Enhanced engraftment of genetically modified bone marrow stromal cells

by

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Abstract

Transplantation of marrow stromal cells (MSC) shows promise as a vehicle for gene therapy. However, engraftment efficiency must be improved. To enhance homing and hence engraftment, we expressed VLA-4 on MSC to elicit interactions with adhesion molecules on marrow stem and stromal cells. Electroporation was used to transfect murine stromal cells with VLA-4 α chain cDNA. Cell surface VLA-4 expression and functional binding to VCAM-Ig were demonstrated by flow cytometry. Male MSC were infused into untreated or irradiated female Balb/c recipients with or without co-infusion of marrow cells. Male donor cells were detected by FISH or PCR for Y chromosome specific sequences. The highest level of MSC engraftment was with transfected MSC co-infused with marrow cells. The data show that stromal cell engraftment can be improved by almost 50 fold over infusion of unmodified stromal cells alone and suggest that this approach may have clinical utility.

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List of Abbreviations

α4 integrin: Alpha-4 chain

β1 integrin: Beta-1 chain

β7 integrin: Beta-7 chain

BMC: Bone marrow cells

BMT: Bone marrow transplantation

FACS: Flow cytometry

FGF: Fibroblast growth factor

FISH: Fluorescence in situ hybridization

FN: Fibronectin

FVIII: factor VIII

Ig: Immunoglobin

IL-1: Interleukin-1

IL-4 Interleukin-4

L929: Mouse fibroblast cell line

LTMC : Long term bone marrow culture

mAb: Monoclonal antibody

Mn: Manganese Ion

P388D1: Mouse monocyte-macrophage cell line

PCR: Polymerase chain reaction

SCF: Stem cell factor

TDY: Testis determining Y region

TNF-α: Tumor necrosis factor-alpha

VCAM-1: Vascular cell adhesion molecule-1

VCAM-Ig: Recombinant vascular cell adhesion molecule fusion protein

VLA: Very late adhesion molecule

Chapter 1

INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis is a complex process in which pluripotent stem cells differentiate and evolve into committed progenitor cells. They in turn proliferate into mature functional blood cells such as erythrocytes, megakaryocytes, monocytes, neutrophils, eosinophils, basophils, T- as well as B-lymphocytes. Hematopoiesis takes place within the bone marrow stromal microenvironment (figure 1).

1.2 Stromal cells

The bone marrow stromal microenvironment is a complex network of cells and extracellular matrix, which maintains the hematopoietic system throughout life. Components of the hematopoietic microenvironment include the cellular part, stromal cells; and the extracellular part, matrix. Stromal cells are characterized by nonhematopoietic features and include cells of reticular, monocytic, epithelial, endothelial, adipocytic, fibroblastic, and smooth muscle type.

Stromal precursor cells can differentiate into osteoblasts, chondroblasts, adipocytes and fibroblasts in culture under appropriate conditions or after implantation into appropriate tissues in vivo (Pereira et al., 1995). After infusion of marrow stromal cells into the hosts, progeny of the cells appears in a variety of tissues, including bone (Pereira et al., 1995), cartilage (Pereira et al., 1998), lung, spleen, and thymus (Keating et al., 1996). In addition, some studies showed that marrow stromal cells can engraft into the central nervous system. The presence of donor-derived astrocytes was observed after systemic infusion of whole marrow into immunodeficient mice (Eglitis et al., 1997). However, stromal cells are less likely to differentiate as opposed to hematopoietic cells and are considered to be quiescent unless they are induced by certain growth factors or cytokines.

Components of the extracellular matrix consist of basal lamina, and interstitial collagen types, fibronectin and proteoglycans which are all identified in the adherent

