Table 1 Studies of AAVS1-targeted Integration in Cultured Cells

Authors	Year	Cells	Transfection[1] (*n* Plasmid[2])	Rep Expression: Promoter (Protein)	Transgene (*cis* Element)	AASV1/Total[3] (% Targeted)	Analysis of Integration
Shelling *et al.*	1994	HeLa/293	C (1)	p5 (Rep78/68)	Neo (ITR)	9/12 (75)	Southern
Balagué *et al.*	1997	293	C (1)	p5 (Rep78)	Neo (ITR)	5/9 (45)	Southern
Surosky *et al.*	1997	293	C (2)	RSV (Rep78/68)	LacZ (ITR)	6/7 (86)	Southern/FISH
Pieroni *et al.*	1998	HeLa	C (1)	p5 (Rep78/68)	GFP/Neo (ITR)	15/100 (15)	Southern/FISH
		Huh-7	L (1)	p5 (Rep78/68)	GFP/Neo (ITR)	5/55 (9)	Southern/PCR
Lamartina *et al.*	1998	HeLa	L (1)	CMV (Rep78)	Neo (ITR)	9/37 (24)	Southern
		HeLa	L (1)	CMV (Rep68)	Neo (ITR)	8/37 (22)	Southern
		HeLa	L (1)	Rep68 protein[4]	Neo (ITR)	N.A.[5]	PCR
Tsunoda *et al.*	2000	HeLa	L (1)	p5 (Rep78/68)	Neo (ITR/p5)	22/36 (61)	Southern/PCR
Rinaudo *et al.*	2000	HeLa	C (2)	CMV (Rep78/68-PR[6])	Neo (ITR)	7/28 (25)	Southern
Kogure *et al.*	2001	K562	L (2)	MMTV/IRES (Rep78)	Neo (ITR)	6/17 (22)	Southern/FISH
		K562	E (2)	CMV (Rep78)	Neo (ITR)	8/25 (32)	Southern/FISH
Huttner *et al.*	2003	HeLa	C (rAAV[7])	p5 (Rep78/68)	GFP/Hygro (ITR)	7/10 (70)	Southern/FISH
Urabe *et al.*	2003	293	C (2)	CMV (Rep78)	Neo (ITR)	4/17 (24)	Southern/FISH
		293	C (2)	CMV (Rep68)	Neo (ITR)	8/20 (40)	Southern/FISH
Philpott *et al.*	2004	HeLa	E (1)	p5 (Rep78/68)	GFP (ITR/p5)	82/95 (86)	Southern
		HeLa	E (1)	p5 (Rep78/68)	GFP (p5)	44/47 (94)	Southern
		HeLa	E (2)	T7 (Rep78/68)	CAT (p5)	9/10 (90)	Southern
Wong, Jr. *et al.*	2006	293	C (rAAV2[7])	CMV (Rep78/68-VP22[8])	SEAP (ITR)	N.A.	PCR
Feng *et al.*	2006	293	C (2)	p5 (Rep78/Rep68)	GFP/Neo (RBS)	7/19 (37)	Southern/PCR

[1]Method used: C, calcium phosphate method; L, lipofection; E, electroporation.
[2]Number of plasmids used for transfection. "1" indicates that a *rep* gene and a transgene cassette were on the same plasmid. "2" indicates that a *rep* plasmid and a transgene plasmid were cotransfected.
[3]Number of clones with AAVS-targeted integration/total number of clones analyzed.
[4]Purified recombinant Rep68 protein was transfected instead of a Rep68 expression cassette.
[5]N.A., not available.
[6]PR (ligand binding domain of the progesterone receptor) was fused to Rep78/68.
[7]AAV vector transduction was used instead of plasmid transfection.
[8]VP22 (a tegument protein of herpes simplex virus) was fused to Rep78/68.

In order to reduce the frequency of the chromosomal integration of the *rep* gene, Surosky *et al.* used a two-plasmid system: one plasmid is for the expression of the Rep protein and the other is an ITR-linked transgene plasmid (Surosky *et al.*, 1997). The idea is that the frequency of integration of a *rep* plasmid is lower than that of an ITR-plasmid when the Rep protein is supplied *in trans*. They concluded that six out of seven LacZ-positive colonies had the LacZ transgene into the AAVS1 site as revealed by Southern blot analysis. Fluorescent *in situ* hybridization (FISH) confirmed that two out of six clones had a LacZ signal on chromosome 19. They also showed that Rep78 or Rep68 alone was sufficient for promoting AAVS1-specific integration and that one ITR sequence could target integration to the AAVS1 site. The full sequence of one AAVS1-integrant was determined and it was thus shown that the whole plasmid was integrated at AAVS1 in tandem array.

