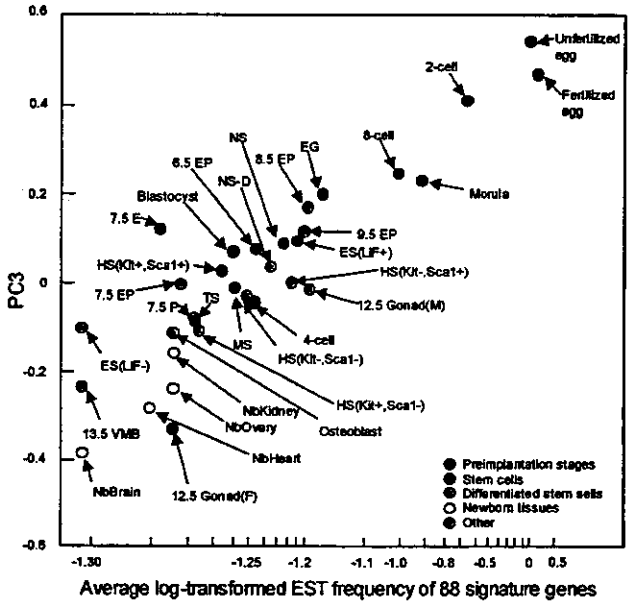


**Figure 5.** Relationship between PC3 and Average Expression Levels of 88 Signature Genes

A list of 88 genes associated with developmental potential: *Birc2, Bmp15, Btg4, Cdc25a, Cyp11a, Dtx2, E2f1, Fmn2, Folr4, Gdf9, Krt2–16, Mitcl, Oas1d, Oas1e, Obox3, Prkab1, Rfpl4, Rgs2, Rnf35, Rnpc1, Slc21a11, Spin, Tcl1, Tcl1b1, Tcl1b3, 1810015H18Rik, 2210021E03Rik, 2410003C07Rik, 2610005B21Rik, 2610005H11Rik, 3230401D17Rik, 4833422F24Rik, 4921528E07Rik, 4933428G09Rik, 5730419I09Rik, A030007L17Rik, A930014I12Rik, E130301L11Rik, AA617276, Bcl2l10, MGC32471, MGC38133, MGC38960, D7Ertd784e,* and 44 genes with only NIA U numbers (see Dataset S10).

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"transcriptional control" category include eight genes, such as MAD homologue 4 interacting transcription coactivator 1 (*Mitc1*), *Drosophila* Deltex 2 homologue (*Dtx2*), and oocyte-specific homeobox 5 (*Obox5*); the "RNA binding" category includes five genes such as RNA-binding region containing I (*Rnpc1*) and 2′-5′-oligoadenylate synthetase 1D (*Oas1d*); the "signal transduction" category includes ten genes, such as AMP-activated protein kinase (*Prkab1*) and regulator of G-protein signaling 2 (*Rgs2*); and the "proteolysis" category includes six genes, such as Ret finger protein-like 4 (*Rfpl4*) and ring finger protein 35 (*Rnf35*). These categories were diverse, and the domination of any specific categories was not observed.

Although all 88 genes shared the general trend of continuous decrease of expression levels from eggs to terminally differentiated tissues, these genes can be further subdivided by their expression patterns. First, 53 genes were those identified as preimplantation specific, particularly unfertilized and fertilized egg-specific genes, which include already well-known genes for their functions in oogenesis and zygotic gene activation, such as *Gdf9, Bmp15, Rfpl4, Fmn2, Tcl1, Obox5,* and *Oosp1*. Second, ten genes were represented as ESTs in both preimplantation embryos and postimplantation embryos, including *Cyp11a* and *D7Ertd784e*. Third, 25 genes were represented well as ESTs in preimplantation embryos, postimplantation embryos, and stem cells, including *Mitc1*, actin-binding Kelch family protein, *Dtx2, Cdc25a, Spin, Rgs2, Prkab1,* and *Birc2*. The seemingly continuous decrease of the expression of these genes is therefore not caused by passive dilution of transcripts that are abundant in oocytes, but is most likely caused by a specific mechanism that actively regulates the expression levels of these genes.

## Concluding Remarks

The sequence information and cDNA clones collected in this work provide the most comprehensive database and resources for genes functioning in early mouse embryos and stem cells. All cDNA clones developed in this project have been made available through the American Type Culture Collection (ATCC). The subset of these cDNA clones have been rearrayed into the condensed clone sets, the NIA Mouse 15K cDNA Clone Set (Tanaka et al. 2000; Kargul et al. 2001) and the 7.4K cDNA Clone Set (VanBuren et al. 2002), which have been made available through designated academic distribution centers. Many genes that are uniquely or predominantly expressed in mouse early embryos and stem cells have been recently incorporated into a 60mer oligonucleotide microarray (Carter et al. 2003). Sequence information has been made available at public sequence databases (e.g., dbEST [Boguski et al. 1993]). Finally, all the information discussed here, as well as the graphical interfaces of the Mouse Gene Index, is available on our Web site at http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html.

Although the full appreciation of these resources is yet to be realized, the initial assessment of the first comprehensive transcriptome of early mouse embryos and stem cells has already provided three major points presented in this report.

First, approximately 1,000 putative genes that were newly identified using our cDNA collection most likely represent mouse genes unidentified previously, as they either encode ORFs greater than 100 amino acids or have multiple exons. The RT–PCR analysis of 19 selected genes confirmed the notion that novel cDNAs from our libraries tend to be expressed specifically in cells and tissues that we used in this project. These gene candidates will be a rich source of genes that are expressed at low levels, but play major roles in ES cells and adult stem cells as well as in early embryos.

Second, the analysis provided lists of genes specific to particular embryonic stages or stem cells and not expressed in other cell types. For example, we have identified signature genes for the individual preimplantation stages, all preimplantation stages, ES cells, and adult stem cells.

Finally, the PCA of 2,812 genes with relatively abundant expression revealed 88 genes with average expression levels that correlate well to the developmental potentials of cells. These genes may provide the first scale to characterize the developmental potential of cells and tissues at the molecular level.

The developmental potential of cells is a fundamental concept in developmental biology, providing a conceptual framework of sequential transition from totipotent fertilized eggs to pluripotent embryonic cells and stem cells to terminally differentiated cells. It is worth noting that genes associated with developmental potential can be identified only by simultaneous analysis of preimplantation embryos and a variety of stem cells. The analyses of stem cells alone could not provide these broader perspectives (Ivanova et al. 2002; Ramalho-Santos et al. 2002; Tanaka et al. 2002). The 88 genes we have identified here may provide a set of marker

genes for scaling the potential of cells. It is important to note that this scale is an operational construct. As such, further studies of the genes in the list will be required to test whether they provide critical clues to resolve the classic problem of the relation of stem cells to development. But the list could have immediate practical utility in assessing the effectiveness of treatments, gene manipulation, or both to convert differentiated cells such as fibroblasts into more potent cells such as ES—one of the most important goals required to achieve stem cell–based therapy.

## Materials and Methods

**cDNA library construction, clone handling, and sequencing.** Sources of tissue materials and RNA extraction methods are available as associated documents in the GenBank DNA sequence records (see also http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html). cDNA libraries were constructed as described elsewhere (Piao et al. 2001). More details are available in Protocol S1.

**Assembling of a gene index.** See description in the legend to Figure 1 and in Protocol S1.

**Analysis of 19 cDNA clones.** Sequencing of full-length cDNA clones and RT–PCR analysis were done by the standard methods. More details are available as Protocol S1.

**Identification of differentially expressed genes.** Most methods for selecting differentially expressed genes from EST frequencies are based on the assumption that each cDNA clone is a random sample from the mRNA pool in the cell and hence that EST frequencies correspond to the Poisson distribution (Audic and Claverie 1997). Real EST libraries, however, do not satisfy this assumption because even small changes in experimental conditions may affect the stability of particular species of mRNA, which in turn will cause a bias in EST frequency. Thus, a reliable detection of differentially expressed genes requires either library replications or comparison of classes of libraries. Because our EST libraries do not have true replications, we selected the latter approach, which yields genes that are specifically expressed in one class of tissues/stages and do not express in other tissues/stages. Some cDNA clones were represented by 5′ EST, some were by 3′ EST, and some were by both 5′ EST and 3′ EST. To avoid counting the same cDNA clone twice by 5′ EST and 3′ EST, all EST frequency analysis was done at the cDNA clone level.