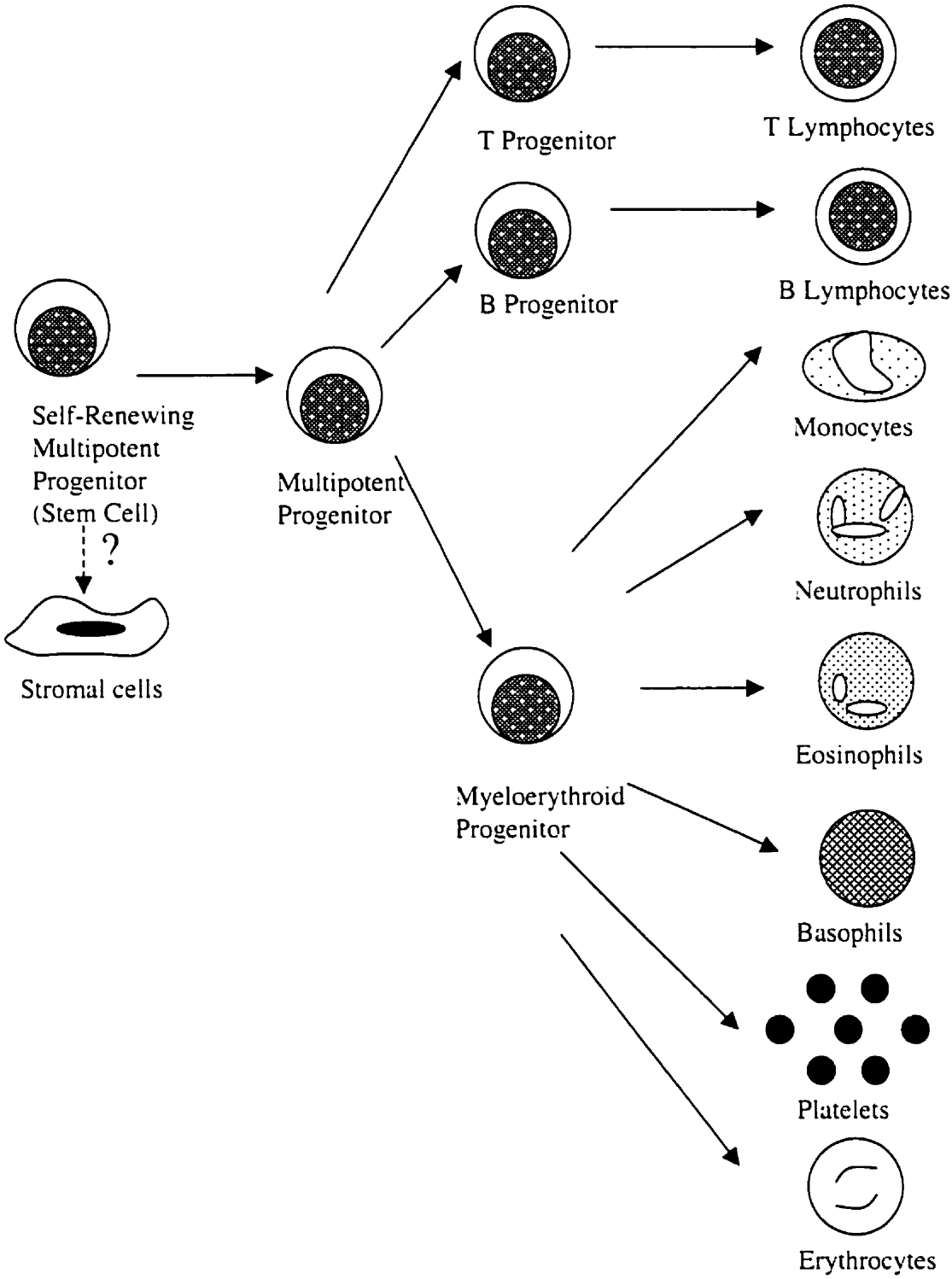


Fig 1. Differentiation pathways of hematolymphoid cells. The primary site of early hematopoietic differentiation in the adult animal is the marrow, which contains microenvironments necessary for the maintenance and differentiation of pluripotent stem cells. The differentiation and proliferation of myeloid and erythroid precursors are dependent on stromal elements that reside in marow and, to a lesser extent, in spleen. The origin of stromal cells remains to be elucidated (Morrison et al., 1995, and Muller-Sieburg, 1989).

layers of long term bone marrow cultures (LTMC) (Clark & Keating, 1995). LTMC has been used to study stem cell regulation (Dexter et al., 1977 and Greenberger, 1984). Extracellular matrix has been shown to be involved in different biological functions such as cell adhesion, binding and presentation of various cytokines and regulation of cell growth.

