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Cyclin D2 Promotes the Proliferation of Human Mesenchymal Stem Cells

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Abstract

Background: Human mesenchymal stem cells (hMSCs) hold promise for use in cell-based therapies and tissue engineering. Although hMSCs are thought to be stable *ex vivo*, it is possible that they undergo an undesirable transformation to a phenotype of unlimited proliferation during *ex vivo*. In this study, we searched for the factor required for unlimited proliferation of hMSCs.

Methods: Changes in gene expression were evaluated between hMSCs and Ewing's sarcoma cell lines, which may be derived from hMSCs, using GeneChip Human Genome U133 plus 2.0 Array. A gene up-regulated by at least 10-fold in Ewing's sarcoma cell lines, Cyclin D2, was overexpressed in hMSCs by a lentiviral vector.

Results: Overexpression of Cyclin D2 in hMSCs altered cell morphology and promoted cell proliferation. Expression of transforming growth factor-β2 (TGF-β2), which induces senescence in hMSCs, was down-regulated in Cyclin D2-overexpressing hMSCs. Furthermore, Gene Ontology analysis revealed that Cyclin D2 overexpression activated expression of genes associated with proliferation and interphase.

Conclusions: Cyclin D2 promotes hMSC proliferation and is a candidate biomaker for hMSC transformation.

Keywords: hMSCs; Ewing's sarcoma; Cyclin D2; Cell proliferation

Introduction

Mesenchymal stem cells (MSCs) self-replicate and differentiate into a variety of cell types such as osteoblasts, chondrocytes, adipocytes, and smooth muscle cells [1-5]. These capacities have made MSCs useful in studies of bone and cartilage regeneration [6-8]. One of the sources of human MSCs (hMSCs) is adult bone marrow, although they occur at a rate of one per one-hundred-thousand nucleated cells [6], and the available volume of bone marrow is limited. To secure the numbers of hMSCs required for tissue regeneration, the cell must be expanded *ex vivo*. Although hMSCs are stable *ex vivo*, it is possible that they undergo transformation to an unlimited proliferation phenotype during expansion.

Previous studies have demonstrated that Ewing's sarcoma is derived from MSCs [9-12]. Ewing's sarcoma is a malignancy that primarily affects children and young adults, with a peak incidence between the ages of 14 and 20 years. It arises mainly in bone and less commonly in soft tissues. The t(11;22)(q24;q12) chromosomal translocation generating EWS-FLI-1 fusion gene is found in 85% of cases [13]. EWS-FLI-1 knockdown inhibits cell proliferation in Ewing's sarcoma cells [14,15]. Thus, EWS-FLI-1 expression is believed to play a key role in Ewing's sarcoma development. However, EWS-FLI-1 expression does not transform normal murine and human fibroblasts [16,17], suggesting EWS-FLI-1 promotes malignant transformation in selective cells.

Several reports have demonstrated that EWS-FLI-1 expression transforms murine MSCs; indeed, tumors form when these cells are injected into immunodeficient mice [9,12]. In contrast, EWS-FLI-1 expression in hMSCs does not accelerate cell proliferation and transformation (10). EWS-FLI-1 expression in hMSCs induces a gene expression profile that closely mimics that of Ewing's sarcoma [9-11] without affecting proliferation. Therefore, MSCs are thought to be the origin of Ewing's sarcoma, but because EWS-FLI-1 alone cannot transform hMSCs, we believe other factors are required for transformation.

The most important safety concern when using hMSCs in cell-based therapies and tissue engineering is the occurrence of unlimited proliferation during *ex vivo* culture. To identify the factors required for unlimited hMSC proliferation, we compared the gene expression profiles of hMSCs and Ewing's sarcoma cell lines and found that Cyclin D2 expression was extremely high in the Ewing's sarcoma cell lines. Overexpression of Cyclin D2 promotes proliferation of hMSCs, suggesting that Cyclin D2 is a candidate biomaker for hMSC transformation.

Materials and Methods

Cell culture

hMSCs derived from bone marrow were purchased from Lonza (Walkersville, MD) and cultured in MSCGM BulletKit, a mesenchymal stem cell basal medium with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B (Lonza Walkersville, MD). Ewing's sarcoma cell lines (Hs 822.T, Hs 863.T, RD-ES, and SK-ES-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA). Hs 822.T and Hs 863.T were cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Intergene). RD-ES was cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. SK-ES-1 was cultured in McCoy's 5a medium modified (Gibco) supplemented with 15% FBS. 293T (human kidney; ATCC) was cultured in DMEM supplemented with 10% FBS.

