Bantel-Schaal U, zur Hausen H. (1988) Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. *Virology* **164**(1): 64–74.

Batchu RB, Hermonat PL. (1995) The trans-inhibitory Rep78 protein of adeno-associated virus binds to TAR region DNA of the human immunodeficiency virus type 1 long terminal repeat. *FEBS Lett* **367**(3): 267–71.

Batchu RB, Kotin RM, Hermonat PL. (1994) The regulatory rep protein of adeno-associated virus binds to sequences within the c-H-ras promoter. *Cancer Lett* **86**(1): 23–31.

Batchu RB, Shammas MA, Wang JY, *et al.* (2002) Adeno-associated virus protects the retinoblastoma family of proteins from adenoviral-induced functional inactivation. *Cancer Res* **62**(10): 2982–5.

Batchu RB, Shammas MA, Wang JY, *et al.* (1999) Interaction of adeno-associated virus Rep78 with p53: implications in growth inhibition. *Cancer Res* **59**(15): 3592–5.

Batchu RB, Shammas MA, Wang JY, *et al.* (2001) Dual level inhibition of E2F-1 activity by adeno-associated virus Rep78. *J Biol Chem* **276**(26): 24315–22.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Bishop AL, Hall A. (2000) Rho GTPases and their effector proteins. *Biochem J* **348** (Pt 2): 241–55.

Brister JR, Muzyczka N. (1999) Rep-mediated nicking of the adeno-associated virus origin requires two biochemical activities, DNA helicase activity and transesterification. *J Virol* **73**(11): 9325–36.

Casper JM, Timpe JM, Dignam JD, *et al.* (2005) Identification of an adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. *J Virol* **79**(1): 28–38.

Cathomen T, Collete D, Weitzman MD. (2000) A chimeric protein containing the N terminus of the adeno-associated virus Rep protein recognizes its target site in an in vivo assay. *J Virol* **74**(5): 2372–82.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**(5466): 669–72.

Chiorini JA, Yang L, Safer B, *et al.* (1995) Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. *J Virol* **69**(11): 7334–8.

Chiorini JA, Zimmermann B, Yang L, *et al.* (1998) Inhibition of PrKX, a novel protein kinase, and the cyclic AMP-dependent protein kinase PKA by the regulatory proteins of adeno-associated virus type 2. *Mol Cell Biol* **18**(10): 5921–9.

Ciechanover A, Shkedy D, Oren M, *et al.* (1994) Degradation of the tumor suppressor protein p53 by the ubiquitin-mediated proteolytic system requires a novel species of ubiquitin-carrier protein, E2. *J Biol Chem* **269**(13): 9582–9.

Collaco RF, Kalman-Maltese V, Smith AD, *et al.* (2003) A biochemical characterization of the adeno-associated virus Rep40 helicase. *J Biol Chem* **278**(36): 34011–7.

Costello E, Saudan P, Winocour E, *et al.* (1997) High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression. *Embo J* **16**(19): 5943–54.

Di Pasquale G, Stacey SN. (1998) Adeno-associated virus Rep78 protein interacts with protein kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. *J Virol* **72**(10): 7916–25.

Duan D, Sharma P, Yang J, *et al.* (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* **72**(11): 8568–77.

Dutheil N, Yoon-Robarts M, Ward P, *et al.* (2004) Characterization of the mouse adeno-associated virus AAVS1 ortholog. *J Virol* **78**(16): 8917–21.

Elliott G, O'Hare P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**(2): 223–33.

Feng D, Chen J, Yue Y, *et al.* (2006) A 16bp Rep binding element is sufficient for mediating Rep-dependent integration into AAVS1. *J Mol Biol* **358**(1): 38–45.

Francois A, Guilbaud M, Awedikian R, *et al.* (2005) The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. *J Virol* **79**(17): 11082–94.

Fu H, Subramanian RR, Masters SC. (2000) 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* **40**: 617–47.

Glauser DL, Saydam O, Balsiger NA, *et al.* (2005) Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. *J Virol* **79**(19): 12218–30.

Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* (2002) Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med* **346**(16): 1185–93.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al.* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**(5644): 415–9.

Hamilton H, Gomos J, Berns KI, *et al.* (2004) Adeno-associated virus site-specific integration and AAVS1 disruption. *J Virol* **78**(15): 7874–82.

Han SI, Kawano MA, Ishizu K, *et al.* (2004) Rep68 protein of adeno-associated virus type 2 interacts with 14-3-3 proteins depending on phosphorylation at serine 535. *Virology* **320**(1): 144–55.

Heister T, Heid I, Ackermann M, *et al.* (2002) Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. *J Virol* **76**(14): 7163–73.

Hermanns J, Schulze A, Jansen-Dblurr P, *et al.* (1997) Infection of primary cells by adeno-associated virus type 2 results in a modulation of cell cycle-regulating proteins. *J Virol* **71**(8): 6020–7.

Hermonat PL. (1991) Inhibition of H-ras expression by the adeno-associated virus Rep78 transformation suppressor gene product. *Cancer Res* **51**(13): 3373–7.

Hermonat PL. (1994) Down-regulation of the human c-fos and c-myc proto-oncogene promoters by adeno-associated virus Rep78. *Cancer Lett* **81**(2): 129–36.

Hermonat PL, Santin AD, Batchu RB. (1996) The adeno-associated virus Rep78 major regulatory/transformation suppressor protein binds cellular Sp1 *in vitro* and evidence of a biological effect. *Cancer Res* **56**(22): 5299–304.

Hermonat PL, Santin AD, Batchu RB, *et al.* (1998) The adeno-associated virus Rep78 major regulatory protein binds the cellular TATA-binding protein *in vitro* and *in vivo*. *Virology* **245**(1): 120–7.

Hickman AB, Ronning DR, Kotin RM, *et al.* (2002) Structural unity among viral origin binding proteins: crystal structure of the nuclease domain of adeno-associated virus Rep. *Mol Cell* **10**(2): 327–37.

Hickman AB, Ronning DR, Perez ZN, *et al.* (2004) The nuclease domain of adeno-associated virus rep coordinates replication initiation using two distinct DNA recognition interfaces. *Mol Cell* **13**(3): 403–14.

Holscher C, Kleinschmidt JA, Burkle A. (1995) High-level expression of adeno-associated virus (AAV) Rep78 or Rep68 protein is sufficient for infectious-particle formation by a rep-negative AAV mutant. *J Virol* **69**(11): 6880–5.

Huttner NA, Girod A, Schnittger S, *et al.* (2003) Analysis of site-specific transgene integration following cotransduction with recombinant adeno-associated virus and a rep encodingplasmid. *J Gene Med* **5**(2): 120–9.

Im DS, Muzyczka N. (1990) The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**(3): 447–57.

Jing XJ, Kalman-Maltese V, Cao X, *et al.* (2001) Inhibition of adenovirus cytotoxicity, replication, and E2a gene expression by adeno-associated virus. *Virology* **291**(1): 140–51.

Kearns WG, Afione SA, Fulmer SB, *et al.* (1996) Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Ther* **3**(9): 748–55.

Khleif SN, Myers T, Carter BJ, *et al.* (1991) Inhibition of cellular transformation by the adeno-associated virus rep gene. *Virology* **181**(2): 738–41.

King JA, Dubielzig R, Grimm D, *et al.* (2001) DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *Embo J* **20**(12): 3282–91.

Klein-Bauernschmitt P, zur Hausen H, Schlehofer JR. (1992) Induction of differentiation-associated changes in established human cells by infection with adeno-associated virus type 2. *J Virol* **66**(7): 4191–200.

Kogure K, Urabe M, Mizukami H, *et al.* (2001) Targeted integration of foreign DNA into a defined locus on chromosome 19 in K562 cells using AAV-derived components. *Int J Hematol* **73**(4): 469–75.

Kotin RM, Linden RM, Berns KI. (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by nonhomologous recombination. *Embo J* **11**(13): 5071–8.

Kotin RM, Siniscalco M, Samulski RJ, *et al.* (1990) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **87**(6): 2211–5.

Kyostio SR, Wonderling RS, Owens RA. (1995) Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. *J Virol* **69**(11): 6787–96.

Labow MA, Graf LH Jr, Berns KI. (1987) Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. *Mol Cell Biol* **7**(4): 1320–5.

Lamartina S, Roscilli G, Rinaudo D, *et al.* (1998) Lipofection of purified adeno-associated virus Rep68 protein: toward a chromosome-targeting nonviral particle. *J Virol* **72**(9): 7653–8.

Leza MA, Hearing P. (1989) Independent cyclic AMP and E1A induction of adenovirus early region 4 expression. *J Virol* **63**(7): 3057–64.

Lieber A, Steinwaerder DS, Carlson CA, *et al.* (1999) Integrating adenovirus-adeno-associated virus hybrid vectors devoid of all viral genes. *J Virol* **73**(11): 9314–24.

Linden RM, Ward P, Giraud C, *et al.* (1996a) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **93**(21): 11288–94.