To detect genes specific to a particular group of libraries, we first estimated the correlation between log-transformed clone frequencies, $\log(1000*n_i/N + 0.05)$, where $n_i$ is the abundance of clone $i$ in the library and $N$ is the total number of clones, with membership indicated (0 or 1) in a particular group (see Dataset S6). The first three group classifications are targeted on oocytes. The next two classifications include all preimplantation stages with and without blastocysts. There are four classifications attempting to differentiate between pluripotent cells and other tissues. The final nine classifications capture various groups of stem cells. Results of these analyses are given in Dataset S7 and a subset of the data is shown in Figure 3. We analyzed only positive correlations because we were interested in genes that are overexpressed in tissues of interest, and $P$-values were estimated using a one-tailed $t$-test. Because $P$-values cannot be used for simultaneous assessment of multiple hypotheses, we determined significant genes using the FDR method (Benjamini and Hochberg 1995). The FDR was set to 0.1, which corresponds to the average proportion of false positives equal to 10%.

As this study is focused on embryo- and stem cell–specific genes, we analyzed EST frequencies in public databases (Boguski et al. 1993) to exclude those genes that are predominantly expressed in adult tissues. A total of 3,338,847 public ESTs have been grouped into the following categories: NIA Collection, Preimplantation, Embryo, Embryonic Stem Cells, Fetus, Neonate, Adult, Adult Gonad, Adult Stem Cells, Adult Tumor, and Unclassified/Pooled Tissues (Dataset S11). Of 29,810 mouse genes, 5,425 genes were not represented by ESTs, 11,574 genes were expressed predominantly in adult tissues (EST frequency in adult tissues exceeds one-third of the maximum EST frequencies in all tissues), and 12,811 were genes expressed in embryos or in gonads, tumors, and stem cells. By removing 2,055 gonad-specific and 56 tumor-specific genes (20 times more ESTs in gonad or tumors than in other tissues), we obtained 10,700 genes that are predominantly expressed in embryos and stem cells (Dataset S12). Only ESTs matching to these genes were analyzed for differential expression.

**PCA of clone frequencies.** For the PCA shown in Figure 4, we selected 2,812 genes that had transcript frequencies of greater than or equal to 0.1% in at least one library (see Dataset S9). Clone/EST frequencies were log-transformed as $\log(1000*n_i/N + 0.05)$, where $n_i$ is the number of clones in U-cluster $i$ in the library, and $N$ is the total number of all clones in this library.

Statistical significance of gene contribution to PC3 (see Figure 5) was evaluated using correlation between log-transformed clone frequencies in various libraries and library position on the PC3 axis. $P$-values, estimated using a one-tailed $t$-distribution, characterize the significance of correlation for a single clone. To control the proportion of false positives, we used FDR, which was set to 0.1.

## Supporting Information

To view this Supporting Information with dynamic Web links, see http://lgsun.grc.nia.nih.gov/Supplemental-Information/.

The NIA Mouse Gene Index has recently made available to the public (http://lgsun.grc.nia.nih.gov/geneindex/). The Web interface provides a view of transcripts and genes on the mouse genome sequence. Unique IDs (U plus 6 digits, e.g., U018631) have been assigned to individual genes in the gene index. "U numbers" in the following datasets have direct links to corresponding genes in the NIA Mouse Gene Index. Clicking the "U number" in the datasets will lead to a Web page of the NIA public Web site.

**Dataset S1.** List of NIA Mouse cDNA Libraries and the Number of ESTs Generated

View online at DOI: 10.1371/journal/pbio.0000074.sd001 (22 KB XLS).

**Dataset S2.** Summary of Gene Counts in the NIA Mouse Gene Index

In addition to the list here, the Web interface at http://lgsun.grc.nia.nih.gov/geneindex provides a view of transcripts and genes on the mouse genome sequence.

View online at DOI: 10.1371/journal/pbio.0000074.sd002 (36 KB XLS).

**Dataset S3.** List of 977 Genes Unique to the NIA Mouse cDNA Collection

These are not found in RefSeq, Ensembl, and RIKEN. For sequence information, see Dataset S4.

View online at DOI: 10.1371/journal/pbio.0000074.sd003 (268 KB XLS).

**Dataset S4.** Sequence Information of 977 Genes in the FASTA Format

View online at DOI: 10.1371/journal/pbio.0000074.sd004 (685 KB TXT).

**Dataset S5.** Primer Sequences for RT–PCR Analysis

View online at DOI: 10.1371/journal/pbio.0000074.sd005 (30 KB DOC).

**Dataset S6.** Classification of cDNA Libraries for the Analysis of Differentially Expressed Genes

This table describes how cDNA libraries were logically grouped for further EST analysis, where membership to a group is indicated with a 1 and nonmembership is indicated with a 0.

View online at DOI: 10.1371/journal/pbio.0000074.sd006 (19 KB XLS).

**Dataset S7.** List of Genes Overexpressed in Preimplantation Embryos and Stem Cells

This table identifies the genes overexpressed in each group of cells/tissues described in Dataset S6.

View online at DOI: 10.1371/journal/pbio.0000074.sd007 (510 KB XLS).

**Dataset S8.** Comparison of the Gene Lists Identified in Dataset S7 with the Published Data

View online at DOI: 10.1371/journal/pbio.0000074.sd008 (23 KB DOC).

**Dataset S9.** List of 2,812 Genes Used for PCA to Investigate the Global Feature of Gene Expression Patterns

View online at DOI: 10.1371/journal/pbio.0000074.sd009 (633 KB XLS).

**Dataset S10.** List of 88 Genes Correlated with Developmental Potential of Cells

View online at DOI: 10.1371/journal/pbio.0000074.sd010 (72 KB XLS).

**Dataset S11.** Comprehensive Data about EST Frequencies of Genes in NIA Mouse cDNA Libraries and in Public Sequence Databases

View online at DOI: 10.1371/journal/pbio.0000074.sd011 (13.9 MB XLS).

**Dataset S12.** List of 10,699 Genes Predominantly Expressed in Embryos and Stem Cells

These genes were identified by the analysis of NIA EST and public EST datasets.

View online at DOI: 10.1371/journal/pbio.0000074.sd012 (3.2 MB XLS).

**Protocol S1.** Supplemental Materials and Methods

View online at DOI: 10.1371/journal/pbio.0000074.sd013 (59 KB DOC).

**Video S1.** 3D View of Results Obtained by PCA of Log-Transformed EST Frequencies in NIA Mouse cDNA Libraries

Red spheres represent libraries and yellow boxes represent genes. Gene names can be legible at closer distance. (For Windows, Media Player or Real Player is required to view. For Macintosh, Quicktime Player is required.) A virtual reality modeling language (VRML) formatted version is also available on our Web site (http://lgsun.grc.nia.nih.gov/Supplemental-Information). The VRML version allows users to freely rotate and zoom the image in 3D space. Genes are also hyperlinked to the NIA Mouse Gene Index Web site (mentioned in Dataset S2).

View online at DOI: 10.1371/journal/pbio.0000074.sv001 (3.9 MB AVI).