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Microarray analysis

Total RNA was extracted from hMSCs and Ewing's sarcoma cell lines with the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA quantity and quality were assessed on an Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA); 100 ng total RNA was used to generate biotin-modified amplified RNA (aRNA) with GeneChip 3′IVT Express Kit (Affymetrix, Santa Clara, CA). Reverse transcription (RT) of first-strand complementary DNA (cDNA) with the T7 promoter sequence was performed with the T7 oligi(dT) primer. Second-strand cDNA synthesis was used to convert the single-stranded cDNA into a double-stranded DNA template by using DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA. In vitro transcription of biotin-modified aRNA with IVT Labeling Master Mix generated multiple copies of biotin-modified aRNA from the double-stranded cDNA templates. The aRNA was purified and quantified; after fragmentation, it was hybridized to GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). The arrays were stained with phycoerythrin and washed at the GeneChip Fluidics station 450 (Affymetrix). The microarrays were scanned and data extracted using GeneChip scanner 3000 7G (Affymetrix); image analysis was performed using the Affymetrix GeneChip Command Console Software and digitized with the Affymetrix Expression Console.

Data processing and pathway analysis

Data analysis was performed with GeneSpring GX 11.0 software (Agilent Technologies, Santa Clara, CA). Raw data were normalized to the 50th percentile per chip and the median per gene. Differentially expressed genes were analyzed using Ingenuity Pathway Analysis (IPA) 9.0 (Ingenuity Systems, Redwood City, CA). Fisher's exact test was used to calculate a P-value. Activation z-score was calculated as a measure of functional and translational activation in Networks and Upstream regulators analysis. An absolute z-score >2 was considered significant.

Real-time RT-PCR

Total RNA was reverse-transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies Co., Carlsbad, CA). Real-time RT-PCR was performed with LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science, Basel, Switzerland) in a Roche LightCycler instrument (software version 4.0). mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for Cyclin D2 and p16 were 5′-TACTTCAAGTGCGTGCAGAAGGAC-3′ and 5′-TCCCA-CACTTCCAGTTGCGATCAT-3′ (Cyclin D2) and 5′-CACTCAC-GCCCTAAGC-3′ and 5′-GCAGTGTGACTCAAGAGAA-3′ (p16). The primers for transforming growth factor-β2 (TGF-β2) and GAPDH were from Light Cycler Primer Sets (Search LC GmbH, Heidelberg, Germany).

Cloning and expression of Cyclin D2

Cyclin D2 cDNA was amplified by RT-PCR of mRNA extracted from SK-ES-1 using 5′-GAATTCGCCACCATGGAGCTGCTGT-GCCACGAGG-3′ (forward; *Eco*R I site underlined) and 5′-CTC-GAGTCACAGGTCGATATCCCGCACG-3′ (reverse; *Xho* I site underlined). The amplified products were cloned into pTA2 (ToYoBo, Osaka, Japan) and verified by sequencing. The verified Cyclin D2 cDNA was cloned into the *Eco*R I and *Xho* I sites of pLVSIN-CMV Pur (TaKaRa, Shiga, Japan). Lentiviral vector was prepared with the Lenti-XTM Packaging System (TaKaRa) according to manufacturer protocols.

Viral infection

hMSCs were infected with the lentiviral vector containing Cyclin D2 (hMSCs/CyclinD2) or empty vector (hMSCs/Empty) at 37°C for 24 h. Infected cells were selected with 1 μg/mL puromycin for 14 days and the bulk of the resistant cells was used in subsequent experiments.

Western blotting

hMSCs/CyclinD2 and hMSCs/Empty were lysed in RIPA buffer (Wako, Osaka, Japan). Cyclin D2 was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a membrane (Immunobilon-P^{SQ}; Millipore, Billerica, MA). Blots were blocked and probed overnight at 4°C with a mouse monoclonal antibody against Cyclin D2 (MBL, Nagoya, Japan). Blots were incubated with peroxidase-conjugated anti-mouse IgG (Abcam) and bound antibodies were visualized with Chemi-Lumi One Super Chemiluminescence (Nacalai Tesque, Kyoto, Japan).

Cell proliferation

Proliferation of hMSCs/CyclinD2 and hMSCs/Empty was measured with the TetraColor ONE reagent (Seikagaku Co., Tokyo, Japan). Cultures were incubated for 2 h in medium containing the reagent. Absorbance was read at 450 nm (reference at 600 nm) on a plate reader (SH-9000, Corona Electric Co., Ibaraki, Japan).

Results

Cyclin D2 expression in Ewing's sarcoma cell lines versus hMSCs

To identify the factors required for proliferation of hMSCs, we compared the gene expression profiles of hMSCs and four Ewing's sarcoma cell lines (Hs 822.T, Hs 863.T, RD-ES, and SK-ES-1) (Figure 1a). Hs 822.T and Hs 863.T had similar expression profiles, as did RD-ES and SK-ES-1. The expression profiles of Hs 822.T and Hs 863.T were more similar to those of hMSCs than to those of the other Ewing's sarcoma (RD-ES and SK-ES-1). Therefore, we first compared the expression profiles of hMSCs, Hs 822.T, and Hs 863.T. We identified 44 genes that differed by at least 10-fold between hMSCs and Ewing's sarcoma cell lines (data not shown). These were narrowed to 9 genes by selecting genes that also differed from hMSCs by more than 10-fold in RD-ES and SK-ES-1 (Table 1). CCND2 (Cyclin D2) stood out in this group of 9 genes, because it represents a family of key cell-cycle regulators. Indeed, aberrant expression of