Linden RM, Winocour E, Berns KI. (1996b) The recombination signals for adeno-associated virus site-specific integration. *Proc Natl Acad Sci USA* **93**(15): 7966–72.

Marcello A, Massimi P, Banks L, *et al.* (2000) Adeno-associated virus type 2 rep protein inhibits human papillomavirus type 16 E2 recruitment of the transcriptional coactivator p300. *J Virol* **74**(19): 9090–8.

McKenna NJ, Lanz RB, O'Malley BW. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**(3): 321–44.

Mehrle S, Rohde V, Schlehofer JR. (2004) Evidence of chromosomal integration of AAV DNA in human testis tissue. *Virus Genes* **28**(1): 61–9.

Meneses P, Berns KI, Winocour E. (2000) DNA sequence motifs which direct adeno-associated virus site-specific integration in a model system. *J Virol* **74**(13): 6213–6.

Muramatsu S, Handa A, Kajigaya S, *et al.* (1998) Transcription-positive cofactor 4 enhances rescue of adeno-associated virus genome from an infectious clone. *J Gen Virol* **79** (Pt 9): 2157–61.

Muzyczka N, Berns KI. (2001) Parvoviridae: The Viruses and Their Replication, 4th ed. In: *Fields' Virology*, Knipe DM, Howley PM, Griffin DE, *et al.* (eds.) Lippincott Williams & Wilkins, Philadelphia, pp. 2327–2359.

Nada S, Trempe JP. (2002) Characterization of adeno-associated virus rep protein inhibition of adenovirus E2a gene expression. *Virology* **293**(2): 345–55.

Nakai H, Yant SR, Storm TA, *et al.* (2001) Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J Virol* **75**(15): 6969–76.

Nam CH, Rabbitts TH. (2006) The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Mol Ther* **13**(1): 15–25.

Needham PG, Casper JM, Kalman-Maltese V, *et al.* (2006) Adeno-associated virus rep protein-mediated inhibition of transcription of the adenovirus major late promoter in vitro. *J Virol* **80**(13): 6207–17.

Ni TH, McDonald WF, Zolotukhin I, *et al.* (1998) Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. *J Virol* **72**(4): 2777–87.

Ni TH, Zhou X, McCarty DM, *et al.* (1994) *In vitro* replication of adeno-associated virus DNA. *J Virol* **68**(2): 1128–38.

Nony P, Tessier J, Chadeuf G, *et al.* (2001) Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. *J Virol* **75**(20): 9991–4.

O'Gorman S, Fox DT, Wahl GM. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* **251**(4999): 1351–5.

Oelze I, Rittner K, Sczakiel G. (1994) Adeno-associated virus type 2 rep gene-mediated inhibition of basal gene expression of human immunodeficiency virus type 1 involves its negative regulatory functions. *J Virol* **68**(2): 1229–33.

Palombo F, Monciotti A, Recchia A, *et al.* (1998) Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J Virol* **72**(6): 5025–34.

Pereira DJ, Muzyczka N. (1997) The adeno-associated virus type 2 p40 promoter requires a proximal Sp1 interaction and a p19 CArG-like element to facilitate Rep transactivation. *J Virol* **71**(6): 4300–9.

Philip R, Brunette E, Kilinski L, *et al.* (1994) Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol Cell Biol* **14**(4): 2411–8.

Philpott NJ, Giraud-Wali C, Dupuis C, *et al.* (2002a) Efficient integration of recombinant adeno-associated virus DNA vectors requires a p5-rep sequence in cis. *J Virol* **76**(11): 5411–21.

Philpott NJ, Gomos J, Berns KI, *et al.* (2002b) A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. *Proc Natl Acad Sci USA* **99**(19): 12381–5.

Philpott NJ, Gomos J, Falck-Pedersen E. (2004) Transgene expression after rep-mediated site-specific integration into chromosome 19. *Hum Gene Ther* **15**(1): 47–61.

Pieroni L, Fipaldini C, Monciotti A, *et al.* (1998) Targeted integration of adeno-associated virus-derived plasmids in transfected human cells. *Virology* **249**(2): 249–59.

Recchia A, Parks RJ, Lamartina S, *et al.* (1999) Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. *Proc Natl Acad Sci USA* **96**(6): 2615–20.

Recchia A, Perani L, Sartori D, *et al.* (2004) Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. *Mol Ther* **10**(4): 660–70.

Rinaudo D, Lamartina S, Roscilli G, *et al.* (2000) Conditional site-specific integration into human chromosome 19 by using a ligand-dependent chimeric adeno-associated virus/Rep protein. *J Virol* **74**(1): 281–94.

Ryan JH, Zolotukhin S, Muzyczka N. (1996) Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. *J Virol* **70**(3): 1542–53.

Samulski RJ, Zhu X, Xiao X, *et al.* (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *Embo J* **10**(12): 3941–50.

Satoh W, Hirai Y, Tamayose K, *et al.* (2000) Site-specific integration of an adeno-associated virus vector plasmid mediated by regulated expression of rep based on Cre-loxP recombination. *J Virol* **74**(22): 10631–8.

Saudan P, Vlach J, Beard P. (2000) Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. *Embo J* **19**(16): 4351–61.

Sauer B, Henderson N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* **85**(14): 5166–70.

Schnepp BC, Jensen RL, Chen CL, *et al.* (2005) Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol* **79**(23): 14793–803.

Shelling AN, Smith MG. (1994) Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene. *Gene Ther* **1**(3): 165–9.

Smith RH, Kotin, RM. (1998) The Rep52 gene product of adeno-associated virus is a DNA helicase with 3′-to-5′ polarity. *J Virol* **72**(6): 4874–81.

Smith RH, Kotin RM. (2000) An adeno-associated virus (AAV) initiator protein, Rep78, catalyzes the cleavage and ligation of single-stranded AAV ori DNA. *J Virol* **74**(7): 3122–9.

Smith RH, Kotin RM. (2002) Adeno-associated virus. *In Mobile DNA II* (Craig, N., Craigie, R., Gellert, M., Lambowitz, A. M., Ed.). ASM Press, Washington, D. C., pp. 905–923.

Smith RH, Spano AJ, Kotin RM. (1997) The Rep78 gene product of adeno-associated virus (AAV) self-associates to form a hexameric complex in the presence of AAV ori sequences. *J Virol* **71**(6): 4461–71.

Song S, Lu Y, Choi YK, *et al.* (2004) DNA-dependent PK inhibits adeno-associated virus DNA integration. *Proc Natl Acad Sci USA* **101**(7): 2112–6.

Stracker TH, Cassell GD, Ward P, *et al.* (2004) The Rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. *J Virol* **78**(1): 441–53.

Su PF, Chiang SY, Wu CW, *et al.* (2000) Adeno-associated virus major Rep78 protein disrupts binding of TATA-binding protein to the p97 promoter of human papillomavirus type 16. *J Virol* **74**(5): 2459–65.

Suomalainen M, Nakano MY, Boucke K, *et al.* (2001) Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. *Embo J* **20**(6): 1310–9.

Surosky RT, Urabe M, Godwin SG, *et al.* (1997) Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J Virol* **71**(10): 7951–9.

Takeuchi T, Kozuka T, Nakagawa K, *et al.* (2000) Adeno-associated virus type 2 nonstructural protein Rep78 suppresses translation *in vitro*. *Virology* **266**(1): 196–202.

Tan I, Ng CH, Lim L, *et al.* (2001) Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of actin cytoskeleton. *J Biol Chem* **276**(24): 21209–16.

Tessier J, Chadeuf G, Nony P, *et al.* (2001) Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. *J Virol* **75**(1): 375–83.

Thyagarajan B, Olivares EC, Hollis RP, *et al.* (2001) Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol Cell Biol* **21**(12): 3926–34.

Tratschin JD, Miller IL, Smith MG, *et al.* (1985) Adeno-associated virus vector for high-frequency integration, expression, and rescue of genes in mammalian cells. *Mol Cell Biol* **5**(11): 3251–60.

Tsunoda H, Hayakawa T, Sakuragawa N, *et al.* (2000) Site-specific integration of adeno-associated virus-based plasmid vectors in lipofected HeLa cells. *Virology* **268**(2): 391–401.

Tullis GE, Shenk T. (2000) Efficient replication of adeno-associated virus type 2 vectors: a cis-acting element outside of the terminal repeats and a minimal size. *J Virol* **74**(24): 11511–21.

Ueno T, Matsumura H, Tanaka K, *et al.* (2000) Site-specific integration of a transgene mediated by a hybrid adenovirus/adeno-associated virus vector using the Cre/loxP-expression-switching system. *Biochem Biophys Res Commun* **273**(2): 473–8.

Urabe M, Ding C, Kotin RM. (2002) Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* **13**(16): 1935–43.

Urabe M, Hasumi Y, Kume A, *et al.* (1999) Charged-to-alanine scanning mutagenesis of the N-terminal half of adeno-associated virus type 2 Rep78 protein. *J Virol* **73**(4): 2682–93.