**Accession Numbers**

The LocusLink (http://www.ncbi.nih.gov/LocusLink/) accession numbers for the genes discussed in this paper are *1810015H18Rik* (LocusLink ID 69104), *2210021E03Rik* (LocusLink ID 52570), *2410003C07Rik* (LocusLink ID 66977), *2610005B21Rik* (LocusLink ID 72119), *2610005H11Rik* (LocusLink ID 72114), *3230401D17Rik* (LocusLink ID 66680), *4833422F24Rik* (LocusLink ID 74614), *4921528E07Rik* (LocusLink ID 114874), *4933428G09Rik* (LocusLink ID 66768), *5730419I09Rik* (LocusLink ID 74741), *5930405J04Rik* (LocusLink ID 68094), *A030007L17Rik* (LocusLink ID 68252), *A930014I12Rik* (LocusLink ID 77805), *AA617276* (LocusLink ID 100012), actin-binding Kelch family protein (LocusLink ID 246293), *Bcl2l10* (LocusLink ID 12049), *Birc2* (LocusLink ID 11796), *Bmp15* (LocusLink ID 12155), *Btg4* (LocusLink ID 56057), *Cdc25a* (LocusLink ID 12530), *Col18a1* (LocusLink ID 12822), *Cyp11a* (LocusLink ID 13070), *D7Ertd784e* (LocusLink ID 52428), *Dctn1* (LocusLink ID 13191), *Dstn* (LocusLink ID 56431), *Dtx2* (LocusLink ID 74198), *E130301L11Rik* (LocusLink ID 78733), *E2f1* (LocusLink ID 13555), *Fmn2* (LocusLink ID 54418), *Folr4* (LocusLink ID 64931), *Gdf9* (LocusLink ID 14566), *Hic2* (LocusLink ID 58180), *Ing5* (LocusLink ID 66262), *Itga3* (LocusLink ID 16400), *Kars-ps1* (LocusLink ID 85307), KRAB box zinc finger (LocusLink ID 170763), *Krt2–16* (LocusLink ID 16680), *MGC32471* (LocusLink ID 212980), *MGC38133* (LocusLink ID 243362), *MGC38960* (LocusLink ID 235493), *Mitc1* (LocusLink ID 75901), *Mkrn1* (LocusLink ID 54484), *Mov10* (LocusLink ID 17454), *Mtf2* (LocusLink ID 17765), *Oas1d* (LocusLink ID 100535), *Oas1e* (LocusLink ID 231699), *Obox3* (LocusLink ID 246791), *Obox5* (LocusLink ID 252829), *Oosp1* (LocusLink ID 170834), *Pola2* (LocusLink ID 18969), *Prkab1* (LocusLink ID 19079), *Rad54l* (LocusLink ID 19366), *Rcc1* (LocusLink ID 100088), *Rfpl4* (LocusLink ID 192658), *Rgs2* (LocusLink ID 19735), *Rnf35* (LocusLink ID 260296), *Rnpc1* (LocusLink ID 56190), *Scmh1* (LocusLink ID 29871), *Sfmbt* (LocusLink ID 54650), *Slc21a11* (LocusLink ID 108116), *SMARCC2* (LocusLink ID 6601), *Smarcf1* (LocusLink ID 93760), *Smtn* (LocusLink ID 29856), *Spin* (LocusLink ID 20729), *Tcl1* (LocusLink ID 21432), *Tcl1b1* (LocusLink ID 27379), and *Tcl1b3* (LocusLink ID 27378).

The GenBank (http://www.ncbi.nih.gov/Genbank/index.html) accession numbers of new ESTs reported in this paper are AA406988–AA407326, AA409386–AA409982, AA409984–AA410173, AW536060–AW536143, AW537733–AW537828, AW545917–AW545921, BE824469–BE825132, BI076411–BI076872, BM114148–BM121445, BM121647–BM125459, BM194710–BM203257, BM203259–BM214569, BM214575–BM251183, BM293391–BM293823, BU576966–BU576966, CA530650–CA580325, CA870176–CA882792, CA882932–CA896558, CD538085–CD544029, CD544034–CD555913, CD559752–CD565790, CF153424–CF161651, and CF161657–CF175178.

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**Author contributions.** MSHK conceived and designed the experiments. YP, RM, GF, PRM, CAS, UCB, YW, MGC, TH, KA, HA, LS, TST, WLK, TY, SAJ, SP, and MSHK performed the experiments. AAS, YP, RM, DBD, YQ, VV, GF, J. Kelso, WH, and MSHK analyzed the data. RN, KRB, DDT, RJH, DLL, DS, J. Keller, EK, GHK, AU, AV, JR, TK, BLMH, AC, MD, J. Kelso, and WH contributed reagents/materials/analysis tools. AAS, YP, RM, VV, GF, and MSHK wrote the paper. ■

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# Redifferentiation of dedifferentiated chondrocytes and chondrogenesis of human bone marrow stromal cells via chondrosphere formation with expression profiling by large-scale cDNA analysis

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**Abstract**

Characterization of dedifferentiated chondrocytes (DECs) and mesenchymal stem cells capable of differentiating into chondrocytes is of biological and clinical interest. We isolated DECs and bone marrow stromal cells (BMSCs), H4-1 and H3-4, and demonstrated that the cells started to produce extracellular matrices, such as type II collagen and aggrecan, at an early stage of chondrosphere formation. Furthermore, cDNA sequencing of cDNA libraries constricted by the oligocapping method was performed to analyze difference in mRNA expression profiling between DECs and marrow stromal cells. Upon redifferentiation of DECs, cartilage-related extracellular matrix genes, such as those encoding leucine-rich small proteoglycans, cartilage oligomeric matrix protein, and chitinase 3-like 1 (cartilage glycoprotein-39), were highly expressed. Growth factors such as FGF7 and CTGF were detected at a high frequency in the growth stage of monolayer stromal cultures. By combining the expression profile and flow cytometry, we demonstrated that isolated stromal cells, defined by $CD34^-$, c-$kit^-$, and $CD140\alpha^{-\text{ or low}}$, have chondrogenic potential. The newly established human mesenchymal cells with expression profiling provide a powerful model for a study of chondrogenic differentiation and further understanding of cartilage regeneration in the means of redifferentiated DECs and BMSCs.


*Keywords:* Dedifferentiation; Redifferentiation; Chondrocytes; Marrow stromal cells; Regeneration; Stem cells; Transplantation

## Introduction

Chondrocytes differentiate from mesenchymal cells during embryonic development [1], and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix (ECM) molecules, including type II collagen and aggrecan [2–4]. It is rapidly lost during serial monolayer culture since the phenotype of differentiated chondrocytes is unstable in culture [5–8]. This process is referred to as "dedifferentiation" and is a major impediment to use of in mass cell populations for cell therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of

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Table 1
Monoclonal antibodies[a]

| Antigen | Clones | Application | Source |
|---|---|---|---|
| CD14 | MY4 | F | BC |
| CD29, integrin β1 | 4B4 | F | BC |
| CD31, PECAM-1 | 5.6E | F, H | IT |
| CD34 | QBEnd-10 | F | IT |
| CD44, Pgp-1 | J-173 | F, H | IT |
| CD45, LCA | DW124-5-2 | F | BC |
| CD50, ICAM-3 | HP2/19 | F, H | IT |
| CDw90, Thy.1 | F15-42-1 | F, H | CT |
| CD105, endoglin | 1G2 | F | IT |
| CD106, VCAM-1 | 51-10C9 | F | P |
| CD117, c-kit | Nu-ckit | F | CT |
| CD140a, PDGFR α | αR1 | F | P |
| CD144, VE-cadherin | 55-7H1 | F | P |
| CD166, ALCAM | 3A6 | F, H | RDI |

[a] BC, Beckman Coulter; CT, Cytotech; F, fluorescence-activated cell sorting; H, immunohistochemistry; ICAM-3, intercellular adhesion molecule-3; IT, Immunotech; LCA, leukocyte common antigen; P, Pharmingen; PECAM-1, platelet endothelial cell adhesion molecule-1; PDGFR, platelet-derived growth factor receptor; Pgp-1, phagocytic glycoprotein-1/hyaluronate receptor; RDI, Research Diagnostics, Inc.

type II collagen and cartilage proteins [9]. When cultured three-dimensionally in a scaffold such as agarose, collagen, and alginate, redifferentiated chondrocytes reexpressed the chondrocytic differentiation phenotype. We have successfully induced differentiation of chondrocytes and marrow stromal cells by chondrosphere formation.

Mesenchymal stem cells persist in adult bone marrow as a population of cells [10] with the capacity to differentiate to osteogenic [11], chondrogenic [12–14], or adipogenic [15] lineages both in vitro and when implanted subcutaneously in SCID mice [14]. These cells can be isolated from the marrow by standardized techniques and expanded in culture through many generations, while retaining their capacity to differentiate along these pathways when exposed to appropriate culture conditions. This property of mesenchymal stem cells opens up therapeutic opportunities for the treatment of lesions in mesenchymal tissues, and protocols have been devised for the treatment of defects in articular cartilage [16], bone [17], tendon [18], and meniscus [19] and for bone marrow stromal recovery [20] and osteogenesis imperfecta [21]. It also provides an opportunity to study the differentiation of mesenchymal cells in chondrocytes in the developing limb in detail.