An essential feature of LTMC is the presence of an adherent layer composed of mesenchymal cells lacking hematopoietic characteristics. The nature of the stromal cells in LTMC has been defined by morphological criteria, antibody staining of the cytoskeleton and cell surface markers, and by enzyme reactions (Dexter, 1990). Stromal cells express class I histocompatibility antigens but not HLA-DR, are collagen IV positive, but lack the CD45 cell surface antigen which is a characteristic of most hematopoietic cells. Furthermore, stromal reticular cells are alkaline phosphatase positive whereas macrophages are not (Rosendaal M, 1991). Marrow stromal cells enriched with Stro-1, an antibody recognizing a human stromal surface antigen, were found to support hematopoiesis in vitro (Simmons and Torok-Strob, 1991).

Hematopoietic cells can be shown to interact with cellular and extracellular matrix components of the microenvironment through direct cell-cell contact interactions and the release of growth factors and cytokines. Cell adhesion molecules on stromal cells could be important for the release of cytokines as well as for mediating undifferentiated bone marrow cell binding. In situ studies of bone marrow showed that hematopoietic cells are found in close association with stromal cells, suggesting that stromal cells nurture hematopoietic cells (Weiss L, 1976). Studies demonstrating that hematopoiesis in cultures without this adherent layer declines rapidly (Dexter, 1982) indicate that the stromal cell layer plays a crucial role in promoting hematopoietic progenitor cell proliferation and differentiation.

1.3 Stromal cell transplantation

Autologous bone marrow transplants are performed widely for a variety of malignancies and may also provide a convenient means of delivering manipulated cells in gene therapy protocols for a variety of disorders (Gorin et al., 1989 and Gorin et al.,

1991). Anklesaria et al (1989) showed that transformed stromal cells from a murine model engraft and influence hematopoiesis in vivo. Furthermore, some stromal cell lines derived from LTMC transplanted intravenously could partially cure the microenvironmental defect in Sl/Sl^{d} mice (McCulloch et al., 1965). This suggests that marrow stromal cells may be capable of engraftment and thus, provide a vehicle for gene delivery to correct hematopoietic disorders. Stromal cells offer a number of advantages in this regard: they are easy to obtain and maintain in culture (Dexter et al., 1977) are amenable to transfection (Keating et al., 1990) and are transplantable (Wu and Keating, 1991). However, mechanisms governing stromal cell engraftment in transplant recipients remain to be determined.

1.4 Hematopoietic stem cells

Hematopoietic stem cells are a population of cells capable both of self-renewal and differentiation into a variety of hematopoietic lineages (Spangrude et al, 1988 and Wolpert, 1988). Enrichment techniques for human hematopoietic stem cells have used the expression of CD34, present on bone marrow progenitor cells (Saeland et al, 1988 and Mayani et al, 1989).

A stem cell in the bone marrow is thought to be surrounded by a specific combination of stromal elements. Such a special microenvironment is referred to as a niche (Schofield, 1978). As stem cells differentiate into more mature progenitor cells, they may move from one type of niche to another (Mason et al., 1989 and Miyake et al., 1991). In a niche, the microenvironment is thought to regulate stem cell activity by providing specific combinations of cytokines and by establishing direct contact with the stem cell (Zipori, 1988, Gordon, 1988 and Funk et al., 1994). Lack of contact leads to imbalanced maturation of stem cells (Verfaillie, 1992). For successful long term engraftment of bone marrow transplantation recipients, it is necessary that at least some stem cells reach the specific niches. This process is referred to as homing. Coincidental binding to stromal cells may enable them to reach a stem cell niche.

1.5 Homing

Homing represents a cascade of adhesive interactions between hematopoietic cells and bone marrow stroma and/or its extracellular matrix. Homing is a highly selective process that may be mediated through a specific homing receptor or through a method of selective capture, retainment, or survival advantage afforded by the marrow (Cheryl Hardy, 1995 and Papayannopoulou and Craddock, 1997). The VLA4/VCAM-1 adhesion pathway plays a significant role in this process. VCAM-1 is recognized as the dominant bone marrow endothelial addressin in hematopoietic cell homing. Cytokine-activated human endothelial cells express vascular cell adhesion molecule-1 (VCAM-1), which binds lymphocytes (Mariano et al, 1990).