The work by Pieroni *et al.* described the transfection of HeLa cells or Huh-7 cells with a plasmid harboring the *p5-rep* gene and an ITR-flanked GFP/Neo cassette, with the resulting generation of clones with the transgene integrated in AAVS1 (7 to 25%), which were then analyzed by Southern blot. AAVS1-specific integration was confirmed in one Huh-7 clone by PCR amplification of the ITR-AAVS1 junction sequence and in three HeLa cell clones by FISH colocalizaiton of the GFP/Neo and AAVS1 signals (Pieroni *et al.*, 1998).

By using an AAV vector plasmid similar to that of Shelling and Smith, Tsunoda *et al.* tested site-specific integration in HeLa cells (Tsunoda *et al.*, 2000). In addition, their plasmid had a hygromycin resistance gene beside the ITR-flanked *p5-rep* and *Neo* gene cassette. Southern blot analysis showed that 22 out of 36 (61%) clones had the *GFP/Neo* gene in AAVS1. The junction sequence between the ITR and AAVS1 was amplified by PCR and this showed that the junction occurred in the p5 promoter region. A RBS homolog and *trs* homolog exist in the p5 promoter region, where Rep78 or Rep68 binds and regulates the p5 promoter activity. The Rep-mediated amplification of the AAV genome occurs via the RBS homolog (Nony *et al.*, 2001; Tessier *et al.*, 2001; Tullis and Shenk, 2000), which is important for efficient production of AAV progeny. In addition, the p5 element has been shown to be sufficient for AAVS1-specific integration (Philpott *et al.*, 2002a and 2002b). All the junction sequences amplified had a partially deleted p5 portion, not an ITR sequence (Tsunoda *et al.*, 2000). This is probably partially because cell survival depends on the inactivation of the *rep* gene. The Rep protein is cytotoxic and anti-proliferative (see above). Tsunoda *et al.* also speculated that the plasmid backbone was also simultaneously inserted into AAVS1 as

well as the ITR-flanked *rep/Neo* cassette, an observation consistent with the result of Surosky *et al.* (Surosky *et al.*, 1997).

Kogure *et al.* applied the AAVS1-targeting system to K562 cells, a hematopoietic cell line (Kogure *et al.*, 2001). To limit the expression of Rep78, they used a weak promoter derived from the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) driving bicistronic expression of GFP and Rep78. They transfected the *rep* plasmid and an ITR-Neo plasmid into K562 cells, analyzed 17 clones by Southern blot and found that six clones (22%) harbored the *Neo* gene in AAVS1. FISH analysis confirmed that five out of six clones had a *Neo* gene on chromosome 19.

Urabe *et al.* used a CMV-driven *rep78* gene plasmid for AAVS1-specific integration in HEK 293 cells (Urabe *et al.*, 2003). To regulate the expression level of Rep78, they used decreasing amounts of *rep78* plasmid. They observed that approximately 20% of the clones analyzed by Southern blot showed integration of the *Neo* gene into AAVS1. They also compared the efficiency of targeted integration mediated by Rep78 and Rep68, and found that Rep68 was superior to Rep78 in their experiment. Southern analysis of G418-resistant clones obtained from transfection with the *rep68* plasmid showed that eight out of 20 clones (40%) had the integration of the *Neo* gene into AAVS1. The presence of the *Neo* gene on chromosome 19 was confirmed in all six clones.

Philpott *et al.* utilized the p5 promoter instead of the ITR as a *cis* element for AAVS1-specific integration and successfully introduced the *GFP* gene into AAVS1 at a frequency of 94% (out of 47 HeLa cell clones), a result comparable to data obtained with an ITR-plasmid (86%) (Philpott *et al.*, 2004). They observed that clones obtained by transfection of a plasmid harboring both a *rep* cassette and a *GFP* cassette showed a gradual decrease in the transgene expression over 18 weeks, while clones obtained from transfection with two plasmids (one for Rep expression, and the other for transgene delivery) stably expressed the transgene over time.