We have generated cDNA libraries and carried out large-scale sequencing of cDNA constructed with the oligocapping methods [22] with the objective of conducting a global survey of gene expression and differentiation of genes that are specific for redifferentiated chondrocytes and marrow stromal-derived chondrogenic progenitor. We have successfully established human diploid chondrocyte strains and marrow stromal cell strains, and although these strains are mortal, they serve a useful tool as reproducible systems of human chondrogenesis with cell-type-specific profiling patterns.

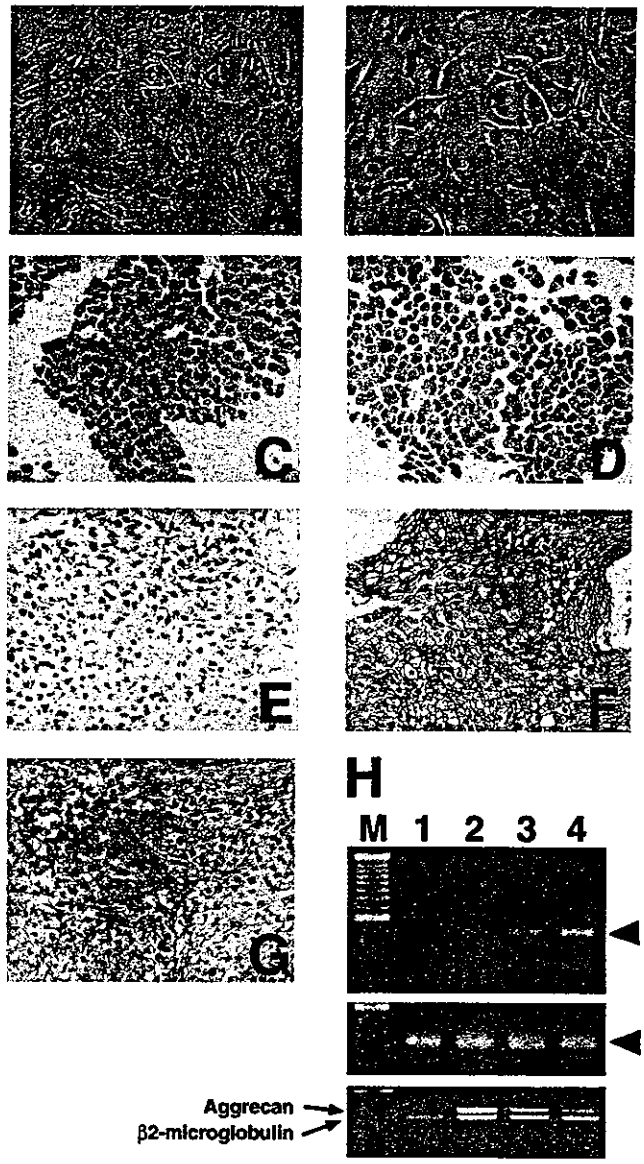



Fig. 1. Redifferentiation of dedifferentiated chondrocytes. Morphology of dedifferentiated chondrocytes in monolayer culture is fibroblast-like (original magnification: A, × 100; B, × 200). Aggregated cells started to accumulate alcian blue-positive substances in their cytoplasms, but not deposit matrix in an extracellular space [C, hematoxylin and eosin (HE) stain; D, Alcian blue stain]. Redifferentiation of the dedifferentiated chondrocytes on 5th day of the chondrosphere culture (E, HE stain; F, Alcian blue stain; G, toluidine blue stain). The differentiated cells produced an extracellular matrix that showed metachromasie with toluidine blue stain. Reverse transcription-polymer chain reaction analysis of gene expression for type II collagen (upper column, arrowhead) and aggrecan (lower column) during redifferentiation (H). β2-Microglobulin was used for an internal control of each RNA (arrowhead in the middle column; lower band indicated by arrow in the lower column). M, molecular markers; lane 1, monolayer culture; lane 2, 1 week of redifferentiation; lane 3, 2 weeks; and lane 4, 3 weeks.

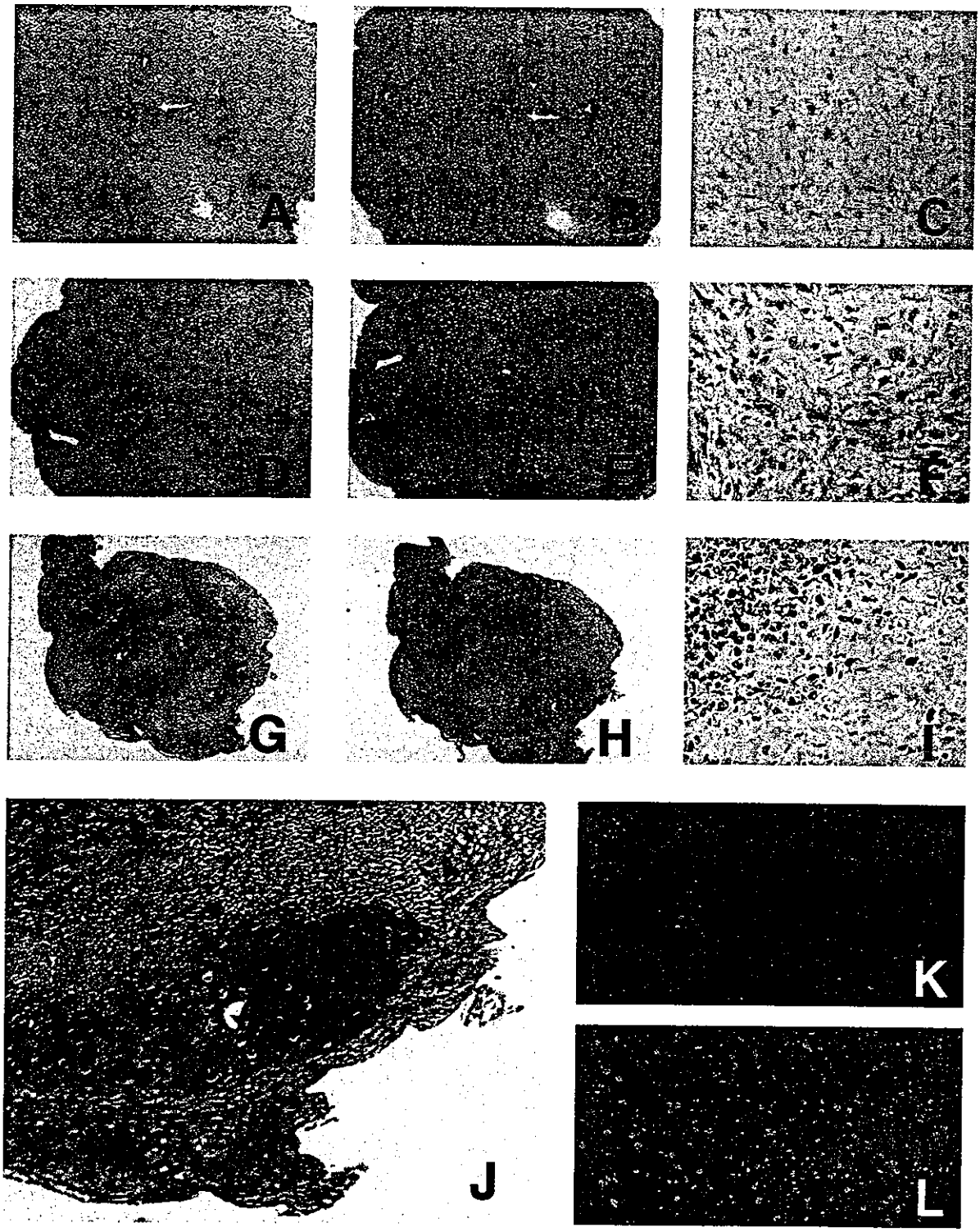



Fig. 2. Chondrosphere culture for a long period. Chondrosphere culture of dedifferentiated chondrocytes at 3 weeks [A, hematoxylin and eosin (HE) stain; B, Alcian blue stain; C, aggrecan], 2 months (D, HE stain; E, Alcian blue stain; F, aggrecan), 11 months (G, HE stain; H, Alcian blue stain; I, aggrecan). (J) High-power view of H. Immunocytochemical analysis was performed for the expression of aggrecan (C, F, and I). Immunocytochemical analysis for the expression of type II collagen in chondrosphere culture of dedifferentiated chondrocytes at just after 2 h of incubation (K) and at 3 weeks (L). Original magnification: A, B, D, E, G, and H, ×40; C, F, and I, ×200; J, ×100.