Hematopoietic stem cell interactions with elements of the underlying stroma are essential for sustained normal hematopoiesis. Adhesion receptors in the integrin family play a role in promoting adhesion of human hematopoietic stem cells to cultured human marrow stromal cells. Stem cells are known to home and adhere to stromal cells in vivo and in vitro whereas there is no evidence for homing of stroma. The mechanisms by which stem cells are restricted within specific areas in the marrow remain undefined (Aizawa and Tavassoli, 1987 and Aizawa and Tavassoli, 1988) and attention has begun to focus on the role of glycoproteins and integrins in this process (Verfaillie et al., 1991 and Williams et al., 1991). Enriched CD34hi progenitor cells express VLA-4 and other integrins. Homogeneous marrow stromal cell monolayers capable of supportable proliferation of CD34hi cells (Teixido et al, 1994), express VCAM-1 and fibronectin which are ligands for VLA-4 and VLA-5. It was demonstrated that VCAM-1 and VLA-4 represent a ligand-receptor pair that allows attachment of lymphocytes to activated endothelium. Moreover, a study (Mariano et al, 1990) showed that VLA-4 binding to VCAM-1 occurs by a mechanism independent of the VLA-4 interaction with fibronectin(FN).

The primary human bone marrow stromal monolayer in culture is a complex mixture of several different cell types including fibroblasts, endothelial cells, and macrophages. One approach to determine the contribution of a specific stromal cell

subtype toward stem cell adhesion and maintenance has been to establish homogeneous fibroblast-like, stromal cell cultures devoid of macrophages from murine marrow.

1.6 α and β chain of VLA molecules

Integrins are heterodimeric molecules made up of nonconvalently associated α and β subunits with a widespread cell distribution and a high degree of conservation throughout evolution (Hynes RO, 1987). Subsequent studies involving cross-linking, cell distribution, monoclonal antibody (mAb) recognition, and peptide mapping have shown that there are at least five distinct heterodimers in the VLA protein family (Hemler et al., 1985 and Hemler et al., 1987). From cell distribution studies, it is clear that nearly all tissues and cell types express one or more VLA complexes, and different lineages appear to have characteristic VLA profiles. (Hemler et al., 1983 and Hemler et al., 1984). The α4 subunit can associate with either the β1 or the β7 subunit. Both α4β1 and α4β7 are expressed on activated T and B lymphocytes and have been implicated in the development and progression of a number of autoimmune diseases (Yang et al., 1992, Abraham et al., 1994 and Burkly et al., 1994).

β1 integrin subfamily predominantly mediates cell attachment to the extracellular matrix (Hynes RO, 1987 and Ruoslahti E, 1988). The β1 integrin family, also termed VLA proteins, includes at least six receptors that specifically interact with fibronectin, collagen, and/or laminin (Hemler ME, 1990). VLA1, 2, and 3 mediate cell binding to collagen, VLA3, 4, and 5 bind fibronectin (FN), and VLA1, 2, and 6 bind laminin (Ruoslahti E, 1991). Within the VLA family, VLA-4 is atypical because it is mostly restricted to lymphoid and myeloid cells (Hemler et al, 1987b). VLA-4 (α4β1) complex is expressed at substantial levels on hematopoietic cells such as normal peripheral B and T cells, thymocytes, and monocytes as well as on marrow blast cells and erythroblasts but not on stromal cells; and plays a role in cell trafficking, activation and development through its interaction with two alternative ligands, VCAM-1 and FN.

1.7 VCAM-1 molecules

The ligand for VLA-4, VCAM-1, has been identified on the surface of activated endothelial cells of marrow stroma. VLA-4 surface expression on K562 cells following transfection of the VLA α4 subunit cDNA resulted in specific cell adhesion to VCAM-1, and anti-VLA-4 antibody completely inhibited this VCAM-1-dependent cell-cell attachment (Elices et al., 1990). Bone marrow stromal cells constitute a population of different cell types that provide the bone marrow microenvironment with cytokines necessary for sustained hematopoiesis and also express membrane ligands for adhesion receptors on hematopoietic cells. Integrin receptors play an essential role in cell-matrix and cell-cell interactions.