Recombinant AAV vector is widely used as a gene transfer vector and is being evaluated for some human applications including coagulation factor IX deficiency, lipoprotein lipase deficiency, and Parkinson's disease. Conventional AAV vectors harbor the gene of interest flanked by the ITRs and are thus devoid of the *rep* gene. Since the *rep* gene product is essential to AAVS1-specific integration, these AAV vectors are not able to preferentially integrate into AAVS1. To achieve the AAVS1-specific integration of the AAV vector genome, Huttner *et al.* infected HeLa cells with an AAV vector after

transfection with a Rep-expression plasmid and reported that seven out of 10 clones showed site-specific integration of the AAV vector genome (Huttner *et al.*, 2003). Wong, Jr. *et al.* also performed Rep-mediated insertion of rAAV by using a fusion protein consisting of Rep and VP22, a tegument protein of herpes simplex virus (Wong *et al.*, 2006). VP22 traffics intercellularly and spreads over adjacent cells (Elliott and O'Hare, 1997). The Rep protein has a nuclear localization signal (NLS) sequence, which inhibits the spread of the Rep-VP22 fusion protein. Removal of the NLS allowed the fusion protein to traffic intercellularly. The advantage of this strategy is that it can be applied to cells easy to transduce with AAV vectors and hard to transfect with plasmid DNA.

7. How to Regulate Rep Expression

The Rep protein affects numerous cellular phenomena as mentioned above. In addition, infection with wild-type AAV or Rep-directed transgene insertion into AAVS1 appears to accompany the disruption and rearrangement of the AAVS1 locus (Balague *et al.*, 1997; Hamilton *et al.*, 2004; Kotin *et al.*, 1990; Shelling and Smith, 1994; Urabe *et al.*, 2003). Thus, it is necessary to regulate the expression level of the Rep protein to as low as possible for applications aimed at AAVS1-specific integration.

The regulation of Rep protein expression at the DNA level includes the bacterial Cre/*loxP* system (Sauer and Henderson, 1988) and the yeast FLP system (O'Gorman *et al.*, 1991). The former was applied to the regulation of the Rep protein (Satoh *et al.*, 2000). The p5 promoter was moved downstream of the *rep* ORF on a plasmid and a *loxP* sequence was placed upstream of the *rep* ORF and another was placed downstream of the p5 promoter. Cre removes a stuffer sequence between the two *loxP* sites and the *rep* cassette is circularized such that the p5 promoter is placed just upstream of the *rep* ORF. Co-transfection of HEK293 cells was performed with a Rep-expression plasmid, a Cre expression plasmid, and an AAV vector plasmid on which a *Neo* gene and an expression cassette for a secreted form of alkaline phosphatase (SEAP) were placed between the ITRs. Only by the use of a *Cre-expression* plasmid could they obtain G418-resistant clones where the *Neo/SEAP* gene was targeted into the AAVS1 locus.

Regulation of the *rep* gene at the transcription level includes the use of a weak promoter. The bacteriophage T7 promoter (Recchia *et al.*, 1999) and the combination of the mouse mammary tumor virus (MMTV) LTR promoter and internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus (Kogure *et al.*, 2001) have been used for Rep expression.

The native p5 promoter is weak enough for Rep expression. However, since the p5 promoter encompasses an imperfect RBS and is thus a *cis* element of AAVS1-specific integration (see above), it is desirable to avoid using the p5 promoter for Rep expression in order to minimize the frequency of *rep* gene integration.

In general, RNA is more labile than DNA. Transfection of RNA encoding Rep proteins may thus reduce the prolonged cytostatic effects of the Rep protein. The delivery of the Rep protein may limit the duration of Rep cytostatic action even more. Lamartina *et al.* transfected HeLa cells with a mixture of a recombinant Rep protein and an ITR-flanked plasmid by the use of a lipid reagent (Lamartina *et al.*, 1998). The analysis of pooled transfected cells by PCR showed that AAVS1 site-specific integration occured in many cells. Three junctions were sequenced and confirmed the site-specific integration.