Urabe M, Kogure K, Kume A, *et al.* (2003) Positive and negative effects of adeno-associated virus Rep on AAVS1-targeted integration. *J Gen Virol* **84**(Pt 8): 2127–32.

Urcelay E, Ward P, Wiener SM, *et al.* (1995) Asymmetric replication in vitro from a human sequence element is dependent on adeno-associated virus Rep protein. *J Virol* **69**(4): 2038–46.

Walz CM, Correa-Ochoa MM, Muller M, *et al.* (2002) Adenoassociated virus type 2-induced inhibition of the human papillomavirus type 18 promoter in transgenic mice. *Virology* **293**(1): 172–81.

Wang H, Lieber A. (2006) A helper-dependent capsid-modified adenovirus vector expressing adeno-associated virus rep78 mediates site-specific integration of a 27-kilobase transgene cassette. *J Virol* **80**(23): 11699–709.

Weger S, Hammer E, Heilbronn R. (2002) Topors, a p53 and topoisomerase I binding protein, interacts with the adeno-associated virus (AAV-2) Rep78/68 proteins and enhances AAV-2 gene expression. *J Gen Virol* **83**(Pt 3): 511–6.

Weger S, Hammer E, Heilbronn R. (2004) SUMO-1 modification regulates the protein stability of the large regulatory protein Rep78 of adeno associated virus type 2 (AAV-2). *Virology* **330**(1): 284–94.

Weger S, Wendland M, Kleinschmidt JA, *et al.* (1999) The adeno-associated virus type 2 regulatory proteins rep78 and rep68 interact with the transcriptional coactivator PC4. *J Virol* **73**(1): 260–9.

Weinberg RA. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**(3): 323–30.

Weitzman MD, Kyostio SR, Kotin RM, *et al.* (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc Natl Acad Sci USA* **91**(13): 5808–12.

Wollscheid V, Frey M, Zentgraf H, *et al.* (1997) Purification and characterization of an active form of the p78Rep protein of adeno-associated virus type 2 expressed in Escherichia coli. *Protein Expr Purif* **11**(3): 241–9.

Wonderling RS, Kyostio SR, Walker SL, *et al.* (1997). The Rep68 protein of adeno-associated virus type 2 increases RNA levels from the human cytomegalovirus major immediate early promoter. *Virology* **236**(1): 167–76.

Wonderling RS, Owens RA. (1996) The Rep68 protein of adeno-associated virus type 2 stimulates expression of the platelet-derived growth factor B c-sis proto-oncogene. *J Virol* **70**(7): 4783–6.

Wonderling RS, Owens RA. (1997) Binding sites for adeno-associated virus Rep proteins within the human genome. *J Virol* **71**(3): 2528–34.

Wong KK Jr, Chatterjee S, Conrad J, *et al.* (2006) AAV2 Rep protein fusions. *US Patent 7122348: http://www.uspto.gov/web/patents/patog/week42/OG/html/1311-3/US07122348-20061017.html.*

Wu F, Garcia J, Sigman D, *et al.* (1991) tat regulates binding of the human immunodeficiency virus trans-activating region RNA loop-binding protein TRP-185. *Genes Dev* **5**(11): 2128–40.

Yamamoto N, Suzuki M, Kawano MA, *et al.* (2007) Adeno-associated virus site-specific integration is regulated by TRP-185. *J Virol* **81**(4): 1990–2001.

Zhan D, Santin AD, Liu Y, *et al.* (1999) Binding of the human papillomavirus type 16 p97 promoter by the adeno-associated virus Rep78 major regulatory protein correlates with inhibition. *J Biol Chem* **274**(44): 31619–24.

[Frontiers in Bioscience 13, 1887-1891, January 1, 2008]

Vector-producing tumor-tracking multipotent mesenchymal stromal cells for suicide cancer gene therapy

Takashi Okada[1], Keiya Ozawa[2]

[1]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, [2]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Japan

TABLE OF CONTENTS

1. ABSTRACT

Suicide cancer gene therapy with retroviral vector-producing cells was in the way of an adjuvant to the surgical resection of recurrent glioblastoma, although any benefit appeared to be marginal. It is likely that this therapeutic approach may have better outcomes if the vectors and transgenes are delivered more efficiently to the tumor cells. We have shown previously that tumor cells engineered by adenovirus-retrovirus hybrid vectors to produce retroviral progeny destroy satellite tumor cells. Whether the systemic delivery of vector-producing cells can effectively treat aggressive tumors remains to be determined. Effective retroviral vector delivery vehicles may be multipotent mesenchymal stromal cells (MSCs), which have been shown to home to tumor cells *in vivo* and deliver cancer-killing gene or immune products with minimal host rejection. Therefore, it may be possible to transduce tumors with recombinant progeny vectors delivered by MSCs. This may be particularly suitable for treating diffuse cancers like glioblastoma multiforme. While this strategy remains to be tested in various orthotopic or metastatic tumor models, it has the potential to greatly improve the outcome of suicide gene therapy.

2. SUICIDE CANCER GENE THERAPY USING VECTOR-PRODUCING CELLS

A gene therapy against glioma has been developed in which a cell line that continuously secretes a retroviral vector is implanted into brain tumors (1). The vector, which expresses the herpes simplex thymidine kinase (HSV-*tk*) transgene, "infects" the local tumor cells, which then become susceptible to tumoricidal metabolites generated by HSV-*tk*-mediated activation of the prodrug ganciclovir (GCV). The therapeutic value of this technique appeared to be enhanced by a bystander effect wherein the transduced tumor cells communicate the apoptosis signal to neighboring cells. However, while this cancer gene therapy system was shown to have some clinical benefit, its efficacy was limited due to the poor efficiency of gene transfer (2). To improve the therapeutic potential of this system, it is necessary to enhance (a) the efficiency of therapeutic gene delivery *in vivo* and (b) the stability of the vector-producing cells.

With regard to therapeutic gene delivery *in vivo*, we have previously described a hybrid vector system where adenoviral vectors are used to deliver retroviral vector andpackaging proteins into cells (3). This system benefits

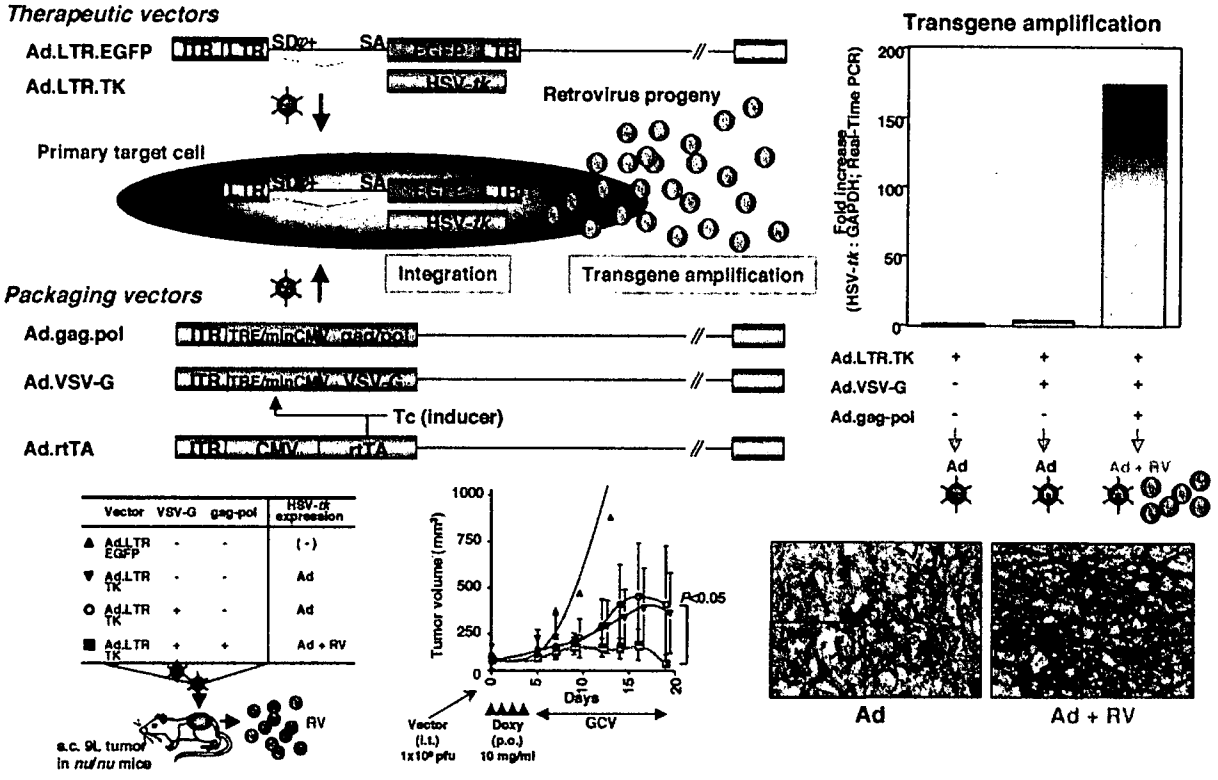

Figure 1. Two adeno-retroviral hybrid vectors containing a retroviral vector genome (Ad.LTR.EGFP or Ad.LTR.TK) were constructed. Co-transduction of rat 9L glioma cells with an adeno-retroviral hybrid vector together with vectors expressing retrovirus packaging proteins (Ad.gag.pol, Ad.VSV-G) as well as an inducer (Ad.rtTA) increased the transduction efficiency. Injection of established subcutaneous 9L tumors on athymic mice with a combination of AVC2.GCTK and packaging vectors followed by GCV treatment resulted in complete regression by 50% of the tumors at day 22, while no tumor regression was observed in control animals. Furthermore, the relative copy number of the HSV-tk gene in tumors treated with the adeno-retroviral vectors was significantly higher than in control tumors. In situ hybridization analysis also suggested dispersion of the HSV-tk product across a wider area of the tumor than in control tumors, which indicates the spread of the in situ-generated retroviruses.