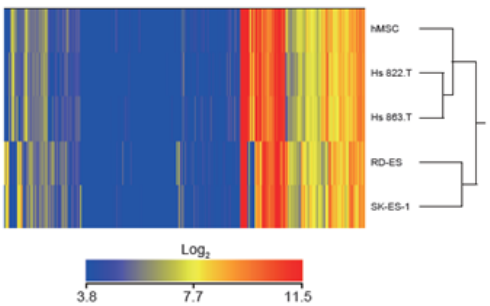

Figure 1a: Expression of Cyclin D2 was higher in Ewing's sarcoma cell lines than in hMSCs. Global gene expression was measured in hMSCs and Ewing's sarcoma cell lines. The whole-genome expression pattern is displayed in hierarchical cluster format. The color key shown at the bottom is in Log_2 scale.

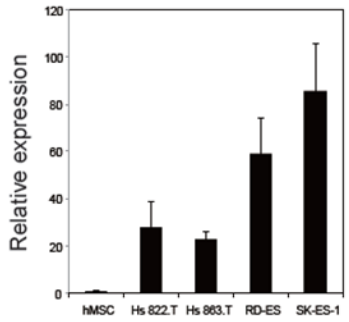

Figure 1b: Expression of Cyclin D2 was higher in Ewing's sarcoma cell lines than in hMSCs. Cyclin D2 expression in hMSCs and Ewing's sarcoma cell lines was determined by real-time RT-PCR with values normalized to GAPDH. Results are plotted relative to hMSCs. Data from triplicate samples (means ± SD) are shown.

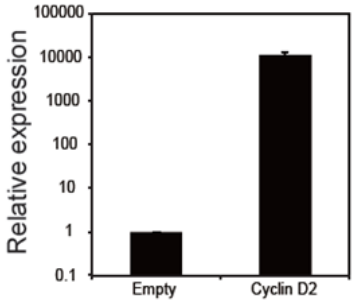

Figure 2a: Over expression of Cyclin D2 promoted hMSC proliferation. At 14 days after infection when puromycin selection was completed, Cyclin D2 mRNA in hMSCs/CyclinD2 and hMSCs/Empty was determined by real-time RT-PCR and normalized to GAPDH. Results are plotted relative to hMSCs/Empty.

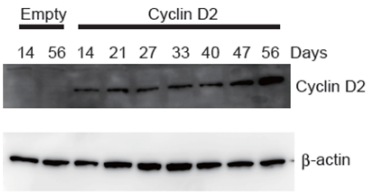

Figure 2b: Overexpression of Cyclin D2 promoted hMSC proliferation. Cyclin D2 protein expression was visualized by western blotting at the indicated days after infection. Equal amounts of protein were loaded in each lane.

Cyclin D2 has been associated with tumor progression in many tumor types [18-21]. We measured Cyclin D2 mRNA expression in hMSCs and Ewing's sarcoma cell lines by real-time PCR and confirmed its extreme induction in Ewing's sarcoma (Figure 1b). Hs 822.T and Hs 863.T exhibited similar Cyclin D2 expression levels; expression was higher in RD-ES and SK-ES-1.

Overexpression of Cyclin D2 promoted hMSC proliferation

We transduced the Cyclin D2 gene into hMSCs by using a lentiviral vector; an empty vector served as a negative control. At 14 days after infection when puromycin selection was completed, Cyclin D2 mRNA expression in hMSCs infected with lentiviral vector containing Cyclin D2 (hMSCs/CyclinD2) was about 10,000-fold higher than in hMSCs infected with empty vector (hMSCs/Empty) (Figure 2a). Cyclin D2 was stably expressed in hMSCs at least 56 days after infection (Figure 2b). Next, we observed the cell morphology of the hMSCs/CyclinD2. Phase contrast microscopy revealed the normal fibroblast-like morphology of hMSCs/Empty (Figure 2c) and smaller spread areas in hMSCs/CyclinD2 (Figures 2c and 2d). Furthermore, proliferation of hMSCs/CyclinD2 was faster than that of hMSCs/Empty, indicating that overexpression of Cyclin D2 promoted hMSC proliferation. However, the proliferation slowed over time and did not result in unlimited proliferation (data not shown).

TGF-β2 expression was down-regulated in hMSCs/CyclinD2, but p16 expression was not

To investigate the effects of overexpression of Cyclin D2 on the cell cycle, we examined the change in cell cycle-associated gene expression over time (p16, p21, Bmi1, TGF-β1, and TGF-β2). We did not detect significant differences in expression of p21, Bmi1, and TGF-β1 between hMSCs/CyclinD2 and hMSCs/Empty (data not shown). However, TGF-β2 expression was lower in hMSCs/CyclinD2 than in hMSCs/Empty (Figure 3a). In addition, the increase in TGF-β2 expression during culture was suppressed in hMSCs/CyclinD2 compared with hMSCs/Empty. In contrast, the increasing rate of p16 expression in hMSCs/CyclinD2 was higher than in hMSCs/Empty, although expression in both cell types was comparable 14 days after infection (Figure 3b).