Regulation of a protein function by a molecular switch is an attractive tool for Rep protein. Rinaudo *et al.* developed a chimeric protein between Rep and the truncated form of the ligand binding domain (LBD) of the progesterone receptor (Rinaudo *et al.*, 2000). The progesterone receptor (PR) associates with heat shock proteins hsp70, hsp90, and several co-chaperone proteins via its LBD. Binding of progesterone promotes conformational changes in PR, resulting in its release from the chaperone complex, and then its nuclear transport (McKenna *et al.*, 1999). The truncated LBD does not bind endogenous progesterone but a synthetic antagonist, RU486 (Rinaudo *et al.*, 2000). In the absence of RU486, C-terminally truncated Rep (residue 1-491) fused to the LBD is predominantly in the cytoplasm, whereas in the presence of RU485, the fusion protein moves into the nucleus. Following cotransfection of HeLa cells with a Rep-LBD expression plasmid and an ITR-flanked *Neo* plasmid and a 24-hour treatment with RU486, they obtained G418-resistant clones. Southern analysis showed that seven out of 28 clones harbored the *Neo* gene in the AAVS1 site. In addition, generation of AAVS1 rearrangement without insertion of the *Neo* gene was markedly reduced. Another regulation system for the Rep protein was reported. The Rep protein functions as a hexameric complex. The Rep domain responsible for DNA binding and nicking fused to a protein that multimerizes can target an ITR-linked DNA into AAVS1. Oligomerization of Rep molecules on the RBS is a prerequisite for Rep enzymatic activities, including nicking, helicase, and ATPase activities. The N-terminal two thirds portion (1–224) of the Rep protein is able to target site-specific integration when it is fused C-terminally to artificial multimerizing proteins (Cathomen *et al.*, 2000).

8. Vehicles to Deliver the Rep Gene into Cells

A number of strategies have been developed to incorporate DNA into target cells. Plasmid transfection with the calcium phosphate precipitation method or with lipofection is the simplest way to introduce DNA as well as electroporation. For "hard-to-transfect" cells, a viral vector is the second choice.

The Rep proteins are cytostatic as mentioned above and transient or regulated expression of the Rep protein is favored. A number of attempts have been made to create an adenoviral vector harboring the *rep* gene, which turned to be unsuccessful due to low yields and instability of recombinant *rep*-Ad vectors. AAV Rep proteins inhibit the replication of adenovirus at different steps. The Rep78 and Rep68 proteins associate with the single-stranded DNA binding protein, an E2A gene product of adenovirus (Stracker *et al.*, 2004). Rep68 has been shown to bind the E2a promoter region (Casper *et al.*, 2005) and suppresses transcription (Casper *et al.*, 2005; Jing *et al.*, 2001; Nada and Trempe, 2002). In addition Rep78 represses E1a, E2a, E4 promoter activity, but trans-activates E1b and E3 promoters. By contrast, in the presence of E1a protein, Rep78 repressed all the promoters (Jing *et al.*, 2001). Rep78 and Rep68 inhibit the transcription from the Ad major late promoter by the association with the TATA-box binding protein and binding to sites adjacent to the TATA box (Needham *et al.*, 2006). The regulation of *rep* gene expression by the Cre/*LoxP* system (Ueno *et al.*, 2000) or the tetracycline inducible system (Recchia *et al.*, 2004) succeeded in generating an Ad vector with the *rep* gene. In addition, a promoter derived from bacteriophage T7 (Recchia *et al.*, 1999) and the locus control region (LCR) of the human β-globin gene (Wang and Lieber, 2006), which functions very weakly in mammalian cells, have been shown to drive expression levels of the Rep protein compatible with Ad vector production.

Another viral vector tested for incorporation of the Rep expression cassette is herpes simplex virus (HSV) vector. The details are presented by Fraefel *et al.* in the following chapter. HSV is also a helper virus for AAV and support AAV replication. HSV appears to tolerate the anti-viral effect of the Rep protein more than the adenovirus. A p5 promoter-driven Rep cassette could be successfully packaged into an HSV mini-amplicon vector although the titer of the recombinant HSVs was low. By using a hybrid amplicon vector with a *rep* gene and a transgene cassette between the ITR sequences, AAVS1-targeted integration of the transgene was achieved, which was confirmed by amplification of junction sequences in HEK293 cells (Heister *et al.*, 2002) or by Southern blot and FISH analysis in fibroblasts obtained from transgenice mice bearing human AAVS1 sequence (Bakowska *et al.*, 2003).

Baculovirus, an invertebrate virus that is widely used for the production of recombinant proteins is also able to harbor the *rep* gene and to mediate AAVS1-specific integration (Palombo *et al.*, 1998). It was reported that the *rep* gene and the ITRs were stable in the baculovirus genome and that titers of *rep*-baculovirus were comparable to wild-type ones (Urabe *et al.*, 2002).

9. Future Direction

Insertion of foreign DNA into a specific chromosome at a predetermined site will become a prerequisite for human gene manipulation in the future. AAV offers an attractive tool to achieve site-specific integration. Currently it is impossible to insert transgene into AAVS1 in all transfected cells. *Ex vivo* gene therapy is a practical strategy to apply for AAVS1-targeted integration since a cell clone that harbors the therapeutic transgene at the AAVS1 site can be selected and expanded for use.