from the efficient gene transfer characteristics of adenoviral vectors as well as the stable and long-term gene expression that is typical of retroviral vectors. We have shown that direct transduction of primary target tumor cells with hybrid adeno-retroviral vectors results in their transient production of recombinant retrovirus particles that then subsequently transduce neighboring tumor cells (3). Moreover, when we transduced established subcutaneous 9L tumors on athymic mice *in situ* with adenovirus vectors that express transcomplementing genes encoding retroviral proteins and retroviral vector RNAs, upon GCV treatment, 50% of the tumors showed complete regression at day 22, while no tumor regression was observed in control animals (Figure 1). This strategy can now be developed further by using cells with tumor-tracking properties as the vector-producing cells, thereby targeting the therapeutic gene to the tumor cells *in vivo*.

3. MULTIPOTENT MESENCHYMAL STROMAL CELLS (MSCs) AS A PLATFORM FOR VECTOR PRODUCTION *IN SITU*

We propose here an improved *in situ* vector production strategy where cells bearing tumor-tracking properties efficiently produce retrovirus- or other virus-based progeny vectors (Figure 2). Candidate tumor-tracking vector-producing cells are adult stem cells. In particular, the fibroblast-like plastic-adherent cells isolated from bone marrow and other sources that are now widely known as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) (4), may be useful as they have been shown to have tumor-seeking properties (5). While the mechanism that induces MSCs to preferentially engraft themselves in tumors remains poorly undertsood, this phenomenon may be mediated by the cytokines released by the tumor or inflammatory tissue. These include hepatocyte growth factor (HGF) (6), vascular endothelial cell growth factor (VEGF) (7), transforming growth factor (TGF) (7), fibroblast growth factor (FGF) (6), platelet-derived growth factor (PDGF) (8), monocyte chemoattractant protein-1 (MCP-1) (9), and IL-8 (9). Moreover, chemokine C-X-C motif receptor 4 (CXCR4), which is present on the surface of an MSC subset, is known to mediate not only the specific migration of MSCs to bone marrow (10), it also governs the migration and homing of a variety of cell types in the developing brain, including neuronal and glial precursors. The only known chemokine that binds with CXCR4 is stromal-cell derived factor-1 (SDF-1). While CXCR4 itself is a major chemokinereceptor on glioma cells and promotes their

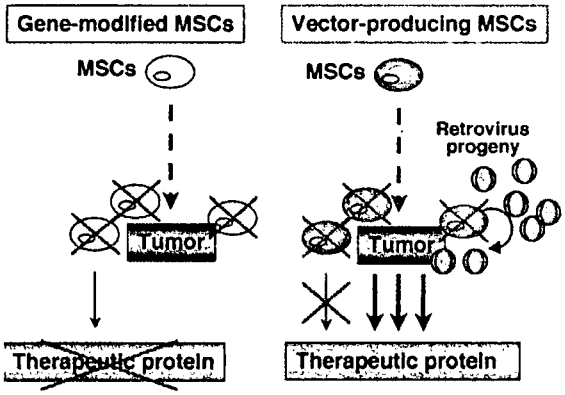

Figure 2. Left panel; Gene-modified MSCs. Although gene-modified MSCs have tumor-seeking properties, the local expression of the therapeutic protein is dependent on the continuing presence of the MSCs. Right panel; Vector-producing MSCs. The retrovirus progeny produced by the MSCs can transduce tumor cells *in situ*, which extends the expression of the therapeutic protein, even when the MSCs die off.

survival (11), high-grade gliomas have recently been found to secrete significant levels of SDF-1 (12). SDF-1 alpha stimulates human glioblastoma cell growth by activating both extracellular signal-regulated kinases 1/2 and Akt. Therefore, CXCR4 expression by MSCs may help them to home to gliomas. When MSCs are infused in mice, they are rapidly and efficiently arrested in the microvasculature (13). Furthermore, these cells are not immunogenic and escape recognition by alloreactive T cells and natural killer cells (14). It also appears that the engraftment of MSCs into the tumor helps them to maintain their stem cell properties *in vivo*. Thus, it appears that MSCs may efficiently engraft human gliomas after intravascular or local delivery and can be used as *in situ* therapeutic vector producers.

Other candidate tumor-tracking cells that may be used as vector-producing cells include endothelial progenitor cells (EPCs). EPCs have been isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are believed to include a hematopoietic stem cell population, and have been shown to incorporate into neovascularization foci (15). Moreover, VEGF promotes adult vasculogenesis by enhancing EPC recruitment and vessel formation at sites of tumor neovascularization (16). Magnetic resonance imaging (MRI) of magnetically labeled endothelial progenitor cells also demonstrated that they traffick to sites of tumor angiogenesis (17).

4. GENETIC MANIPULATION OF MSCs TO FACILITATE THEIR PRODUCTION OF PROGENY VECTORS

MSCs exhibit senescence-associated growth arrest and phenotypic changes during long-term *in vitro* culture. However, overexpression of human telomerase reverse transcriptase (hTERT) in MSCs reconstitutes their telomerase activity and extends their life span (18). Telomerization of MSCs by hTERT overexpression also maintains the stem cell phenotype of MSCs and thus may be useful for generating the numbers of stable MSCs needed for cell differentiation studies and tissue engineering protocols.

To produce therapeutic vectors, the MSCs must be efficiently transduced with viral components. Virus-based transduction techniques have been shown to achieve high gene transduction and transgene expression in many cellular models, and attempts have been made to transduce MSCs with various virus-based vectors such as oncogenic retrovirus- or lentivirus-based vectors. However, the use of integrating viral vectors has several disadvantages, particularly with regard to their safety risks. Many non-viral methods also have limited utility as they are rather inefficient with most primary cells. However, nucleofection, which is a non-viral electroporation-based gene transfer technique, has been shown to be an efficient non-viral transfection technique for MSCs, which then may be used as cellular vehicles for the delivery of biological agents (19). Thus, the Nucleofector technology may be promising as an alternative tool for efficiently transfecting MSCs so that they produce progeny virus.

5. FUTURE DIRECTIONS

Here we propose that current suicide cancer gene therapy strategies may be improved by using vector-producing tumor-tracking MSCs. This strategy is likely to generate *in situ* the vector numbers needed for the killing of solid tumors. We also showed that it may be feasible to produce large-scale preparations of vector-producing cells by transient transduction of MSCs by hybrid adenovirus-based vector infection. It has been shown that the hybrid adenovirus-based vectors that express retroviral proteins can efficiently transduce cells, which then produce progeny vectors (3). However, an impediment for this aim is that MSCs lack the Coxsackie adenovirus receptor (CAR) (20). To overcome this problem, it may be necessary to use a chimeric Ad35 fiber-containing Ad5 vector (21) or a fiber-modified Ad5 vector bearing an RGD-motif peptide in the HI loop of the fiber knob domain (22). Alternatively, it may be possible to use an adaptor molecule that bridges the gap between the viruses and MSCs. Supporting the latter possibility is that we have previously developed a CAR-SCF fusion protein that improves the transduction efficiency of the adenovirus vector with c-kit positive cells (23). Similar CAR-ligand adaptor molecules may be useful for enhancing MSC transduction with the adenovirus vector.

To improve the tumor-targeting properties of the vector-producing cell, how MSCs naturally seek out tumors should be investigated in more detail. In addition, the localization, stability, and vector-producing capacity of gene-manipulated MSCs should be adequately analyzed *in vivo*. Tracking the localization of the MSCs may also help diagnose the recurrence of the disease. Such tracking may be performed by using a molecular imaging technique with MRI. To this end, it has been shown that MSCs labeled with fluorophore particles (IFPs) provide MRI contrast *in vivo* (24). Thus, this type of technology would enable us to

closely study MSC retention, engraftment, and migration in the clinic.