Overexpression of Cyclin D2 altered the expression of genes associated with cell proliferation and interphase

Total RNA was extracted from hMSCs/CyclinD2 and hMSCs/Empty 14 days after infection and analyzed by DNA microarray, which identified 690 genes that were differentially expressed by at least 2-fold between hMSCs/CyclinD2 and hMSCs/Empty

Gene Symbol	Entrez Gene Name	Fold Change			
		Hs822.1	Hs863 T	RD-ES	SK-ES-1
BIEND5	BEN domain containing 5	-16.720	-14.819	27.302	26.690
CCND2	Cyclin D2	38.078	37.702	67.745	247.690
FBLN1	Fibulin1	-18.992	-24.363	-36.755	-198.523
HAPLN 1	Hyaluronan and and proteo glycan link protein 1	-22.460	-11.738	-26.674	-110.084
1GF2BP1	Insulin-Re growth factor 2 mRNA binding protein 1	22.847	22.358	288.588	207.475
SLC24A3	Solute carrier may 24 (sodium/potassium calcum exchanger), member 3	38.867	75.637	324.331	173.842
SSTR1	Somatostain receptor 1	79.594	14.840	22.406	28.783
STEAP4	STEAP family member 4	-24.490	-18.837	-21.747	-33.153
TMEFF2	Transmembrane protein with EGF-like and two follistatin-ike domain 2	37.577	10.463	34.418	197.834

Table 1: Expression of Cyclin D2 was higher in Ewing's sarcoma cell lines than in hMSCs. The nine genes differentially expressed by at least 10-fold between hMSCs and the four Ewing's sarcoma cell lines.

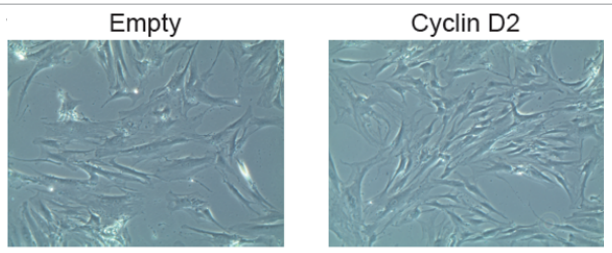

Figure 2c: Overexpression of Cyclin D2 promoted hMSC proliferation. At 14 days after infection, cells were observed by phase contrast microscopy.

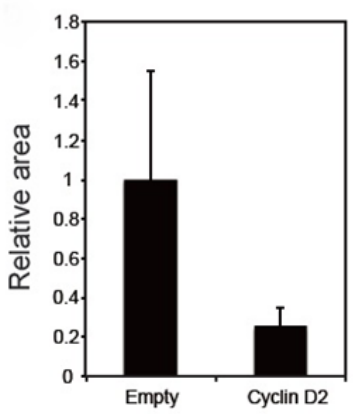

Figure 2d: Overexpression of Cyclin D2 promoted hMSC proliferation. Fifteen random hMSCs/Empty and hMSCs/CyclinD2 cells were measured with Image J 1.43t software (NIH, USA). Values were plotted relative to the averaged area of hMSCs/Empty.

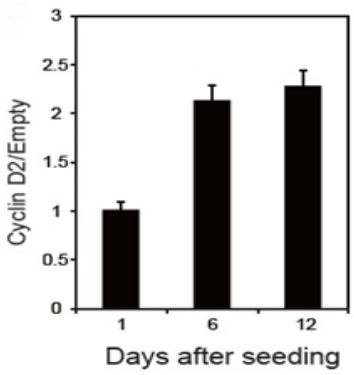

Figure 2e: Overexpression of Cyclin D2 promoted hMSC proliferation. At 14 days after infection, equal numbers of cells were seeded in 24-well plates and proliferation was determined with Tetra Color One 1, 6, and 12 days after seeding. The optical density (Cyclin D2/Empty) of triplicate samples is shown.

(Figure 4a). Gene ontology (GO) analysis revealed these genes are associated with movement, development, growth and proliferation, cell cycle, and intercellular signaling and interactions (Table 2). Specific predictions indicated that proliferation and interphase are activated in hMSCs/CyclinD2. The induced genes that are associated with proliferation and interphase are listed in tables 3 and 4; in summary, 94 of 186 genes and 19 of 50 genes exhibited expression shifts consistent with increased in proliferation and interphase, respectively.

Discussion

Although EWS-FLI-1 expression transformed murine MSCs, expression in hMSCs did not promote cell proliferation. In this study, we found that Cyclin D2 expression was extremely high in the Ewing's sarcoma cell lines and overexpression of Cyclin D2 in hMSCs promoted cell proliferation. GO analysis also predicted that cell proliferation and interphase were activated by overexpression of Cyclin D2.