## Materials and methods

### *Isolation and cell culture of human bone marrow stromal cells (BMSCs) and dedifferentiated chondrocytes (DECs)*

Bone marrow samples and normal human articular chondrocytes were obtained from normal human donors with the approval of the internal Keio Ethics Committee (#13-11). Cells were resuspended in BMSC culture medium [10% fetal bovine serum in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DMEM-HG)] with antibiotics/antimycotics supplements (Gibco BRL, Gaithersburg, MD, USA). Chondrocytes were maintained in chondrocyte growth medium (CGM; Bio Whittacker/Clonetics, Walkersville, MD, USA), and cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$. When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and were replated with one-half of the harvested cells.

### *Chondrosphere culture*

For chondrogenic differentiation, we established a chondrosphere culture modified from the micromass culture. The cells were harvested with trypsin and EDTA, and the suspension was cytocentrifuged to obtain a concentration of $2.0 \times 10^7$ cells/ml. After placing 10-$\mu$l drops containing $2.0 \times 10^5$ cells each on a 35-mm uncoated culture dish, the cells were allowed to attach to the dish surface for 2 h in a humidified atmosphere at 37°C. Each culture was then flooded with 0.5 ml of each medium for each chondrosphere. The medium for BMSCs consisted of high-glucose DMEM supplemented with 10 ng/ml transforming growth factor (TGF)-$\beta$3, $1 \times 10^{-7}$ M dexamethasone, 50 $\mu$g/ml ascorbic acid-2-phosphate, 40 $\mu$g/ml proline, 100 $\mu$g/ml pyruvate, and 50 mg/ml ITS+ Premix (Becton-Dickinson, Bedford, MA; 6.25 $\mu$g/ml insulin, 6.25 $\mu$g/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid). We used CGM and chondrocyte differentiation medium (CDM; Clonetics) for the redifferentiation of dedifferentiated chondrocytes (DECs). On the second day, cells were gently detached from the uncoated dishes, by scraping with a 25-ml pipette, and then passaged on uncoated dishes. The BMSC medium was replaced every day and the DEC medium was replaced every 2 or 3 days. Chondrosphere culture was continued up to 11 months.

### *Histological and immunocytochemical analysis*

Chondrospheres were fixed in 20% formalin, embedded in paraffin, cut into 5-$\mu$m sections, and stained with hematoxylin and eosin, Alcian blue, and toluidine blue. Immunocytochemical analysis was performed as described [23] by using antibodies against aggrecan and type II collagen.

### *RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was prepared from chondrosphere cultures with Isogen (Nippon Gene). RNA for RT-PCR was converted to cDNA with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotec, Uppsala, Sweden) according to the manufacturer's recommendations. PCR amplification conditions for the cDNAs obtained was performed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 45 s, in which the 68°C step was prolonged by 5 s in every cycle after the first 10 cycles. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The PCR primers were as follows: type II collagen (forward): 5′-TTCAGCTATGGAGATGACAATC-3′, type II collagen-(reverse): 5′-AGAGTCCTAGAGTGACTGAG-3′ [24]; aggrecan (forward): 5′-GCCTTGAGCAGTTCACCTTC-3′, aggrecan (reverse): 5′-CTCTTCTACGGGGACAGCAG-3′ [25]; COMP (forward): 5′-CAGGACGACTTTGATGCAGA-3′, COMP (reverse): 5′-AAGCTGGAGCTGTCCTGGTA-3′ [25]; cartilage glycoprotein-39 (forward): 5′-GATAGCCTCCAACACCCAGA-3′, cartilage glycoprotein-39 (reverse): 5′-CTGAGCAGGAGCTGCTTTTT-3′; WISP1 (forward): 5′-GCCCCCAATTCTGCAAGTG-3′, WISP1 (reverse): 5′-TTAGGCTGGAAGGACTGGC-3′; $\beta$2-microglobulin (forward): 5′-CTCGCGCTACTCTCTCTTTCTGG-3′, $\beta$2-microglobulin (reverse): 5′-GCTTACATGTCTCGATCCCACTTAA-3′ [25].

### *Flow cytometric analysis*

Cells were detached and stained for 30 min at 4°C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed on a FACScan analyzer (Becton Dickinson). Antibodies (anti-human CD14, CD29, CD31, CD34, CD44, CD45, CD105, CD144, CD31, CD50, CD117, CD140$\alpha$, CD90, CD106, CD166, and isotype control antibodies) were purchased from Beckman Coulter (Miami, FL, USA), Immunotech (Marseilles Cedex, France), Cytotech (Helleback, Denmark), and Pharmingen Pharmaceutical, Inc. (San Diego, CA, USA) (Table 1).

### *cDNA sequence: oligocapping method sequencing*

cDNA libraries were constructed according to the oligocapping method [22,26]. Each plasmid DNA containing a cDNA insert was extracted with MultiScreen 96-well filter plates (Millipore Corp., Billerica, MA, USA). The 5′ end of the cDNA insert was sequenced with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 1 $\mu$l of Big Dye Terminator Reaction Mix, 1 $\mu$l of 3.2 pmol/$\mu$l of primer (5′-TACGGAAGTGTTACTTCTGC-3′), 3 $\mu$l of buffer (200 mM Tris-Cl, 10 mM $MgCl_2$, pH 9), and 400 ng of plasmid were mixed in a PCR tube, and the PCR cycle was performed (denaturation, 10 s at 96°C; annealing, 5 s at 50°C; elongation, 4 min at 50°C)

with a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). Sequencing analysis was performed with ABI3700 (Applied Biosystems, Foster City, CA, USA).

*Infection of retroviral constructs*

Cloning of the full-length of hTERT cDNA has been described previously [43]. The hTERT cDNA was cloned into pCLXSN with the Gateway System (Invitrogen) to generate pCLXSN-hTERT. Briefly, the cDNA was first cloned into pDONR201 by BP reaction, and then into a destination vector, pDEST-CLXSN, which has a modified cassette containing attR sites and ccdB (Invitrogen) at the multicloning site of pCLXSN (Imgenex Corp., San Diego, CA) [27]. Production of recombinant retroviruses has been described [28]. Briefly, retroviral vector together with a packaging plasmid, pCL-10A1 (Imgenex Corp., San Diego, CA) [27], was transfected into 293T cells by the calcium phosphate method, and the culture fluid was harvested at 60 h post transfection. Production of the LXSN-16E6E7 retrovirus has been described previously [29]. The titers of the recombinant viruses were greater than $1 \times 10^6$ drug-resistant colony forming units per milliliter on HeLa cells, and 1 ml of its culture fluid was added to cells in the presence of polybrene (8 $\mu$g/ml). Following inoculation with viruses, BMSCs were grown in the presence of G418 (100 $\mu$g/ml) or hygromycin B (50 $\mu$g/ml), and a polyclonal drug-resistant cell line was established and further analyzed. For combinations of retroviral infections, cells were sequentially transduced with LXSN-E6E7 and LXSN-hTERT, and selected with G418 and hygromycin B, respectively. The stably transduced cells with an expanded life span were named ThMSC1.

## Results

*Redifferentiation of DECs by chondrosphere culture*

Human DECs were isolated from femoral head cartilage and designated as DECs. We used DECs beyond the 5th passage and induced rechondrogenesis by chondrosphere culture. In monolayer culture, the chondrocytes were flat with a fibroblast-like morphology (Fig. 1A and B), but they became round and in close contact with each other after chondrosphere culture at a high density cell condensation of $2 \times 10^7$ cells/ml and 2-h incubation (Fig. 1C and D). On day 5, the cells produced an ECM that exhibited metachromasia upon toluidine blue stain (Fig. 1E, F, and G). Expression of type II collagen and aggrecan genes commenced on day 7 (Fig. 1H).