Although previous studies have illuminated the exciting possibilities of suicide cancer gene therapy, in most cases the therapies that were used delivered rather limited clinical benefits. For the sake of safety as well as improving the therapeutic effect of suicide cancer gene therapy, it is important that the suicide gene-expressing vector is accurately delivered to the tumor. This may be achieved by using MSCs to initiate virus production near tumor cells *in situ*. These viruses then transduce the tumor cells, which themselves produce virus progeny, thereby amplifying the transgene expression of the tumor. While the therapeutic benefit of this strategy remains to be tested in various orthotopic or metastatic tumor models, it may be promising for detecting and eradicating evasive tumors *in vivo*.

6. REFERENCES

1. Ram, Z., Culver, K.W., Oshiro, E.M., Viola, J.J., DeVroom, H.L. *et al*: Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 3, 1354-1361 (1997)

2. Rainov, N.G: A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 11, 2389-2401 (2000)

3. Okada, T., Caplen, N.J., Ramsey, W.J., Onodera, M., Shimazaki, K. *et al*: *In situ* generation of pseudotyped retroviral progeny by adenovirus-mediated transduction of tumor cells enhances the killing effect of HSV-tk suicide gene therapy *in vitro* and *in vivo*. *J Gene Med* 6, 288-299 (2004)

4. Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S. and Keating, A: Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 7, 393-395 (2005)

5. Nakamizo, A., Marini, F., Amano, T., Khan, A., Studeny, M. *et al*: Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 65, 3307-3318 (2005)

6. Yamamoto, S., Wakimoto, H., Aoyagi, M., Hirakawa, K. and Hamada, H: Modulation of motility and proliferation of glioma cells by hepatocyte growth factor. *Jpn J Cancer Res* 88, 564-577 (1997)

7. Tille, J.C. and Pepper, M.S: Mesenchymal cells potentiate vascular endothelial growth factor-induced angiogenesis *in vitro*. *Exp Cell Res* 280, 179-191 (2002)

8. Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. and Betsholtz, C: Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 126, 3047-3055 (1999)

9. Wang, L., Li, Y., Chen, X., Chen, J., Gautam, S.C., Xu, Y. and Chopp, M: MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematology* 7, 113-117 (2002)

10. Wynn, R.F., Hart, C.A., Corradi-Perini, C., O'Neill, L., Evans, C.A., Wraith, J.E., Fairbairn, L.J. and Bellantuono, I: A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 104, 2643-2645 (2004)

11. Zhou, Y., Larsen, P.H., Hao, C. and Yong, V.W. CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* 277, 49481-49487 (2002)

12. Barbero, S., Bonavia, R., Bajetto, A., Porcile, C., Pirani, P., Ravetti, J.L., Zona, G.L., Spaziante, R., Florio, T. and Schettini, G: Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. *Cancer Res* 63, 1969-1974 (2003)

13. Bentzon, J.F., Stenderup, K., Hansen, F.D., Schroder, H.D., Abdallah, B.M., Jensen, T.G. and Kassem, M: Tissue distribution and engraftment of human mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene. *Biochem Biophys Res Commun* 330, 633-640 (2005)

14. Le Blanc, K., Rasmusson, I., Sundberg, B., Gotherstrom, C., Hassan, M., Uzunel, M. and Ringden, O: Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363, 1439-1441 (2004)

15. Asahara, T. and Kawamoto, A: Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 287, C572-579 (2004)

16. Li, B., Sharpe, E.E., Maupin, A.B., Teleron, A.A., Pyle, A.L., Carmeliet, P. and Young, P.P: VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. *Faseb J* 20, 1495-1497 (2006)

17. Arbab, A.S., Pandit, S.D., Anderson, S.A., Yocum, G.T., Bur, M., Frenkel, V., Khuu, H.M., Read, E.J. and Frank, J.A: Magnetic resonance imaging and confocal microscopy studies of magnetically labeled endothelial progenitor cells trafficking to sites of tumor angiogenesis. *Stem Cells* 24, 671-678 (2006)

18. Simonsen, J.L., Rosada, C., Serakinci, N., Justesen, J., Stenderup, K., Rattan, S.I., Jensen, T.G. and Kassem, M: Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. *Nat Biotechnol* 20, 592-596 (2002)

19. Aluigi, M., Fogli, M., Curti, A., Isidori, A., Gruppioni, E. *et al*: Nucleofection is an efficient nonviral transfection technique for human bone marrow-derived mesenchymal stem cells. *Stem Cells* 24, 454-461 (2006)

20. Hung, S.C., Lu, C.Y., Shyue, S.K., Liu, H.C. and Ho, L.L:. Lineage differentiation-associated loss of adenoviral susceptibility and Coxsackie-adenovirus receptor expression in human mesenchymal stem cells. *Stem Cells* 22, 1321-1329 (2004)

21. Olmsted-Davis, E.A., Gugala, Z., Gannon, F.H., Yotnda, P., McAlhany, R.E., Lindsey, R.W. and Davis, A.R: Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. *Hum Gene Ther* 13, 1337-1347 (2002)

22. Tsuda, H., Wada, T., Ito, Y., Uchida, H., Dehari, H., Nakamura, K., Sasaki, K., Kobune, M., Yamashita, T. and

Hamada, H: Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. *Mol Ther* 7, 354-365 (2003)
23. Ito, A., Okada, T., Mizuguchi, H., Hayakawa, T., Mizukami, H., Kume, A., Takatoku, M., Komatsu, N., Hanazono, Y. and Ozawa, K: A soluble CAR-SCF fusion protein improves adenoviral vector-mediated gene transfer to c-Kit-positive hematopoietic cells. *J. Gene Med.* 5, 929-940 (2003)
24. Hill, J.M., Dick, A.J., Raman, V.K., Thompson, R.B., Yu, Z.X. *et al*: Serial cardiac magnetic resonance imaging of injected mesenchymal stem cells. *Circulation* 108, 1009-1014 (2003)

Abbreviations: MSCs: mesenchymal stromal cells; GCV: ganciclovir; EPCs endothelial progenitor cells

Key Words: Cancer gene therapy, Multipotent mesenchymal stromal cells, Vector, Review

Send orrespondence to: Takashi Okada M.D., Ph.D., Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8551, Japan, Tel: 81-42-346-1720, Fax: 81-42-346-1750, E-mail: t-okada@ncnp.go.jp

http://www.bioscience.org/current/vol13.htm

Progress in Hematology

International Journal of
HEMATOLOGY

Mechanisms of Immunomodulation by Mesenchymal Stem Cells

Katsutoshi Ozaki, Kazuya Sato, Iekuni Oh, Akiko Meguro, Raine Tatara, Kazuo Muroi, Keiya Ozawa

Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan

Received January 11, 2007; accepted February 9, 2007

Abstract

Mesenchymal stem cells (MSCs) have been identified in animals, especially in bone marrow. As stem cells, they have the ability to differentiate into multiple cell types. This potential raises exciting therapeutic possibilities. A recent report described the successful use of MSCs for the treatment of graft-versus-host disease; however, the scientific community has yet to define the molecular mechanisms of immunomodulation by MSCs. This review summarizes what is known and discusses the conflicting data with regard to the mechanisms of immunomodulation by MSCs.
Int J Hematol. 2007;86:5-7. doi: 10.1532/IJH97.07003

Key words: Mesenchymal stem cells; Immunomodulation; GVHD

1. Introduction

Previous research has demonstrated that mesenchymal stem cells (MSCs) are capable of differentiating into multilineage cells [1]. MSCs have great potential as a cell source for regenerative therapy. For example, the ability of MSCs to differentiate into osteoblasts was used in the treatment of a human disease, osteogenesis imperfecta [2]. Prior to the clinical use of MSCs for the treatment of graft-versus-host disease (GVHD), basic research indicated these cells to be immunomodulatory. Di Nicola et al reported that human MSCs suppress T-cell proliferation induced by allogeneic peripheral blood lymphocytes, allogeneic dendritic cells, or phytohemagglutinin [3]. Finally, Le Blanc et al described a patient with severe steroid-resistant GVHD following bone marrow transplantation who showed marked improvement following 2 infusions of MSCs [4]. This report was the first indication of the efficacy of MSCs as a cell therapy for GVHD. Recently, a high response rate (70%) to treatment of GVHD (grade III-IV) with MSC infusion was reported [5,6]. These data strongly support the hypothesis that MSCs are effective for treatment of GVHD. Despite this clinical success, the molecular mechanisms involved in MSC therapy have not been determined.

Correspondence and reprint requests: Prof. Keiya Ozawa, Division of Hematology, Department of Medicine, Jichi Medical University, Yakushiji 3311-1, Shimotsuke-shi, Tochigi 329-0498, Japan.