Some challenges to develop better AAVS1-targeted integration systems include: 1) increasing the frequency of AAVS1-specific integration; 2) decreasing the frequency of disruption of non-AAVS1 sites; and 3) reducing the cytotoxicity of the Rep protein. Better understanding of AAV biology will help us to refine the system.

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Review

Cell and gene therapy using mesenchymal stem cells (MSCs)

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Abstract

Mesenchymal stem cells (MSCs) are considered to be a promising platform for cell and gene therapy for a variety of diseases. First, in the field of hematopoietic stem cell transplantation, there are two applications of MSCs: 1) the improvement of stem cell engrafting and the acceleration of hematopoietic reconstitution based on the hematopoiesis-supporting ability; and 2) the treatment of severe graft-versus-host disease (GVHD) based on the immunomodulatory ability. Regarding the immunosuppressive ability, we found that nitric oxide (NO) is involved in the MSC-mediated suppression of T cell proliferation. Second, tumor-bearing nude mice were injected with luciferase-expressing MSCs. An in vivo imaging analysis showed the significant accumulation of the MSCs at the site of tumors. The findings suggest that MSCs can be utilized to target metastatic tumors and to deliver anti-cancer molecules locally. As the third application, MSCs may be utilized as a cellular vehicle for protein-supplement gene therapy. When long-term transgene expression is needed, a therapeutic gene should be introduced with a minimal risk of insertional mutagenesis. To this end, site-specific integration into the AAVS1 locus on the chromosome 19 (19q13.4) by using the integration machinery of adeno-associated virus (AAV) would be particularly valuable. There will be wide-ranging applications of MSCs to frontier medical treatments in the near future.
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Keywords: Cancer gene therapy; GVHD; Mesenchymal stem cells; Site-specific integration; Tumor targeting

1. Introduction

In bone marrow, there are different types of tissue stem cells (adult stem cells); i.e. hematopoietic stem cells and mesenchymal stem cells (MSCs). MSCs account for a small population of cells in bone marrow as a non-hematopoietic component with the capacity to differentiate into a variety of cell lineages, including adipocytes, osteocytes, chondrocytes, muscles, and stromal cells [1]. Recent studies demonstrated that MSCs are capable of supporting hematopoiesis and of regulating immune response [2]. In addition, since MSCs can be readily isolated and expanded *in vitro*, they are expected to be a source of cell therapy. Interestingly, MSCs have the ability to accumulate at the site of: i) tissue/organ damage; ii) inflammation; and iii) cancer when administered *in vivo*. Therefore, MSCs can be utilized for: i) regenerative therapy; ii) treatment of graft-versus-host disease (GVHD) and Crohn disease; and iii) platform of cancer gene therapy (targeted delivery of anti-cancer agents). Another unique feature of MSCs is little or low immunogenicity due to the lack of expression of co-stimulatory molecules. This phenomenon makes it possible to administer MSCs without HLA matching for cell therapy. A single lot of expanded MSCs from one healthy donor can be utilized for treatment of many patients. Although clinical applications of MSCs have been conducted for the suppression of severe acute GVHD in allogeneic stem cell transplantation [3,4] and for regenerative therapy [5,6], molecular mechanisms underlying the biological effects of

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MSCs remains obscure. Finding key molecules for differentiation, immunosuppression, and hematopoietic support of MSCs would be valuable for further augmenting the efficacy of MSCs in a wide range of clinical applications. In this regard, development of the technology for genetic manipulation of MSCs is also important research project. Site-specific integration of a therapeutic gene into a safe locus in the genome should be investigated from the safety standpoint.

2. Microarray analysis of genes responsible for differentiation of mesenchymal stem cells

Genes regulating the differentiation of MSCs remain obscure and it is technically difficult to do high-throughput analysis using primary MSCs, because such cells contain heterogeneous populations. To overcome the problems related to the heterogeneity of primary MSCs, we utilized MSC-like cell lines. It has been shown that 10T1/2 cells, derived from C3H mouse embryo cells, differentiate into adipocytes, osteocytes, and chondrocytes with a treatment of 5-azacytidine. We previously established two sub-lines from 10T1/2, designated as A54 for a preadipocyte cell line and M1601 for a myoblast cell line [7]. Under appropriate culture conditions, A54 and M1601 cells terminally differentiate into adipocytes and myotubes, respectively, while parental 10T1/2 cells remain undifferentiated under the same culture conditions. Therefore, 10T1/2 cells can be utilized as a model of MSCs, and A54 and M1601 are used as committed mesenchymal progenitors. Gene expression profiles of these cell lines were compared by microarray analysis before and after differentiation.