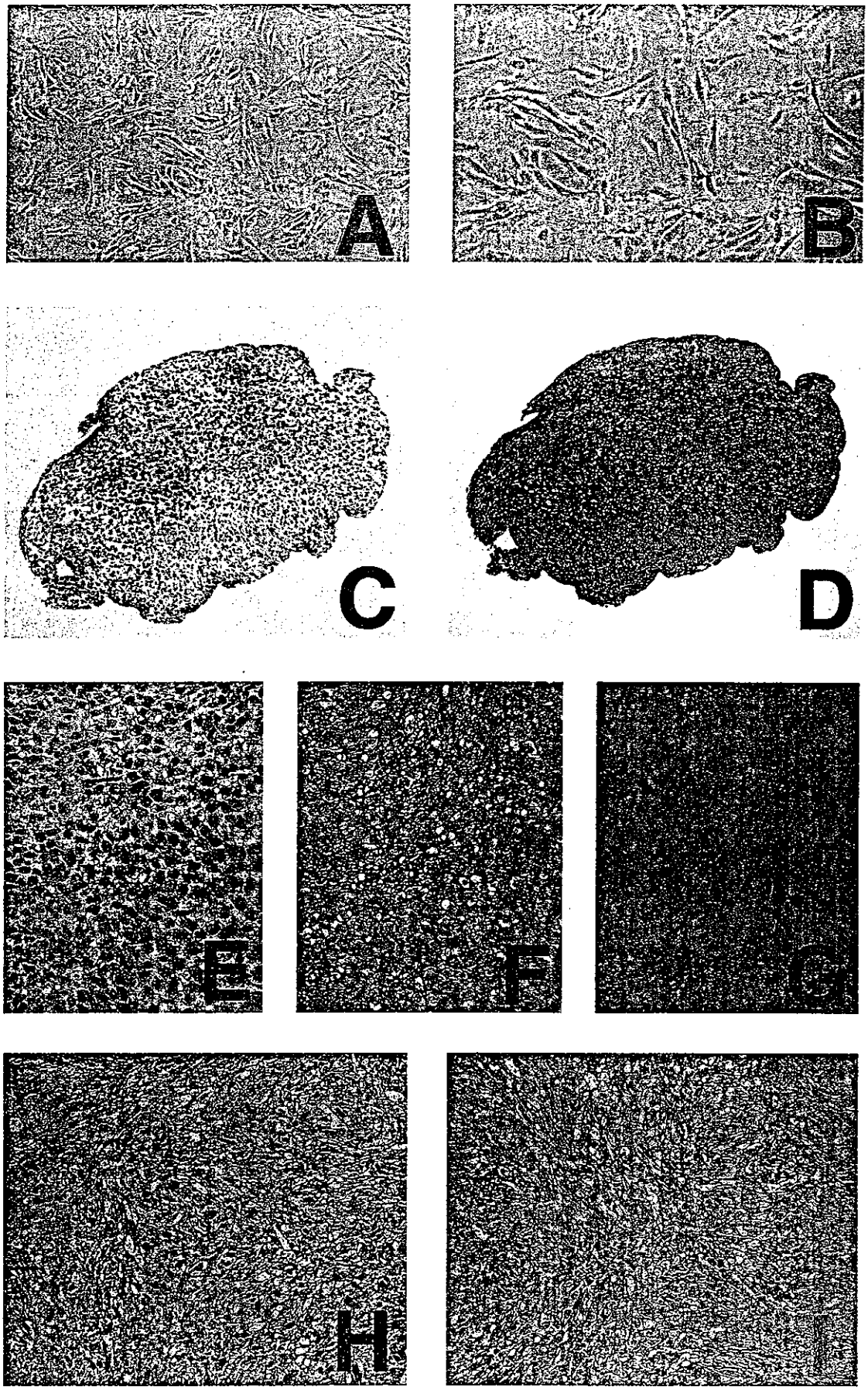
*Chondrosphere culture for a long period*

We performed micromass culture of monolayered DECs in chondrocyte differentiation medium, formed chondrospheres by detaching the micromasses from the dishes, continued chondrosphere culture, and then compared DEC redifferentiation at 3 weeks, 2 months, and 11 months. The cells were equally embedded in ECM at 3 weeks (Fig. 2A, B, and C) and at 2 months (Fig. 2D, E, and F). At 11 months (Fig. 2G, H, and I), the chondrospheres consisted of three morphologically distinct areas, (1) a high cell density area in the center, (2) an ECM-rich area in the inner layer, and (3) a fibrous area with flattened cells in the outer layer (Fig. 2J). The result of the immunocytochemical analysis showed that aggrecan was undetectable at 3 weeks, and started to be detected in both the cell cytoplasm and also ECM at a later stage (Fig. 2C, F, and I). A slight amount of type II collagen was detected at 2 h of chondrosphere culture, and its expression was enhanced by 3 weeks of culture (Fig. 2K and L).

*Chondrosphere culture of BMSCs*

We also generated 7 BMSC strains from two healthy donors, designating them H3-1 to H3-4 and H4-1 to H4-3, and we subjected them to chondrosphere culture, to determine whether they would differentiate into chondrocytes. Expression of chondrocyte-specific genes, such as type II collagen and aggrecan, was induced only when the cells were exposed to TGF-$\beta$3. Thus, we supplemented the chondrosphere culture of BMSCs with TGF-$\beta$3 in subsequent experiments. However, this is not the case for redifferentiation of DECs. The H4-1 cells were cultured to passage 16 in monolayer culture (Fig. 3A and B) and then placed in chondrosphere culture. On day 5 of chondrosphere culture, the H4-1 cells exhibited round morphology and were surrounded by ECM that stained with alcian blue and exhibited metachromasia with toluidine blue (Fig. 3C–G). To compare the chondrosphere culture with pellet culture, we determined expression of type II collagen and aggrecan in H4-1 cells (Fig. 4A). Aggrecan was induced on day 3 after addition of TGF-$\beta$3 in the chondrosphere culture, but not in the pellet culture. On day 5, aggrecan was detected in both cultures. Type II collagen was observed in both cultures on day 3 and day 5. We compared H4-1 with H3-4 at the 7th passage, which is one of other human stromal strains for chondrogenesis. The H3-4 cells were spindle-shaped in morphology (Fig. 3H and I), and aggrecan was detected in them by RT-PCR, but no type II collagen (Fig. 4B).

Chondrogenic differentiation is known to be mediated by mitogen-activated protein (MAP) kinases and blocked by inhibitors [30]. We used two MAPK inhibitors, PD98059, a specific inhibitor of mitogen-or extracellular signal-regulated kinase-1 (MEK1), and SB203580, which inhibits phosphorylation of P38 MAPK. Expression of type II collagen on day 5 was clearly inhibited by SB203580 (Fig. 4C), but not by PD98059 at any time during the chondrogenic differentiation protocol, indicating that chondrocytic differentiation of BMSCs is mediated by the previously reported signal pathway of mesenchymal cells into chondrocytes during embryonic development [9]. Inhibition of


A
B
C
D
E
F
G
H

ERK and p38 kinase partially reduced aggrecan expression (Fig. 4C).

*Cell surface markers in BMSCs and DECs*

The H4-1 chondrogenic progenitors were positive for CD29 (integrin β1), CD44 (Pgp-1/ly-24), CD105 (endoglin), CDw90 (Thy-1), CD106 (VCAM-1), and CD166 (ALCAM), and negative for CD14 (a marker for macrophage and dendritic cells), CD34, CD45 (leukocyte common antigen), CD144 (VE-cadherin), CD31 (PECAM-1), CD50 (ICAM-3), CD117 (c-kit), and CD140α (PDGFR) (Table 2). H3-4 nonchondrogenic stroma cells were positive for CD29, CD34, CD44, CD105, CD140α, CDw90, CD106, and CD166, and negative for CD14, CD45, CD144, CD31, CD50, and CD117. The DECs were positive for CD14, CD29, CD44, CD105, CD90, and CD106, CD140α, and CD166, and negative for CD34, CD45, CD144, CD31, CD50, and CD117.