2. Molecular Mechanisms

Di Nicola et al demonstrated that cell-cell contact is not required for T-cell suppression by MSCs; however, cell-cell contact does induce maximum suppression. Transforming growth factor β (TGF-β) and hepatocyte growth factor reportedly are the primary mediators of this suppression [3]; however, Tse et al have observed that anti–TGF-β, indomethacin (an inhibitor of prostaglandin synthesis), and a specific inhibitor of indoleamine 2,3-dioxygenase (an inducer of tryptophan degradation) does not restore T-cell proliferation, thus suggesting that TGF-β, prostaglandin E_2 (PGE_2), and tryptophan depletion are not essential for T-cell suppression by MSCs [7]. Krampera et al used T-cells from T-cell receptor transgenic mice and demonstrated that cell-cell contact is required for T-cell suppression by MSCs [8]. They also suggested that anti–TGF-β did not affect suppression, in contrast to the previously mentioned results [3]. Djouad et al argued that immunomodulation by MSCs and an MSC-like cell line was caused by an inducible soluble factor, because supernatant from MSCs cocultivated with allogeneic splenocytes suppressed T-cell proliferation; supernatant from cultures of MSCs alone did not have this effect [9]. Another study found that tryptophan degradation induced by indoleamine 2,3-dioxygenase led to T-cell suppression by MSCs [10], again in contrast to the previously cited results [7]. Aggarwal et al reported that human MSCs alter cytokine secretion from dendritic cells, inhibit cytokine production by type 1 helper T-cells (Th1), enhance Th2 cytokine production, induce more regulatory T-cells, and inhibit cytokine production by natural killer cells as well as T-cells [11]. Notably, these inves-

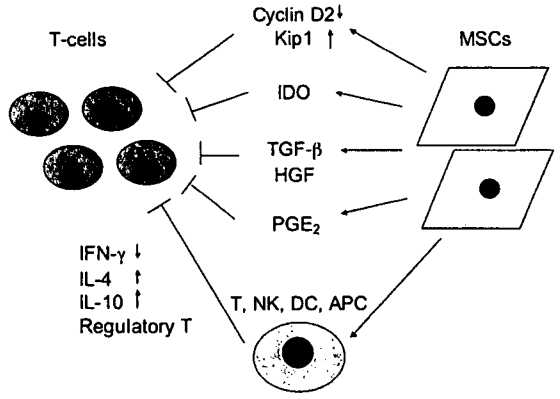

Figure 1. Summary of the research on the molecular mechanism of immunomodulation by mesenchymal stem cells (MSCs). IDO indicates indoleamine 2,3-dioxygenase; TGF-β, transforming growth factor-β; HGF, hepatocyte growth factor; PGE_2, prostaglandin E_2; NK, natural killer cells; DC, dendritic cells; APC, antigen-presenting cells; IFN-γ, interferon-γ; IL-4, interleukin-4.

tigators found that PGE_2 was induced and that an inhibitor of prostaglandin production restored T-cell proliferation [11]. Beyth et al reported that human MSCs altered the function of monocytes and dendritic cells as well as T-cells [12]. They suggested that monocytes and dendritic cells were immunoregulatory antigen-presenting cells in the presence of MSCs. Glennie et al characterized the status of T-cells inhibited by MSCs [13]. They found that although the T-cells were activated, they could not divide or produce interferon -γ (IFN-γ) effectively in the presence of MSCs. These investigators argued that these results represent the "division arrest anergy" of T-cells due to the suppression of cyclin D2 and that the T-cells do not respond to a secondary stimulation [13]. The results of these reports are summarized in Figure 1.

3. Nitric Oxide: A Newly Identified Mediator

Many investigators have sought to determine whether immunomodulation is mediated by soluble factors. This question appears to be easily assessed by means of a Transwell, which separates T-cells from MSCs via a permeable membrane with small pore sizes (eg, 1 μm). Cell-cell contact is avoided, and only soluble factors can penetrate the membrane and affect cells on the other side. The results that have been obtained with this method are ambiguous, however. In our study, MSCs showed a strong suppression effect against T-cell proliferation in the presence of cell-cell contact. The degree of suppression of T-cell proliferation in a Transwell experiment was significantly lower [14]. It is difficult to draw conclusions from these results, but the conflicting reports may have arisen from different interpretations of similar results. The observation that cell-cell contact is required for MSCs to maximally suppress T-cell proliferation could be explained by a distance-dependent effect of a highly unstable soluble factor. Such an effect could be advantageous for the clinical use of MSCs, because a soluble factor that acts only near the inflammatory site would be ideal.

Finally, studies have indicated that nitric oxide (NO) is one of the mediators of T-cell suppression [14]. NO is a highly unstable molecule and is produced by MSCs. T-cell activation is required for MSC production of NO [14]. NO suppresses stat5 phosphorylation and the cell cycle progression of T-cells ([14], Figure 2). CD4 T-cells and CD8 T-cells, but not CD19 B-cells, induced NO synthesis, and MSCs suppressed B-cell proliferation as well as T-cell proliferation, thus suggesting distinct mechanisms of T-cell and B-cell suppression by MSCs [14]. The RAW264.7 macrophage cell line, which is known to secrete NO and was used instead of MSCs, induced lower NO production and less T-cell suppression in a Transwell experiment compared with experiments carried out without a Transwell, as was observed with MSCs [14]. These results suggested that the reduction in T-cell suppression by MSCs observed in Transwell experiments might therefore reflect the characteristics of NO.

Inhibitors of TGF-β, indoleamine 2,3-dioxygenase, prostaglandin production, and NO synthase were used to confirm these results and to compare the importance of NO to the other factors. TGF-β is a well-established immunoregulatory cytokine. Inhibition of TGF-β did not affect the suppression of T-cell proliferation, nor did inhibition of tryptophan depletion [14]. Both inhibitors of prostaglandin production and NO synthesis were comparable in restoring T-cell proliferation, and the combination of 2 inhibitors were not additive, suggesting that these 2 factors work by associated pathways [14]. In fact, given that NO has been reported to be upstream of PGE_2 [15], the induction of PGE_2 may thus result from the induction of NO by MSCs.

One early report suggested that MSC activation is required for the production of a soluble factor that inhibits T-cell proliferation [9]. We observed that T-cell activation was required for suppression by MSCs and that the cocultivation of MSCs with T-cells without T-cell mitogen did not induce NO production [14].

In contrast to these contradictory studies, the inhibition of IFN-γ production by MSCs has been widely accepted by many investigators [8,11,13,14]. One recent report suggested

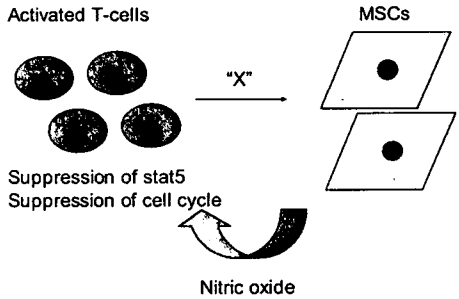

Figure 2. The production and action of nitric oxide (NO). The activation of T-cells is required for the production of NO from mesenchymal stem cells (MSCs). NO suppresses stat5 phosphorylation while also inhibiting cell cycle progression. "X" indicates unknown factors yet to be elucidated.

that MSCs enhance Th2 differentiation in humans [11]. In our laboratory, preferential Th1 suppression was detected in the presence of MSCs. Our MSCs inhibited the production of Th2 cytokine (I.O., unpublished data).

The suppression of stat5 phosphorylation by MSCs is consistent with the fact that stat5 is required for T-cell proliferation [16]. In our laboratory, the inhibition of stat1 phosphorylation by MSCs was also observed (K.O., unpublished data), and the inhibition of cell-cycle initiation was reversible (K.S., unpublished data).

4. Future Directions

How can these contradictions be explained? One explanation may lie in how individual laboratories define MSCs. What constitutes an MSC has not clearly been established. The minimum requirements appear to be the expression of specific cell surface markers and a multiple-differentiation capacity. The source of MSCs varies from one study to another: human or mouse, bone marrow or adipose tissue. MSCs from different tissues or species may yield different results. Because the International Society for Cellular Therapy has proposed minimal criteria for the definition of human MSCs [17], future studies should follow these guidelines.

The clinical use of MSCs for GVHD has been tested in an animal model. Recently, the differing results of 2 independent studies were reported [18,19]. Sudres et al showed that MSCs from C57BL/6 mice had no beneficial effect on the survival of BALB/c recipients that had undergone bone marrow transplantation with $CD3^+$ T-cells from C57BL/6 mice. Even when the investigators increased the number of MSCs to 5×10^5, the survival rate of the experimental animals was equivalent to that of the controls [18]. In contrast, Yanez et al observed the immunosuppressive effects of MSCs against GVHD in a mouse model [19]. They injected 5×10^4 MSCs into each host mouse on days 0, 7, and 14. The survival rates were 80% for the treated group and 0% for the untreated group at day 70; however, infusions of MSCs on days 14, 21, and 28 had no effect [19]. Unfortunately, a statistical analysis of these data was not provided. If MSC treatment is effective in an animal model, investigators can then address how MSCs modulate GVHD in vivo.