Each of parent 10T1/2, A54, and M1601 cell lines showed a distinctive and unique gene expression profile despite morphological similarity (Fig. 1) [8]. Parental 10T1/2 cells had 105 elevated genes including ones encoding Activin, Dlk, Nov, Grb10, p15, and many functionally unknown molecules. Dlk and Nov are known to be involved in Notch signaling pathway and were reported to have the ability to inhibit differentiation into adipocytes and osteoblasts [9]. In preadipocyte A54 cells, 201 genes were up-regulated, including genes known to be involved in adipocyte differentiation such as genes encoding C/EBPα, C/EBPδ, PPAR-γ, PAI-I, and Frizzled-1 [10]. Myoblasts M1601 cells showed 137 up-regulated genes, including ones related to skeletal muscle differentiation such as genes encoding MyoD, MLC1F, α-skeletal actin, myosin heavy chain, and myosin light chain [11] as well as genes related to cardiac muscle differentiation such as genes encoding α-cardiac actin, cardiac troponin C, and troponin T2 [12].

Previous studies have shown that preadipocytes have a higher ability to support hematopoiesis than other kinds of stromal cell components *in vitro* [12,13]. Our results of gene expression profile revealed up-regulation of critical cytokines for hematopoiesis such as SCF and SDF-1 in preadipocyte A54 cells. In addition, many chemokines, such as CXCL-1 and CCL-7, were also up-regulated. Since Ang-1 was reported to be indispensable for the self-renewal of hematopoietic stem cells [14], we performed real-time PCR analysis of Ang-1 along with SCF, SDF-1, CEBP-δ, IGF-1, and CXCL-1. The expression of these genes was highest in A54 cells among the three cell lines. Moreover, protein expression of Ang-1 was only detected in A54 among three cell lines and the level of this protein decreased after adipocyte differentiation.

To examine the effects of these three lines on hematopoiesis, we co-cultured mouse hematopoietic stem cell fraction with these three stromal cell lines. The cells in Lin(−)Sca-1(+) fraction were plated on 10T1/2, A54, or M1601 cells.

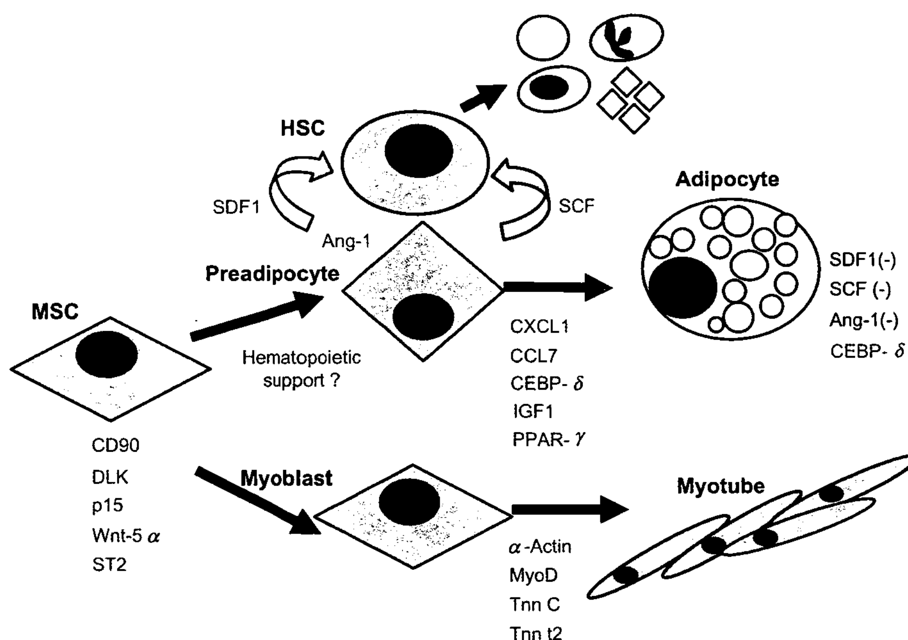

Fig. 1. Proposed model for the hierarchy of the bone marrow stromal system [8].

After 6 days of co-culture, hematopoietic progenitors were detected only on the A54 cells. These results suggest that only A54 cells have the ability to support hematopoietic cell growth among these three cell lines, consistent with the previous report. Hematopoietic cell proliferation was not observed on the layer of the terminally differentiated A54 adipocytes, suggesting that A54 cells lose the ability for hematopoietic cell support after adipocyte differentiation. To understand the molecular mechanisms of this observation, we examined the expression levels of SCF, SDF-1, and Ang-1 during adipocyte differentiation by RT real-time PCR. The expression levels of Ang-1 and SCF decreased immediately after the induction of adipocyte differentiation, and that of SDF-1 decreased gradually. In contrast to this, the level of adipocyte differentiation marker, CEBP-δ, was unchanged.