*Establishment of ThMSC1 cells*

To examine the possibility of using the cells as a source of cells for transplantation, they need to have a long life span and retain differentiated capability even after a longer culture period. To produce such cells, we infected H4-1 cells with human telomerase reverse transcriptase subunit (hTERT), E6, and E7. The infected cells, designated as ThMSC1 cells, grew for at least 6 months and proliferated for more than 40 population doublings. The ThMSC1 cells were spindle-shaped, and they elongated more than H4-1 cells in monolayer culture. The ThMSC1 cells did not express type II collagen or aggrecan after 5 days of exposure to TGF-β3 in chondrosphere culture, suggesting that they had lost their capacity of chondrogenesis.

*Analysis of the cDNA sequences of cDNA libraries*

Profiling of the redifferentiation of DECs (RDEC), H4-1, and ThMSC1 cells was performed; DEC cells at passage 7 were redifferentiated for 7 days of chondrosphere culture and then used for cDNA analysis; H4-1 in monolayer culture at passage 16 and ThMSC1 in monolayer culture at passage 20 were used. The sequenced 5′ ends of 2,628, 2,687, and 671 randomly selected cDNA clones obtained from cDNA libraries of redifferentiation of DECs, H4-1 cells, and ThMSC1 cells, respectively, were sequenced (Table 3), and the cDNA clones were clustered by similarity searches of 5′ end sequences. The threshold of similarity was possession of similarity overlaps with a length ≥50 bp and identity ≥90%. The sequences of representative clones of each group were analyzed with the human RefSeq mRNA (updated September 2001, ftp://ncbi.nlm.nih.gov/refseq/) by a similarity search. The numbers of the groups, each of which could correspond to known genes, are 1,134, 1,150, and 417 for DECs, H4-1 cells, and ThMSC1 cells, respectively. The numbers of genes (i.e., group) having high homology with any of the RefSeq sequences are shown in Table 3. The criterion for high homology was that the representative clone of the group have a similarity overlap with an identity ≥96%. The sequence similarity searches in the above in silico procedures were performed with WU-BLAST 2.0 (http://blast.wustl.edu). The percentages of the novel cDNA clones for redifferentiation of DECs, H4-1 cells, and ThMSC1 cells were 1.0%, 1.0%, and 1.6%, respectively.

The numbers of clones per unigene cluster having high homology (identity over 96%) are shown in Fig. 5A. Approximately 70% of the clusters for redifferentiated DECs and H4-1 cells contained only a single clone, whereas 30% of the clusters contained two or more clones. Fig. 5B shows the distribution of the functional classifications of the genes with identity ≥96% for redifferentiated DECs, H4-1 cells, and ThMSC1 cells. Each slice lists the numbers and percentages of human gene functions assigned to a given category of molecular function, according to the Gene Ontology (available at www.geneontology.org/). Almost all H4-1 and ThMSC1 cells had the same distribution, but redifferentiated DECs and H4-1 cells exhibited a different pattern, especially in "structural protein."

The highly detected genes in redifferentiated DECs, H4-1 cells, and ThMSC1 cells were shown in Table 4. The data showed high expression of two groups, i.e., the ECM-related genes and growth factor-related genes. In redifferentiated DECs, there were 365 clones of ECM-related genes, such as biglycan, chitinase 3-like 1 (cartilage glycoprotein-39), decorin, lamin A/C, and fibromodulin (expression level, 13.9%), and 50 clones of growth factor-related genes, such as insulin-like growth factor binding protein 3 and granulin (expression level, 1.9%). In H4-1 cells, there were 111 clones of ECM related genes, such as EFEMP1, biglycan, lamin A/C, and decorin (expression level, 4.1%), and 155 clones of growth factor-related genes, such as insulin-like growth factor binding protein 3 (IGFBP3) and connective tissue growth factor (CTGF) (expression level, 5.8%).

A marked difference in frequency of ECM-related genes was found between redifferentiated DECs and H4-1 cells

Fig. 3. Chondrogenesis of human marrow stromal cells by chondrosphere culture. H4-1, human marrow stromal cells, in monolayer culture at 16th passage are fibroblast-like in morphology (A). (B) High-power view of A. H4-1 cells at 16th passage for 5 days of chondrosphere culture were round-shaped and surrounded by extracellular matrix (C–G), which showed metachromasie with toluidine blue stain (G). (H and I) Chondrogenesis of H3-4 at 7th passage by a pellet culture. The cells are spindle in morphology. C, E, and H, HE stain; D, F, and I, Alcian blue stain.