In contrast to the success of MSC infusion for the treatment of GVHD, the coinfusion of MSCs during transplantation has shown no beneficial effects on the recovery of neutrophils or platelets, or on the incidence of GVHD [20]. This result may be explained by the timing of infusion. At the time of transplantation, inflammation sites are not present in the body. The infusion of MSCs at a later time may therefore be beneficial for patients with GVHD.

Immunomodulation by MSCs may also induce the suppression of graft-versus-leukemia effects; however, the effects of MSC infusion for this purpose have not yet been reported. Future studies should address this question.

The clinical efficacy of MSCs for the treatment of GVHD remains an important issue. A randomized prospective study is thus warranted to prove the efficacy of MSCs for such treatment.

References

1. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
2. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci U S A*. 2002;99:8932-8937.
3. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*. 2002;99:3838-3843.
4. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*. 2004;363:1439-1441.
5. Ringden O, Uzunel M, Rasmusson I, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation*. 2006;81:1390-1397.
6. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of severe acute graft-versus-host disease [abstract]. *Blood*. 2006;108:226a.
7. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation*. 2003;75:389-397.
8. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood*. 2003;101:3722-3729.
9. Djouad F, Plence P, Bony C, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood*. 2003;102:3837-3844.
10. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood*. 2004;103:4619-4621.
11. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105:1815-1822.
12. Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood*. 2005;105:2214-2219.
13. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood*. 2005;105:2821-2827.
14. Sato K, Ozaki K, Oh I, et al. Nitric oxide plays a critical role in suppression of T cell proliferation by mesenchymal stem cells. *Blood*. 2007;109:228-234.
15. Kim SF, Huri DA, Snyder SH. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science*. 2005;310:1966-1970.
16. Moriggl R, Topham DJ, Teglund S, et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity*. 1999;10:249-259.
17. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells: the International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315-317.
18. Sudres M, Norol F, Trenado A, et al. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol*. 2006;176:7761-7767.
19. Yanez R, Lamana ML, Garcia-Castro J, Colmenero I, Ramirez M, Bueren JA. Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. *Stem Cells*. 2006;24:2582-2591.
20. Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant*. 2005;11:389-398.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 956–962
BBRC
www.elsevier.com/locate/ybbrc

Interferon-γ and NF-κB mediate nitric oxide production by mesenchymal stromal cells

I. Oh, K. Ozaki *, K. Sato, A. Meguro, R. Tatara, K. Hatanaka, T. Nagai, K. Muroi, K. Ozawa *

Division of Hematology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan

Received 31 January 2007
Available online 21 February 2007

Abstract

Mesenchymal stromal cells (MSCs) have been shown to have an immunosuppressive effect. Previously, we demonstrated that nitric oxide (NO) is one of the immunomodulatory mediators of MSCs. We herein show that primary mouse bone marrow MSCs and three cell lines that mimic MSCs suppress both differentiation and proliferation in Th1 condition, whereas the suppression in Th2 condition is mild. NO production is inversely correlated with T cell proliferation in Th1 and Th2 conditions. NO is highly induced in Th1 and minimally induced in Th2. Moreover, an inhibitor of NO synthase restores both proliferation and interferon-γ (IFN-γ) production in Th1 condition. Furthermore, an anti-IFN-γ antibody strongly inhibits NO production and an inhibitor of NF-κB reduces the level of induction of inducible NO synthase (iNOS) in MSCs. Taken together, our results suggest that NO plays a significant role in the modification of Th1 and Th2 differentiation by MSCs, and that both IFN-γ and NF-κB are critical for NO production by MSCs.

Keywords: Mesenchymal stem cells; Nitric oxide production; Interferon-γ; NF-κB; Th1 differentiation; Th2 differentiation; Immunosuppression; 10T1/2; iNOS

Although many reports indicate that mesenchymal stromal cells (MSCs) suppress T cell proliferation, the molecular mechanisms involved are poorly understood [1–6]. Human MSCs were reported to suppress Th1 differentiation and augment Th2 differentiation [4]. In this study, we investigated whether mouse bone-marrow-derived MSCs have the same effect on Th1 and Th2 differentiation while were also trying to identify the underlying molecular mechanisms of these effects.

Naïve helper T cells primarily differentiate into either Th1 or Th2 cells. Th1 cells produce IFN-γ and interleukin-2 (IL-2) while Th2 cells produce IL-4, IL-5, and IL-13. In addition, it appears that a disruption of the balance between Th1 and Th2 differentiation is associated with the development of immune diseases [7,8].

Previously, two sub-lines of the parental C3H10T1/2 (10T1/2) cell line were established by treatment of that line with 5-azacytidine. These were characterized as a preadipocyte cell line and a myoblast cell line, and were designated A54 and M1601, respectively [9]. A54 and M1601 differentiate into adipocyte and myotube under defined conditions [9]. Other studies suggest that 10T1/2 cells have the same T cell suppressive effect as primary MSCs [10]. In addition, the 10T1/2 cell line has been used as a model of MSCs [11–14]. In the present study, these three cell lines were used as MSC-like cells and demonstrated that their phenotype is similar to that of primary MSCs.

Nitric oxide (NO) is known to suppress T cell proliferation [15–18]. Previously, we demonstrated that NO is one of the major mediators of T cell suppression by mouse MSCs [19]. It is possible that NO also plays a critical role

* Corresponding authors. Fax: +81 285 44 5258.
E-mail addresses: ozakikat@jichi.ac.jp (K. Ozaki), kozawa@ms2.jichi.ac.jp (K. Ozawa).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.054

in Th1/Th2 differentiation and the present study demonstrates that NO contributes to preferential Th1 suppression by MSCs. Moreover, the results indicate that IFN-γ and NF-κB are key regulators of NO production by MSCs.

Materials and methods

Reagents. Anti-mouse CD3/CD28 beads (Dynal Biotech., Oslo, Norway) were used at 10 μl per 10^6 cells. PE-anti-IFN-γ and PE-anti-IL-4 antibodies (BD Biosciences, San Diego, CA) were used for intracellular staining of cytokines. Murine IL-4 and IL-12 were purchased from R&D Systems (Minneapolis, MN). The neutralizing antibodies for IFN-γ and IL-4 were purchased from BD Biosciences.

NG-monomethyl-L-arginine (L-NMMA) was from Wako Pure Chemical Industries (Osaka, Japan). Lipopolysaccharide (LPS) was purchased from Sigma–Aldrich (St. Louis, MO). Tumor necrosis factor-α (TNF-α), IL-1β, soluble TNF-α receptor, and neutralizing anti-IL-1β antibody were purchased from PeproTech (Rocky Hill, NJ). CpG oligonucleotide (HyCult biotechnology, Netherlands), poly(I:C), and flagellin (InvivoGen, San Diego, CA) were generous gifts from Dr. Motoharu Matsuura (Jichi Medical University). NF-κB inhibitor, Bay-11-7085 [20] was purchased from Wako.

Primary MSCs and MSC-like cell lines. The C3H10T1/2 cell line was obtained from ATCC (American Type Culture Collection). The A54 (preadipocyte) and M1601 (myoblast) cell lines were 10T1/2-derived cell lines [9]. Primary MSCs were generated, maintained, and characterized as previously described [19].

Intracellular staining. Cytofix/Cytoperm® (BD Biosciences) was used for fixation/permeabilization. Then, cells were stained with anti-IFN-γ or anti-IL-4 antibody (BD Biosciences).

Th1 and Th2 differentiation. Splenic $CD4^+$ T cells were selected using the autoMACS magnetic beads system (Milteny Biotech., Auburn, CA) and stimulated with anti-mouse CD3/CD28 beads (Dynal Biotech.) at an appropriate concentration for 2–3 days with or without MSCs. The purity of the cells, determined by flow cytometry using antibodies against CD4 and B220, was approximately 90%. Stimulation was performed under Th1 (10 ng/ml IL-12 and 10 μg/ml anti-IL-4) or Th2 (100 ng/ml IL-4 and 10 μg/ml anti-IFN-γ) polarizing conditions. For intracellular staining, after extensive washing, Golgistop® (Monensin, 2 μM, BD Biosciences) was added, and then the cells were re-stimulated with PMA and ionomycin for 6 h. For enzyme-linked immunosorbent assay (ELISA), after extensive washing, cells were incubated overnight with plate-bound anti-CD3 antibody and the supernatants were subjected to quantification of IFN-γ or IL-4.

Carboxyfluorescein succinimidyl ester (CFSE) labeling. $CD4^+$ T cells were resuspended at a concentration of 1×10^7 cells/ml in PBS. CFSE (Invitrogen) was added to a final concentration of 10 μM and incubated 10 min at 37 °C. Labeling was stopped by washing with RPMI plus 10% fetal calf serum (FCS). Cells were stimulated with anti-CD3/CD28 beads in the presence or absence of MSCs for 3 days. In some experiments 1 mM of L-NMMA, an inhibitor of NO synthase, was added to the culture.