The analysis of functionally unknown molecules is currently underway. In addition, cell-to-cell contact is also believed to be crucial in the interaction between hematopoietic stem cells and MSCs. We are currently investigating the cellular and molecular events in the interactive communication between hematopoietic stem cells and MSCs.

3. Nitric oxide (NO) plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

There is a case report of severe steroid-resistant GVHD after bone marrow transplantation, in which intravenous infusion of MSCs greatly improved clinical manifestations [3]. Moreover, multi-institutional clinical trial of MSC-treatment of severe grade III–IV acute GVHD in Europe revealed very high overall response rate (about 70%) (Le Blanc et al., ASH meeting 2006). The molecular mechanisms by which MSCs suppress T-cell proliferation are complicated, and whether a soluble factor plays a major role remains controversial. Transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), and prostaglandin E_2 (PGE_2) have been reported to mediate T-cell suppression by MSCs [15–17]. In addition, some reports have shown that a soluble factor is the major mediator of suppression, whereas some reports have demonstrated that T-cell-MSC contact is required for this suppression.

We also investigated the molecular mechanisms using primary murine MSCs, and focused on nitric oxide (NO), because it is known to inhibit T-cell proliferation. NO is produced by NO synthases (NOSs), of which there are 3 subtypes; i.e. inducible NOS (iNOS), endothelial NOS, and neuronal NOS. It has been known that macrophages suppress T-cell proliferation, and that this suppression is caused by NO-mediated inhibition of Stat5 phosphorylation [18]. We investigated whether MSCs can also produce NO and whether NO is involved in their ability to suppress T-cell proliferation [19].

T cells proliferated in response to PMA and ionomycin, which act downstream of the T-cell-receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. Such T-cell proliferation was suppressed by the presence of MSC, suggesting that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx. The expression of the activation markers CD25 and CD69 on CD4 or CD8 T cells did not change even in the presence of MSCs. MSCs suppressed the production of IFN-γ but not IL-2.

Although T cells from $Stat5^{-/-}$ mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25. Because this phenotype is similar to the status of activated T cells in the presence of MSCs, we hypothesized that MSCs suppress Stat5 phosphorylation. Indeed, Stat5 phosphorylation in activated T cells was diminished in the presence of MSCs. We found that MSCs caused a significant and cell-dose-dependent production of NO only when co-cultured with activated T cells. The induction of iNOS was readily detected in MSCs but not in T cells. RT-PCR and Western blot analysis detected iNOS expression in MSCs cocultured with activated splenocytes but not in MSCs or splenocytes when cultured alone. The immunofluorescence studies showed that iNOS was exclusively expressed in $CD45^-$ adherent cells, which correspond to MSCs, but not in $CD45^+$T cells. Next, we investigated the effects of *N*-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells. Importantly, L-NAME restored T-cell proliferation and Stat5 phosphorylation, indicating that NO is involved in the inhibition of T-cell proliferation and Stat5 phosphorylation. Moreover, MSCs from inducible $NOS^{-/-}$ mice had a reduced ability to suppress T-cell proliferation.

In the presence of direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation. In contrast, both NO production and T-cell suppression were reduced in a transwell system, in which T cells were separated from MSCs by a 1-μm-pore membrane. There are two possible explanations for the difference in T-cell suppression between the presence and absence of the transwell system. First, the amount of NO produced in the transwell system was lower than that in the presence of direct interaction. This finding suggests that direct interaction is critical for efficient production of NO as well as for strong suppression of T-cell proliferation. A second possible explanation is that, because NO is highly unstable, it can lose its activity before it reaches T cells in the transwell system.

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs, we compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-methyl-DL-tryptophan (1-MT: inhibitor of IDO) or an anti-TGF-β-neutralizing antibody restored T-cell proliferation as effectively as L-NAME; however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression.

In summary, our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through inhibition of Stat5 phosphorylation was supported by the following facts: (1) NO was readily detected in the medium in the co-culture of MSCs and activated T cells; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from $iNOS^{-/-}$ mice had markedly

reduced ability to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS expression was detected only in MSCs co-cultured with activated T cells.