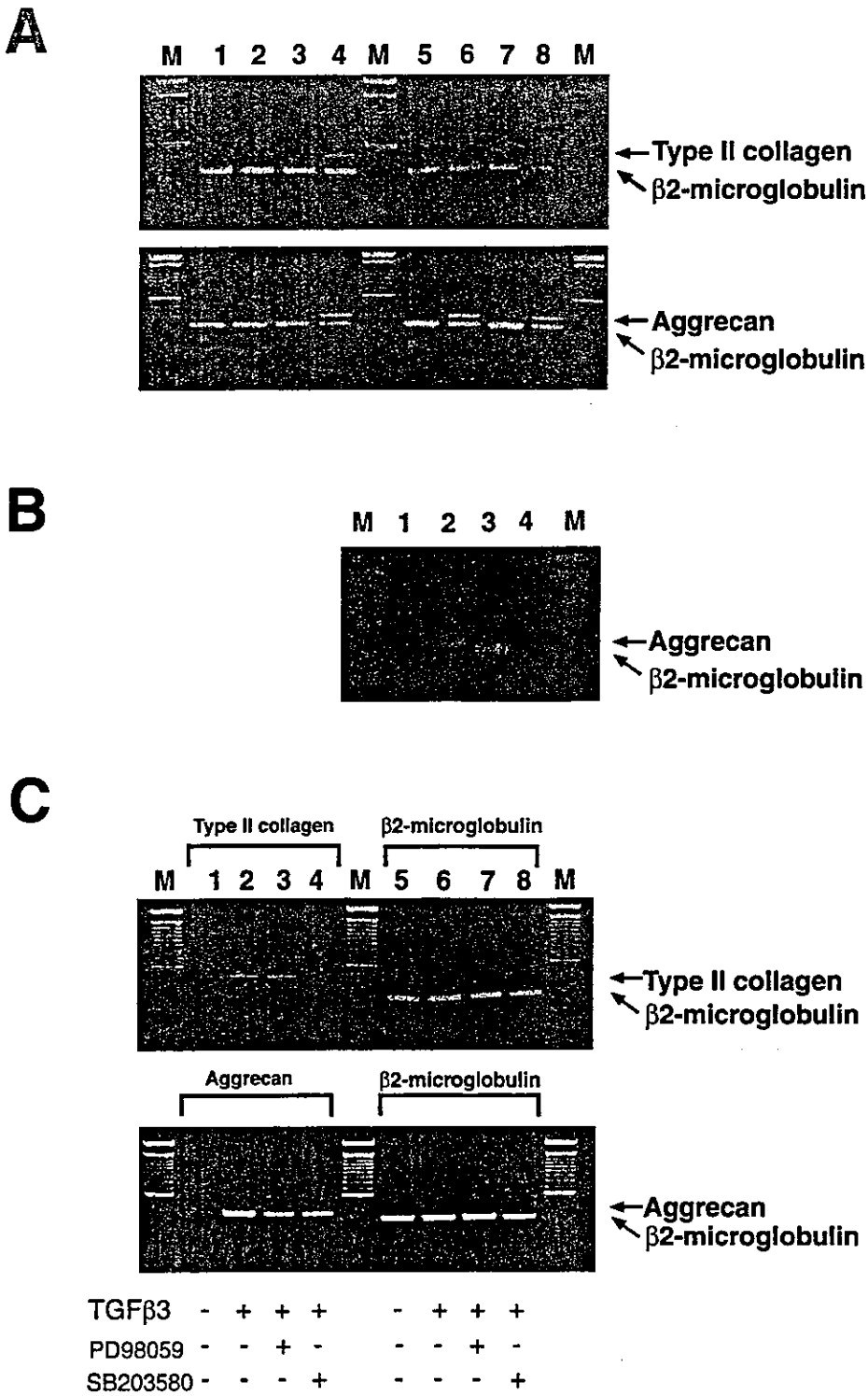



Fig. 4. Regulation of type II collagen and aggrecan by the pellet culture and chondrosphere culture with transforming growth factor (TGF)-β3. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of type II collagen (upper column) and aggrecan (lower column) expression in H4-1 cells. Expression of β2-microglobulin is also shown as a loading control for each lane. Lanes 1 and 5, 3rd and 5th day of pellet culture without TGF-β3, respectively; lanes 2 and 6, 3rd and 5th day of pellet culture with TGF-β3, respectively; lanes 3 and 7, 3rd and 5th day of chondrosphere culture without TGF-β3, respectively; lanes 4 and 8, 3rd and 5th day of chondrosphere culture with TGF-β3, respectively. (B) RT-PCR analysis of aggrecan expression in H3-4 (7th passage) by the pellet culture. RNA was isolated from cells on 7th day of pellet culture. Lane 1, type II collagen; lane 2, aggrecan; lane 3, β2-microglobulin; lane 4 served as a negative control (H20) for β2-microglobulin primers. (C) RT-PCR analysis of type II collagen (lanes 1–4 of upper column) and aggrecan (lanes 1–4 of lower column) expression in H4-1 cells on 5th day of chondrosphere culture. H4-1 cells were differentiated in the chondrosphere culture protocol without TGF-β3 (lanes 1 and 5) or with TGF-β3 (lanes 2 and 6). The cells were exposed to MAPK inhibitors, 20 μM PD98059 (lanes 3 and 7), or 10 μM SB203580 (lanes 4 and 8) throughout the differentiation protocol. Expression of β2-microglobulin is also shown as a loading control (lanes 5–8). M represents 100-bp ladder of molecular markers.

Table 2
Flow cytometric analysis of cell surface markers in H4-1, H3-4, and dedifferentiated chondrocytes (DEC)[a]

| Cells | CD14 | CD29 | CD34 | CD44 | CD45 | CD105 | CD144 |
|---|---|---|---|---|---|---|---|
| H4-1 | − | + | − | ++ | − | + | − |
| H3-4 | − | ++ | ++ | ++ | − | ++ | − |
| DEC | + | ++ | − | ++ | − | ++ | − |
| | CD31 | CD50 | CD117 | CD140a | CD90 | CD106 | CD166 |
| H4-1 | − | − | − | − | + | + | ++ |
| H3-4 | − | − | − | + | ++ | + | ++ |
| DEC | − | − | − | + | ++ | + | ++ |

[a] Peak intensity was estimated in comparison with isotype controls. (++), strongly positive (more than 10 times of the isotype control); (+), weakly positive; (−), negative (less than twice of the isotype).

(Table 5). The number of clones for ECM-related genes, which accounted for most of the structural proteins in Gene Ontology, was 3.4-fold higher than H4-1 cells (Fig. 5C). The percentage of the ECM-related genes was 15.0% in redifferentiated DECs, as follows: proteoglycans, 230 clones; cartilage oligomeric matrix protein (COMP), 85 clones; cartilage glycoprotein-39, 58 clones; EFEMP1, 13 clones; and collagens 5 clones. The proteoglycans in the redifferentiated DECs were mostly leucine-rich small proteoglycans, such as biglycan, 145 clones; decorin, 31 clones; fibromodulin, 23 clones; lumican, 12 clones, and other proteoglycans were osteoglycin, 7 clones; aggrecan, 6 clones; and tenascin C, syndecan 4, testican, fibrillin 1, osteonectin, and osteoglycin, 1 clone each.

The frequency of ECM-related genes in H4-1 cells was much lower (4.3%) than in redifferentiated DECs. The proteoglycans in H4-1 cells were biglycan, 26 clones; decorin, 12 clones; lumican, 5 clones; osteonectin, 5 clones; fibromodulin, 3 clones; and testican and glypican, 1 clone each.

Growth factors, such as TGF-$\beta$, fibroblast growth factor 7 (FGF7), vascular endothelial growth factor C (VEGFC), growth differentiation factor 10 (GDF10), and platelet-derived growth factor C (PDGFC) (1 clone each) were observed in redifferentiated DECs. Granulin, a putative growth factor, was detected at 20 clones in redifferentiated DECs. Connective tissue growth factor (CTGF), 24 clones; FGF7, 7 clones; granulin, 7 clones; and PDGFC, 1 clone; inhibin $\beta$A, 1 clone were detected in H4-1 cells. Among the growth factor-related genes, insulin-like growth factor binding protein 3 (IGFBP3) was highly observed in redifferentiated DECs (30 clones) and in H4-1 (131 clones). Gremlin or bone morphogenetic protein (BMP) antagonist 1 (CKTSF1B1), a member of cysteine knot superfamily, was detected at 6 clones in H4-1 cells. In the CCN family, WNT1-inducible signaling pathway protein 1 (WISP1) and WISP2 were observed in H4-1 cells (1 clone each). Among the Wnt family and signal pathway, frizzled-related protein (FRZB) was seen at 5 clones; dickkopf homolog 3, 3 clones; Wnt5a, 1 clone, in redifferentiated DECs.

Among the cell surface markers, CD29, CD44, CD46, CD54, CD55, CD68, CD90 (Thy-1), and CD105 were detected in redifferentiated DECs and CD10, CD24, CD29, CD44, CD55, CD59, CD73, CD90, CD97, CD105, CD119, and CD164 in H4-1 cells, while CD29, CD44, CD90, and CD105 were highly expressed in both redifferentiated DECs and H4-1 cells.

In the integrin family, integrin $\alpha$11, $\beta$1 (CD29) and $\beta$5 were seen in redifferentiated DECs, while integrin $\alpha$5 and $\beta$1 were detected in H4-1 cells. In the cadherin family, cadherin 11 was expressed in redifferentiated DECs, and cadherin 11 and 13 were observed in H4-1 cells. Among the cellular adhesion molecules cerebral cell adhesion molecule (C-CAM), junctional adhesion molecules, and intercellular adhesion molecule 1 (ICAM-1 or CD54) were expressed in redifferentiated DECs, while vascular cell adhesion molecule 1 (VCAM1) and melanoma adhesion molecule were expressed in H4-1 cells. Among the matrix metalloproteinase-related genes, matrix metalloproteinase 1 (MMP 1), MMP 2, MMP 3, tissue inhibitor of metalloproteinase 1 (TIMP 1), TIMP 3, a disintegrin, and metalloproteinase domain 15 (ADAM 15) were detected in redifferentiated DECs, while MMP 2, TIMP 3, and ADAM 9 were observed in H4-1 cells. Among the cytokine receptors, oncostatin M receptor was detected at 3 clones and the tumor necrosis factor receptor superfamily was observed at 6 clones. Among transcriptional factors, v-ets avian erythroblastosis virus E26 oncogene-related gene (ERG) was detected at 1 clone, v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ETS2), 2 clones, retinoic acid receptor gamma (RARG), 2 clones, SRY-box 9 gene (Sox 9) gene, 1 clone, in redifferentiated DECs. The sequences of the cDNAs were deposited in Genbank (AU279383–AU280837; total 1,455 clones).

Table 3
General analysis of RDEC, H4-1 (BMSC), and ThMSC1 cDNA libraries[a]

| | RDEC | H4-1 (BMSC) | ThMSC1 |
|---|---|---|---|
| Total number of cDNA clones successfully sequenced at the 5′ end | 2,628 | 2,687 | 671 |
| Number of clusters | 1,134 | 1,150 | 417 |
| Number of clusters with high similarity to RefSeq mRNA[b] | 804 | 849 | 308 |
| Number of clusters with low similarity to RefSeq mRNA | 334 | 301 | 109 |
| Percentage of clones with low similarity to RefSeq mRNA in the cDNA library[c] | 13% | 11% | 12% |

[a] "High" and "low" similarity means ≥96% and <96%, respectively. RDEC, redifferentiated dedifferentiated chondrocytes; BMSC, bone marrow stromal cells.

[b] Each cluster represents a RefSeq mRNA.

[c] Each percentage represents the number of clones with low similarity to RefSeq mRNA divided by the total number of cDNA clones sequenced.