Cyclin D2 is a member of the family of D-type cyclins that mediate cell cycle regulation, differentiation, and oncogenic transformation [22,23]. D-type cyclins inactivate retinoblastoma (Rb) by phosphorylation, inducing release of E2F. Free E2F activates genes involved in the activation and maintenance of DNA synthesis. Thus, overexpression of Cyclin D2 generally has growth-promoting effects. Consistent with this notion, overexpression of Cyclin D2 in HeLa

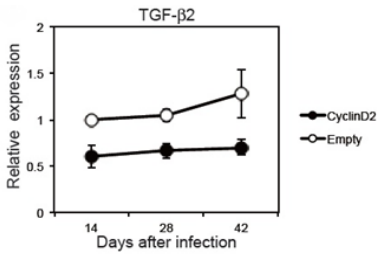

Figure 3a.TGF-β2 expression over time. TGF-β2 mRNA expression was determined by real-time RT-PCR at 14, 28, and 42 days after infection. Results are plotted relative to hMSCs/Empty at 14 days after infection. A representative of three independent experiments is shown

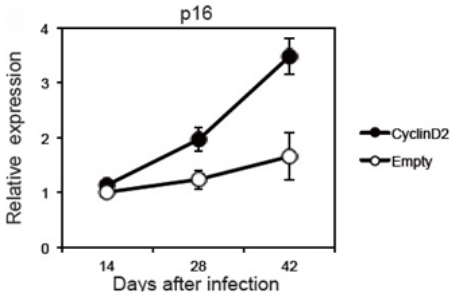

Figure 3b. p16 expression over time. p16 mRNA expression was determined by real-time RT-PCR at 14, 28, and 42 days after infection. Results are plotted relative to hMSCs/Empty at 14 days after infection. A representative of three independent experiments is shown.

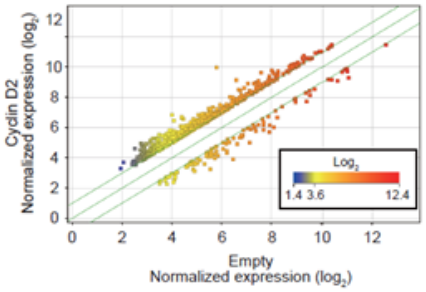

Figure 4a. Global gene expression in hMSCs/CyclinD2. Global gene expression patterns were compared in hMSCs/CyclinD2 and hMSCs/Empty. Genes differentially expressed by at least 2-fold are shown (690 genes). The color key shown at the bottom is in Log_2 scale

Top 5 functional category	Function annotation	p-Value	Activation z-score	No. of genes
Cellular Movement	cell movement of prostate cancer	7.94E-06	0.918	14
	migration of prostate cancer cells	1.54E-05	1.339	12
	recruitment of cells	6.57E-05	1.55	28
Cellular Development	proliferation of tumor cell lines	9.04E-06	1.15	92
	proliferation of cancer cells	1.96E-05	1.302	30
	differentiation of connective tissues	4.35E-05	-0.379	41
	proliferation of tumor cells	4.61E-05	1.036	37
Cellular Growth and Proliferation	proliferation of tumor cell lines	9.04E-06	1.15	92
	proliferation of cancer cells	1.96E-05	1.302	30
	proliferation of tumor cells	4.61E-05	1.036	37
	proliferation of cells	9.27E-05	3.142	186
Cell Cycle	interphase	1.34E-05	2.19	50
Cell-To-Cell Signaling and Interaction	recruitment leukocytes	7.13E-05	1.159	26

Table 2: Global gene expression in hMSCs/CyclinD2. Gene ontology (GO) analysis of the 690 genes was performed with Ingenuity Pathway Analysis (IPA) 9.0. Top five functional categories and the specified categories are listed. An absolute z-score >2 was considered as significant.