Vascular cell adhesion molecule-1 (VCAM-1) is a member of the immunoglobulin (Ig) superfamily (Osborn et al., 1989) which interacts with α4β1 (VLA-4) and α4β7 (Lobb and Hemler, 1994). VCAM-1 is constitutively or inducibly expressed on a variety of cell types including endothelial, neural, smooth muscle, dendritic and marrow stromal cells and regulates cell activation as well as adhesion (Anwar et al., 1994 and Romanic and Madri, 1994). Alternatively, VLA-4 binding to the extra-cellular matrix protein FN may control cell adhesion (Hines et al., 1994) and development (Teixido el al., 1992 and Salomon et al., 1994).

VCAM-1 was first identified as an adhesion molecule inducible on endothelial cells by inflammatory cytokines such as IL-1, IL-4 and TNF-α as adhesion of peripheral blood CD34+ cells to human bone marrow endothelial cells was observed only after IL-1 prestimulation of the endothelial cells (Rood et al., 2000). It was shown that IL-1 stimulation of stromal cells caused increased VCAM-1 expression and increased attachment by CD34hi bone marrow cells. CD34hi cells utilized VLA-4 to adhere to purified VCAM-1 and CD34hi cells may also utilize multiple integrin-mediated adhesion pathways to localize within specialized microenvironment niches created by marrow stromal cells (Teixido et al, 1992).

1.8 Importance of the VLA-4 and VCAM-1 interaction

Members of the integrin family of cell adhesion receptors mediate both cell-extracellular matrix and cell-cell adhesion and are involved in cell migration and localization during embryonic development, tissue organization, cell differentiation, inflammation, and metastasis (Hemler, 1991, Hynes, 1987, Rouslahti, 1988 and Hemler et al., 1990). The integrin, VLA-4 is the adhesion molecule used by CD34hi cells to attach to stroma and interacts with VCAM-1 which is constitutively expressed by bone marrow stromal cells. For example, the VLA/VCAM adhesion pathway has been implicated in important physiological processes involving migration and localization of immune cells (Elices et al., 1990 and Rice et al., 1990) and some tumor cells (Taichman et al, 1991). Adhesion of circulating tumor cells to endothelial cells in target blood vessels play an important role in the process of metastasis (Papadimitriou et al., 1999). It has also been shown that this adhesion pathway plays a role in mediating adhesion of stem cells to bone marrow stroma, which could be critical for initiation and maintenance of hematopoiesis in the bone marrow (Joauin et al., 1992). Interaction between human mast cells and endothelial cells is also mediated by VLA-4/VCAM-1 pathway (Mierke et al., 2000).

Several studies demonstrated a relationship between expression of VLA-4 and the development of metastasis (Holzmann et al., 1989). Therefore, an abnormal increase in the expression of α4 integrins may be one of the molecular mechanisms in advanced metastasis of cancer cells. It is reported that VLA-4 plays a critical role in erythropoiesis, and that the interaction between VCAM-1 and VLA-4 contributes to the formation of erythroblastic islands (Williams et al., 1991 and Sadahira et al., 1995). Thus an alteration in VLA-4 expression may even affect hematopoietic cells in vivo.

The α4 integrins are one of several families of molecules which control the migration of leukocytes into tissues during both inflammatory responses and normal lymphocyte recirculation (Springer, 1994, Garofalo et al, 1995 and Hamamura et al, 1996). This role has been clearly demonstrated by the effect of alpha4 specific mAbs in animal models of inflammation and autoimmune diseases (Lobb and Hemler, 1994). Several of these studies also demonstrated a role for the VLA-4 ligand, VCAM-1, which