IFN-γ or IL-4 concentration. Commercially available ELISA kits were used for the quantification of IFN-γ or IL-4 in supernatants after Th1 and Th2 differentiation, according to the manufacturer's instruction (BD Biosciences).

Measurement of NO production. The concentration of NO in cell culture supernatants was determined by using a Griess reagent kit (Wako Pure Chemical Industries) according to the manufacturer's instructions.

Flow cytometric analysis. Cells were incubated with Fc block® (BD Biosciences) to inhibit non-specific binding of antibodies to Fc receptors.

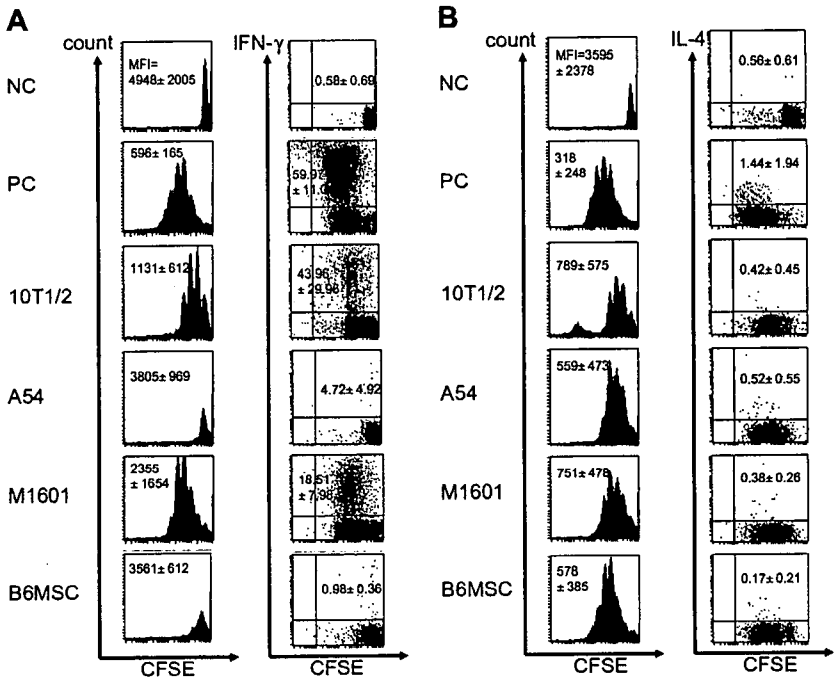

Fig. 1. Th1 and Th2 differentiation in the presence of MSCs. (A) A simultaneous flow cytometric analysis of T cell differentiation and proliferation in Th1 condition. Splenic $CD4^+$ T cells were selected with autoMACS , stained with CFSE, and stimulated in Th1 condition for 72 h. To prevent cytokine secretion, monensin (2 μM) was added during the last 6 h. Then, cells were extensively washed and intracellular-stained with anti-IFN-γ antibody (right panel). The divided cells show a lower intensity of CFSE staining (left panel). (B) A simultaneous flow cytometric analysis of T cell differentiation and proliferation in Th2 condition. Cell preparation was the same as (A) but cells were stimulated in Th2 condition and stained with anti-IL-4 antibody. The numbers indicated in left panels are mean ± SD of mean fluorescein intensity and that in right panels are mean ± SD of the percentage of cells in upper right quadrant. The small isolated CFSE-negative-peak in 10T1/2 in (B) was detached 10T1/2 cells during the washing step. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen and without MSCs.

Then, cells were stained in FACS buffer (PBS supplemented with 10% FBS) with appropriate concentrations of antibodies for 30 min on ice, washed with FACS buffer, and analyzed with a BD LSR cytometer (BD Biosciences), and the data were analyzed using CELLQUEST software (BD Biosciences).

[^{3}H]Thymidine incorporation, immunoblot analysis, detection of inducible NO synthase (iNOS) expression. We performed these assays as described previously [19].

Results

Effects of MSCs on Th1 differentiation

Using primary MSCs and three MSC-like cell lines, the effects of co-cultivation of MSCs on Th1 and Th2 differentiation were examined. Fig. 1A, left panel illustrates that primary MSCs and the A54 cell line strongly suppressed proliferation in Th1 condition. The 10T1/2 and M1601 cells also showed modest suppression. Consistent with this, [^{3}H]thymidine incorporation demonstrated strong suppression of T cell proliferation in the presence of primary MSCs and the A54 cell line and modest suppression in the presence of the 10T1/2 and M1601 cell lines (Supplementary Fig. 1A).

Simultaneously, the production of IFN-γ in the presence of MSCs was examined. In addition to the suppression of T cell proliferation, IFN-γ production is also strongly suppressed in the presence of primary MSCs and the A54 cell line (Fig. 1A, right panel). This suppression was not due to the suppressed number of T cells because the suppression of IFN-γ was also observed with an ELISA, in which an equal number of differentiated cells were used (Supplementary Fig. 1C).

Effects of MSCs on Th2 differentiation

In contrast to Th1 differentiation, a modest suppression of Th2 cell proliferation was observed by a flow cytometric analysis (Fig. 1B, left panel). Specifically, primary MSCs and the A54 cell line showed significantly less suppression compared to the suppression in Th1 condition (Fig. 1B, left panel vs. Fig. 1A, left panel). Consistent with this, [^{3}H]thymidine incorporation assay demonstrated a less suppression of T cell proliferation in Th2 as compared to Th1 in the presence of primary MSCs and A54 cell line (Supplementary Fig. 1B vs. 1A). IL-4 production in the presence of MSCs was suppressed in Th2 condition, as measured by both flow cytometric analysis (Fig. 1B, right panel) and ELISA, in which the same number of differentiated cells were used (Supplementary Fig. 1D).

Nitric oxide production in Th1/Th2 differentiation

Since nitric oxide (NO) is one of the primary mediators of T cell suppression by MSCs [19], it is possible that NO is also involved in suppression of proliferation in Th1 condition. We found a reverse correlation between NO production and T cell proliferation in Th1/Th2 conditions (Fig. 2A and B), where NO production was highly induced in the presence of MSCs in Th1 but it was induced only minimally in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Th1 (Fig. 1A), produce high levels of NO in Th1 condition (Fig. 2A). These results suggest that NO also plays a major role in the preferential suppression of Th1 proliferation by MSCs.

Inhibition of NO synthase restores proliferation and differentiation of Th1 cells

To confirm the role of NO, a specific NO synthase inhibitor, NG-monomethyl-L-arginine (L-NMMA) was used. The presence of this inhibitor restored the T cell proliferation and IFN-γ production in Th1 condition (Fig. 3A and B), demonstrating that NO is a mediator of suppression of both T cell growth and IFN-γ production in Th1 condition. However, L-NMMA does not completely restore the T cell proliferation by primary MSCs, suggesting that there are other factors involving in this suppression, as demonstrated in the previous study [19].

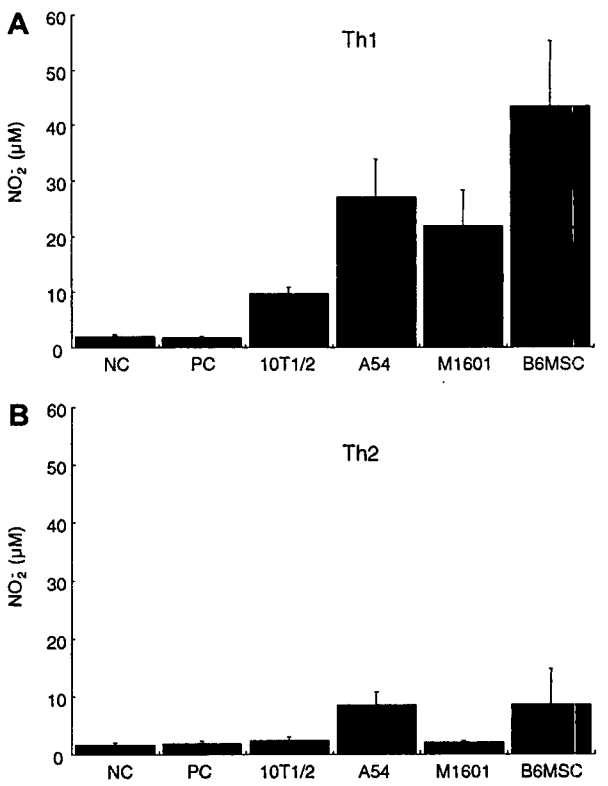

Fig. 2. NO production in Th1 and Th2 conditions in the presence of MSCs. (A) NO production in Th1 condition in the presence of MSCs. (B) NO production in Th2 condition in the presence of MSCs. Splenic $CD4^+$ T cells (1×10^6) were stimulated in either Th1 or Th2 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^5) for 48 h. The concentration of NO_2 in supernatants were determined by the Greiss assay. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen and without MSCs.