ID	Genes in dataset	Fold Change	Prediction (based on expression direction)	ID	Genes in dataset	Fold Change	Prediction (based on expression	ID	Genes in dataset	Fold Change	Prediction (based on Expression direction)
209292_at	ID4	3.609	Increased	201195_s_at	SLC7A5	2.083	direction)	203083_at	THBS2	2.096	Decreased
206271_at	TLR3	2.023	Increased	222749_at	SUFU	-3.251	Increased	203468_at	CDK10	2.228	Decreased
203373_at	SOCS2	3.513	Increased	204508_s_at	CA12	3.237	Increased	1552721_a_at	FGF1	-2.054	Decreased
206649_s_at	TFE3	2.069	Increased	203764_at	DLGAP5	2.188	Increased	204052_s_at	SFRP4	2.249	Decreased
201292_at	TOP2A	2.434	Increased	214012_at	ERAP1	5.288	Increased	205548_s_at	BTG3	2.073	Decreased
204766_s_at	NUDT1	2.158	Increased	204614_at	SERPINB2	-2.048	Increased	242989_at	STRN	2.145	Decreased
209321_s_at	ADCY3	2.329	Increased	201416_at	SOX4	3.11	Increased	213905_x_at	BGN	2.09	Decreased
206693_at	IL7	2.399	Increased	214581_x_at	TNFRSF21	-2.049	Increased	228780_at	POU3F3	-2.721	Decreased
205345_at	BARD1	2.029	Increased	223570_at	MCM10	2.545	Increased	205016_at	TGFA	-3.032	Decreased
226377_at	NFIC	2.026	Increased	219743_at	HEY2	2.13	Increased	1569791_at	STK4	2.396	Decreased
212148_at	PBX1	2.514	Increased	209970_x_at	CASP1	3.111	Increased	216008_s_at	ARIH2	2.331	Decreased
202213_s_at	CUL4B	2.093	Increased	202684_s_at	RNMT	2.503	Increased	221577_x_at	GDF15	2.143	Decreased
224954_at	SHMT1	2.105	Increased	242979_at	IRS1	2.329	Increased	202153_s_at	NUP62	2.009	Decreased
209960_at	HGF	2.732	Increased	225141_at	NFATC3	2.553	Increased	202556_s_at	MCRS1	2.165	Decreased
217371_s_at	IL15	2.953	Increased	218030_at	GIT1	3.032	Increased	203395_s_at	HES1	2.898	Decreased
205887_x_at	MSH3	2.1	Increased	218750_at	TAF1D	2.304	Increased	203184_at	FBN2	2.145	Decreased
1568865_at	FNTB	2.556	Increased	201795_at	LBR	2.383	Increased	203904_x_at	CD82	2.382	Decreased
208296_x_at	TNFAIP8	2.118	Increased	210045_at	IDH2	2.018	Increased	206558_at	SIM2	2.745	Decreased
226534_at	KITLG	2.22	Increased	1553810_a_at	KIAA1524	2.457	Increased	203665_at	HMOX1	2.358	Decreased
214981_at	POSTN	2.095	Increased	209160_at	AKR1C3	2.281	Increased	203543_s_at	KLF9	2.577	Decreased
206026_s_at	TNFAIP6	2.008	Increased	232424_at	PRDM16	2.334	Increased	1557729_at	GRK5	2.098	Decreased
210135_s_at	SHOX2	2.194	Increased	1554509_a_at	FAM188A	-2.494	Increased	236028_at	IBSP	-3.459	Decreased
204457_s_at	GAS1	2.134	Increased	217494_s_at	PTENP1	-2.849	Increased	221539_at	EIF4EBP1	2.487	Decreased
221884_at	MECOM	2.004	Increased	216205_s_at	MFN2	-2.34	Increased	202430_s_at	PLSCR1	2.324	Decreased
209919_x_at	GGT1	2.63	Increased	200644_at	MARCKSL1	3.121	Increased	204054_at	PTEN	2.145	Decreased
229468_at	CDK3	2.022	Increased	217991_x_at	SSBP3	3.21	Increased	209617_s_at	CTNND2	-2.01	Decreased
1563182_at	ACVR1C	-2.241	Increased	211653_x_at	AKR1C1/ AKR1C2	2.791	Increased	231697_s_at	VMP1	2.741	Decreased
227404_s_at	EGR1	2.543	Increased	228302_x_at	CAMK2N1	-3.3	Increased	210143_at	ANXA10	2.124	Decreased
210933_s_at	FSCN1	2.227	Increased	234040_at	HELLS	2.476	Increased	206233_at	B4GALT6	-2.767	Decreased
205357_s_at	AGTR1	2.987	Increased	218413_s_at	ZNF639	2.019	Increased	209705_at	MTF2	2.504	Decreased
209925_at	OCLN	-2.024	Increased	242907_at	GBP2	2.488	Increased	209802_at	PHLDA2	2.17	Decreased
205732_s_at	NCOA2	2.484	Increased	219377_at	GAREM	2.237	Increased	219685_at	TMEM35	-2.404	Decreased
215404_x_at	FGFR1	2.421	Increased	212865_s_at	COL14A1	2.516	Increased	219047_s_at	ZNF668	2.244	Decreased
235521_at	HOXA3	2.05	Increased	203572_s_at	TAF6	2.79	Decreased	64474_g_at	DGCR8	2.201	Decreased
218807_at	VAV3	2.555	Increased	232231_at	RUNX2	2.12	Decreased	205159_at	CSF2RB	-4.605	Decreased
202202_s_at	LAMA4	3.771	Increased	202931_x_at	BIN1	2.056	Decreased	236012_at	PSMF1	2.172	Affected