is expressed on cytokine-induced endothelium (Osborn L, 1990). Vascular endothelial cells constitute the lining of blood vessels and normally exhibit a low affinity for circulating leukocytes (Harlan, 1985). The release of cytokines at sites of inflammation, and in response to immune reactions, causes their activation and results in the increased expression of a host of surface antigens (Collins et al., 1986, Bevilacqua et al., 1987, and Leeuwenbery et al., 1989). This cytokine-induced adhesion molecule, VCAM-1, which binds lymphocytes (Osborn et al, 1989) appears to play an important role in leukocyte recruitment to extravascular tissues and VLA-4 is its key receptor on circulating lymphocytes. VLA-4 recognizes ligands on activated endothelial cells (Takada et al, 1989), contributes to cytotoxic T cell adhesion to B cell targets (Bedhnarczyk J and McIntyre BW, 1989), participate in the homotypic aggregation of lymphocytes, and may be involved in the generation of intracellular signals important for regulation of T cell activation (Groux et al., 1989).

In addition, adhesion of human B cell precursors to marrow stroma is mediated by VLA-4 to VCAM-1. This adhesion pathway has been demonstrated to play a role in mediating the adhesion of stem cells to bone marrow stroma, which could be critical for the initiation and maintenance of hematopoiesis.

The α4 integrins are required for normal development of both B and T precursor cells in bone marrow (Arroyo et al, 1996), and long term bone marrow cultures have demonstrated the involvement of VLA-4 in lymphopoiesis (Miyake et al.1991), underscoring the importance of VLA-4-mediated adhesion during hematopoiesis. It is well established that VLA-4 on stem and precursor cells as well as on several leukemic cells mediates attachment to the ligands on bone marrow stromal cells (Teixido et al, 1992 and Ryan et al., 1991). This interaction is also important for the differentiation and proliferation of hematopoietic progenitor cells. In addition, VLA-4 has been shown to mediate T and B lymphocyte attachment to the heparin II binding fragment of human plasma fibronectin (FN) (Wayner et al., 1989).

In vitro studies also, have suggested a role for VLA-4 in the localization and activation of cells. For example, T cell proliferation and programmed cell death can be regulated through the VLA/VCAM pathway (Damle et al., 1993; Burkly et al., 1991).

VLA-4 supports the adhesion of germinal center B cells to VCAM-1 follicular dendritic cells (Freedman et al., 1990) and inhibits the death of these B cells (Koopman et al., 1994). VLA-4 engagement also induces cytokine expression in macrophages and eosinophils (Yurochko et al., 1992).

However, a high affinity state of VLA-4 molecule is induced by manganese ions or certain activating anti-β1 monoclonal antibodies (Jakubowski et al., 1995). This is consistent with the published data on the metal ion requirements for α4β1-dependent adhesion of cells to VCAM-1, which show that manganese ion can support increased cell adhesion to VCAM-1(Masumoto and Hemler, 1993). In addition, divalent cations bind to the VLA-4 receptor and regulate its function. However, the steps of the adhesive process which they affect are currently undefined. These effects may reflect an induced conformation with higher ligand binding affinity that favors ligand binding, and also affect post-binding steps, such as cytoskeletal alterations which strengthen adhesion and promote cell spreading (Hynes, 1992).

1.9 Studies of VLA/VCAM interaction in vitro

Hematopoietic precursors interact with stromal cells via members of the integrin family of cell adhesion molecules. These cell to cell interactions have been studied, for example, by using monoclonal antibodies to block the function of an alpha 4 epitope (VLA-4), which abrogates lymphopoiesis and suppresses myelopoiesis in murine LTMC. Some myeloid (U937) and lymphoid cell lines (preB Ramos, Nalm 6: T cell Jurkat) showed different degrees of adhesion to resting and activated stromal monolayers, and each was partly or almost completely inhibited by anti-VLA-4 and anti-VCAM-1 antibodies (Joaquin et al, 1992). The anti-α4 integrin monoclonal antibodies prevent binding of granulocyte-macrophage progenitors to stroma. Antibodies against VLA-4 prevent experimental automimune encephalomyelitis, an inflammatory condition of the central nervous system with similarities to multiple sclerosis (Yednock et al., 1992). Preincubation of human CD34+ progenitors with anti-VLA-4 blocking antibodies resulted in a profound reduction of human cell lodgement in the sheep hematopoietic

compartment after in utero transplantation, with a corresponding increase of human cells in the peripheral blood circulation (Zanjani et al., 1999).