In our scenario (Fig. 2), when MSCs are administered to the patients with severe acute GVHD, MSCs are considered to accumulate at the site of inflammation. Upon interaction with activated T cells, MSCs express iNOS and produce NO, which suppresses T-cell proliferation via inhibition of STAT5 phosphorylation. Systemic adverse effects of NO do not occur due to local production of NO with very short half-life. This is a very important point, because conventional treatment of acute GVHD causes severe systemic immunosuppression, which sometimes leads to life-threatening infections. Since MSC treatment causes just local immunosuppression, it should be much safer.

4. Interferon-γ and NF-κB mediate nitric oxide production by mesenchymal stem cells

Human MSCs were reported to suppress Thl differentiation and augment Th2 differentiation. Therefore, we investigated whether mouse bone-marrow-derived MSCs and the 10T1/2 cell lines have the same effect on Thl and Th2. We found a reverse correlation between NO production and T cell proliferation in Thl/Th2 conditions, where NO production was highly induced in the presence of MSCs in Thl but it was only minimally induced in Th2. In particular, primary MSCs and the A54 preadipocyte cell line, which induce strong T cell suppression in Thl, produce high levels of NO in Thl condition. These results suggest that NO also plays a major role in the preferential suppression of Thl proliferation by MSCs.

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that support Th2 differentiation, anti-IFN-γ antibody and IL-4, were investigated. As a result, anti-IFN-γ antibody clearly inhibited the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN-γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated T cells had the ability to induce NO production by MSCs. IFN-γ is critical for NO production; however, in a T cell-free environment, IFN-γ alone does not induce NO production from primary MSCs. IFN-γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs, suggesting that both the IFN-γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. The addition of flagellin induced NO production in combination with IFN-γ. While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO. Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN-γ signal. In addition to these factors, IL-1β and TNF-α induce NO when provided in combination with IFN-γ. As NF-κB is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1β, and TNF-α, we hypothesized that activation of NF-κB is required for NO induction by MSCs. Bay-11-7085, a specific inhibitor of NF-κB, suppressed induction of iNOS in MSCs, thus suggesting that NF-κB is involved in NO production by MSCs as well as IFN-γ [20].

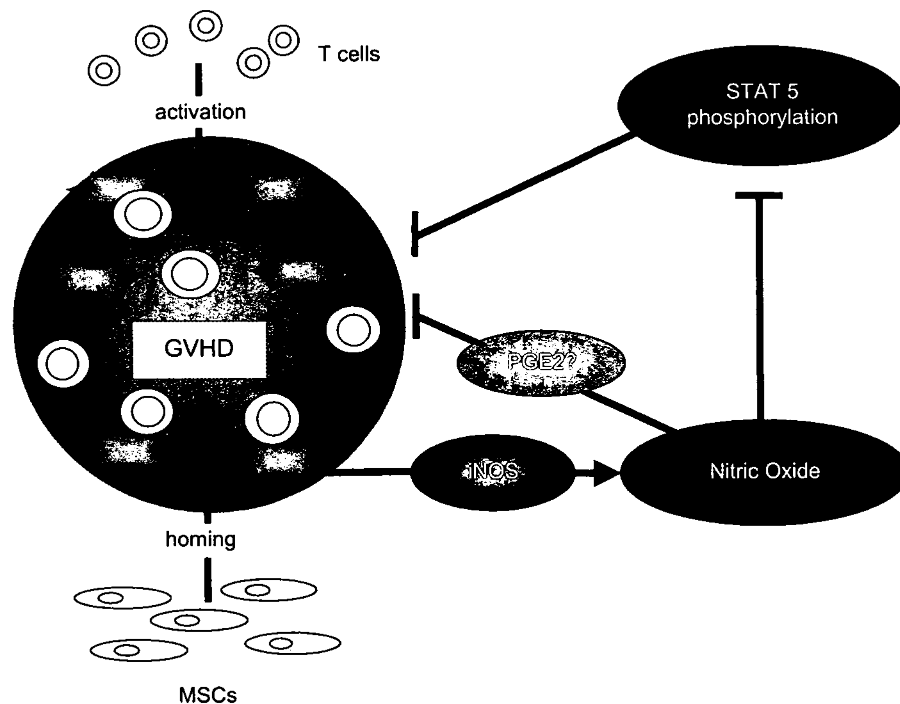

Fig. 2. MSC treatment of acute GVHD and the molecular mechanisms of T-cell suppression. MSCs are considered to accumulate at the site of inflammation and systemic adverse effects may not appear due to the local production of NO, which has very short half-life.