205500_at	C5	2.375	Increased	214433_s_at	SELENBP1	2.103	Decreased	235833_at	PPAT	3.266	Affected
204128_s_at	RFC3	2.06	Increased	228766_at	CD36	-4.325	Decreased	221261_x_at	MAGED4/ MAGED4B	3.294	Affected
200951_s_at	CCND2	18.529	Increased	235300_x_at	RCHY1	2.569	Decreased	204983_s_at	GPC4	-2.902	Affected
222073_at	COL4A3	-2.278	Increased	222999_s_at	CCNL2	2.019	Decreased	231837_at	USP28	2.365	Affected
226731_at	ITGA1	2.816	Increased	208791_at	CLU	-2.492	Decreased	1554606_at	CEP120	2.141	Affected
227125_at	IFNAR2	2.053	Increased	203973_s_at	CEBPD	2.099	Decreased	224325_at	FZD8	2.488	Affected
222036_s_at	MCM4	2.31	Increased	209383_at	DDIT3	2.065	Decreased	217650_x_at	ST3GAL2	2.003	Affected
204061_at	PRKX	2.226	Increased	219266_at	ZNF350	2.143	Decreased	224022_x_at	WNT16	-3.268	Affected
228962_at	PDE4D	2.562	Increased	206825_at	OXTR	-2.396	Decreased	208962_s_at	FADS1	3.311	Affected
212672_at	ATM	2.024	Increased	205027_s_at	MAP3K8	2.11	Decreased	202948_at	IL1R1	2.512	Affected
225572_at	CREB1	2.243	Increased	205891_at	ADORA2B	2.166	Decreased	50277_at	GGA1	2.292	Affected
203046_s_at	TIMELESS	2.156	Increased	231947_at	MYCT1	-2.144	Decreased	1555843_at	HNRNPM	2.611	Affected
213943_at	TWIST1	2.021	Increased	212430_at	RBM38	2.254	Decreased	214157_at	GNAS	-2.307	Affected
209465_x_at	PTN	2.357	Increased	204159_at	CDKN2C	2.004	Decreased	215987_at	RAPGEF2	2.046	Affected
213506_at	F2RL1	4.829	Increased	212401_s_at	CDK11A/ CDK11B	2.086	Decreased	216237_s_at	MCM5	2.753	Affected
231559_at	NNMT	2.208	Increased	205080_at	RARB	2.058	Decreased	206086_x_at	HFE	2.218	Affected
225740_x_at	MDM4	2.283	Increased	214727_at	BRCA2	2.489	Decreased	229807_s_at	MAZ	2	Affected
226636_at	PLD1	2.022	Increased	202718_at	IGFBP2	-2.099	Decreased	231002_s_at	RABEP1	2.266	Affected
227048_at	LAMA1	2.296	Increased	213811_x_at	TCF3	2.395	Decreased	209753_s_at	TMPO	2.116	Affected
230462_at	NUMB	2.763	Increased	202526_at	SMAD4	2.16	Decreased	201627_s_at	INSIG1	3.156	Affected
201727_s_at	ELAVL1	2.216	Increased	1567013_at	NFE2L2	3.005	Decreased	218019_s_at	PDXK	2.152	Affected
205204_at	NMB	2.09	Increased	234339_s_at	GLTSCR2	2.573	Decreased	204639_at	ADA	2.051	Affected
232044_at	RBBP6	2.324	Increased	206332_s_at	IFI16	2.24	Decreased	236223_s_at	RIT1	2.823	Affected
205394_at	CHEK1	2.071	Increased	228967_at	EIF1	2.161	Decreased	208913_at	GGA2	2.062	Affected
57532_at	DVL2	2.03	Increased	235593_at	ZEB2	2.106	Decreased	201286_at	SDC1	2.164	Affected
208937_s_at	ID1	2.256	Increased	1556583_a_at	SLC8A1	-2.156	Decreased	201106_at	GPX4	2.087	Affected

'Increased' means the genes up- or down-expression is predicted to promote proliferation of cells. 'Decreased' means the up- or down-expression is predicted to inhibit. 'Affected' means IPA could not predict whether the expression change promote or inhibit.

Table 3: 'Proliferation of cells' genes differentially expressed by at least 2-fold between hMSCs/CyclinD2 and hMSCs/Empty.

ID	Genes in dataset	Fold Change	Prediction (based on expression direction)
206649_s_at	TFE3	2.069	Increased
209960_at	HGF	2.732	Increased
217371_s_at	IL15	2.953	Increased
232231_at	RUNX2	2.12	Increased
235300_x_at	RCHY1	2.569	Increased
226534_at	KITLG	2.22	Increased
235423_at	ORC2	3.145	Increased
203973_s_at	CEBPD	2.099	Increased
227404_s_at	EGR1	2.543	Increased
205027_s_at	MAP3K8	2.11	Increased
212401_s_at	CDK11A/CDK11B	2.086	Increased
213811_x_at	TCF3	2.395	Increased
206332_s_at	IFI16	2.24	Increased
212672_at	ATM	2.024	Increased
235593_at	ZEB2	2.106	Increased
205394_at	CHEK1	2.071	Increased
208937_s_at	ID1	2.256	Increased
223570_at	MCM10	2.545	Increased
242979_at	IRS1	2.329	Increased
212148_at	PBX1	2.514	Decreased
204159_at	CDKN2C	2.004	Decreased
1552721_a_a	FGF1	-2.054	Decreased
205016_at	TGFA	-3.032	Decreased
201727_s_at	ELAVL1	2.216	Decreased
203395_s_at	HES1	2.898	Decreased

203665_at	HMOX1	2.358	Decreased
204054_at	PTEN	2.145	Decreased
219312_s_at	ZBTB10	2.171	Affected
229861_at	RFFL	2.017	Affected
228302_x_at	CAMK2N1	-3.3	Affected
235764_at	PRDM5	2.205	Affected
1554509_a_a	FAM188A	-2.494	Affected
232424_at	PRDM16	2.334	Affected
221539_at	EIF4EBP1	2.487	Affected
204986_s_at	TAOK2	-2.03	Affected
221577_x_at	GDF15	2.143	Affected
225740_x_at	MDM4	2.283	Affected
203046_s_at	TIMELESS	2.156	Affected
200951_s_at	CCND2	18.529	Affected
1567013_at	NFE2L2	3.005	Affected
215404_x_at	FGFR1	2.421	Affected
209753_s_at	TMPO	2.116	Affected
209383_at	DDIT3	2.065	Affected
229468_at	CDK3	2.022	Affected
204457_s_at	GAS1	2.134	Affected
218833_at	ZAK	2.125	Affected
214048_at	MBD4	2.383	Affected
205345_at	BARD1	2.029	Affected
206693_at	IL7	2.399	Affected
209292_at	ID4	3.609	Affected

'Increased' means the genes up- or down-expression is predicted to increase the function of interphase. 'Decreased' means the up- or down-expression is predicted to decrease. 'Affected' means IPA could not predict whether the expression change increase or decrease

Table 4. 'Interphase' genes differentially expressed by at least 2-fold between hMSCs/CyclinD2 and hMSCs/Empty.

cells, in which Rb is inactivated by human papillomavirus E6 and E7 proteins [24], did not promote cell proliferation (data not shown). On the other hand, increased expression of Cyclin D2 inhibits proliferation of primary human fibroblasts [25], indicating that Cyclin D2 has both positive and negative roles in the cell cycle, depending on cell type. We found that Cyclin D2 in hMSCs has a positive role in the cell cycle (Figure 2e).

TGF-β2 expression was suppressed in hMSCs/CyclinD2 compared with hMSC/Empty during culture (Figure 3a). We previously demonstrated that hMSC growth is reduced and TGF-β2 expression increases during long-term culture [26]. We also reported that fibroblast growth factor-2 (FGF-2) stimulates hMSC growth by suppressing the up-regulation of TGF-β2 [27]. It is unclear how overexpression of Cyclin D2 suppresses the TGF-β2 increase, but this suppression may be involved in the acceleration of hMSCs/CyclinD2. In contrast, the expression of p16, which is up-regulated with aging [28], was increased in both cell types during culture, indicating that not only hMSCs/Empty but also hMSCs/CyclinD2 were aging normally. The rate of increase in hMSCs/Cyclin D2 was higher than in hMSCs/Empty (Figure 3b), suggesting that the promotion of cell proliferation in hMSCs/CyclinD2 induced cellular senescence and enhanced p16 expression. p16 is a tumor suppressor gene [29]; thus, this increase in p16 expression probably prevented unlimited proliferation. Consistent with this notion, some Ewing's sarcomas contain a homozygous deletion of the p16 locus [16], possibly facilitating subsequent transformation.

In this study, overexpression of Cyclin D2 promoted proliferation of hMSCs but did not lead to unlimited proliferation. Other factors are required for the unlimited proliferation of hMSCs. IGF2BP1 was aberrantly expressed in Ewing's sarcoma (Table 1), consistent with a previous report of an association between increased IGF2BP1 expression and tumor progression in patients with lung cancer [30]. Thus, we attempted to transduce the IGF2BP1 gene into hMSCs, but IGF2BP1 expression was up-regulated by only 2-fold and transduction efficiency was low (data not shown). The cause for this inefficiency is unclear. Because the growth kinetics of IGF2BP1-transformed *E. coli* is quite slow (data not shown), it is likely that overexpression of IGF2BP1 is deleterious for hMSCs.

We did not tested whether the other genes listed in Table 1 affect proliferation of hMSCs, because these genes were not thought to directly affect the proliferation. Furthermore, not all Ewing's sarcomas express EWS-FLI-1: indeed, EWS-FLI-1 mRNA was not detected in Hs 822.T and Hs 863.T (data not shown). Thus, we did not transduce the EWS-FLI-1 gene into hMSCs. However, it is possible that the cooperation of these proteins is important for the development of Ewing's sarcoma. Thus, it would be interesting to transduce these genes into hMSCs in addition to Cyclin D2.

Conclusion

Cyclin D2 promotes hMSC proliferation and is a candidate biomaker for hMSC transformation.

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