The stromal ligand for the VLA-4 integrin is thought to be either an alternately spliced form of fibronectin (Guan and Hynes, 1990) or the VCAM-1 molecule which is an endothelial cell surface protein on stromal cells (Simmons et al., 1992 and Rosen et al., 1992). Hematopoietic cells expressing VLA-4 may interact with VCAM-1 for appropriate adhesion with the marrow microenvironment. Furthermore, after chemotherapy and / or total body irradiation, the observed extravasation of stem cells into peripheral blood might result from an interaction with VCAM-1 on activated venule endothelium (Sienna et al., 1989).

1.10 Regulatory role of VLA-4 in signal transduction

In many cellular systems, integrins are not only involved in migration and homing, but also regulate signaling pathways (Rosales et al., 1995 and Clark et al., 1995). Structural features of integrins such as the extracellular I(nsert) domain, metal ion-dependent adhesion sites (MIDAS), and the intracellular XGFFKR and LLviXhDR motifs of the α and β chains, respectively, have been characterized (Juliano et al., 1993 and Clark et al., 1995). These structural integrin characteristics are important for affinity modulation, ligand binding, and the induction of integrin-associated intracellular signals (Schwartz et al., 1995 and Juliano et al., 1993). For VLA-4, it was shown that chimeras of the extracellular domain of α4 and the intracellular domains of either α2, α4, or α5 chains functionally mediate binding of VLA-4 to VCAM-1 and FN (Kassner et al., 1993).

Several signal transducing molecules, including many PTK, have been shown to be involved in integrin signaling (Parsons et al., 1996) and most data have been obtained with focal adhesion kinase $pp125^{FAK}$. This PTK is rapidly phosphorylated and activated after adhesion of T-lymphoblastic cells to FN or VCAM-1 (Nojima et al., 1995), and adhesion of NK cells to FN (Rabinowich et al., 1995). Increases in phosphorylation were partially blocked by anti-VLA-4 or anti-VLA-5 monoclonal antibodies (Nojima et al., 1995).

In tonsillar B cells, ligation of the α4, α5 and β1 chains of VLA-4 and VLA-5, respectively, or adhesion to FN induce a similar pattern of tyrosine phosphorylated proteins, one of which is $pp125^{FAK}$ (Manie et al., 1996). It indicated that $pp125^{FAK}$ is involved in VLA-4 and VLA-5 mediated adhesion pathways in hematopoietic cells. Investigation of the roles of $pp125^{FAK}$ and other signal transducing molecules and transcription factors involved in adhesion and adhesion-associated regulation of hematopoietic progenitor cells and stromal cells awaits exploration.

1.11 Rationale

There are other VLA proteins, each composed of an unique alpha subunit noncovalently associated with a common beta subunit, such as VLA-2, 3, 5 or 6. These are present on nearly all adherent cells, including stromal cells, and have an endogenous beta chain but lack the alpha chain to form a complete VLA molecule. The very late antigen (VLA-4) integrins likely mediate hematopoietic progenitor cell attachment to bone marrow stroma via VLA-4 on stromal cells and VCAM-1 on the surface of stromal cells. This raises the possibility that stromal cells transfected with the VLA alpha chain will express the heterodimer with the beta chain and generate a functional cell surface VLA-4 molecule that can participate in VLA-4/VCAM-1 interactions (figure 2). Doublet cell formation between stromal cells with VCAM-1 expression and bone marrow cells with endogenous VLA-4 expression may provide a means of stromal cell homing. The stromal–hematopoietic cell doublet may then home by hematopoietic cell interaction with the marrow microenvironment (figure 3).

Previous studies indicate that marrow stromal cells can be readily transfected by electroporation to express transgenes (Keating et al., 1990). Since stromal cells are amenable to transfection, it is expected that the alpha chain of the human integrin molecule, VLA-4, can be transfected into murine stromal cells which can be used to enhance adhesion and engraftment in recipient mice. Human and mouse α4 subunits are highly homologous to each other. Furthermore, binding of human/mouse chimeric receptors to recombinant VCAM, a ligand for α4β1, is poor unless VLA-4 molecules are being induced by the addition of divalent cations, Mn^{2+}. It has been shown previously

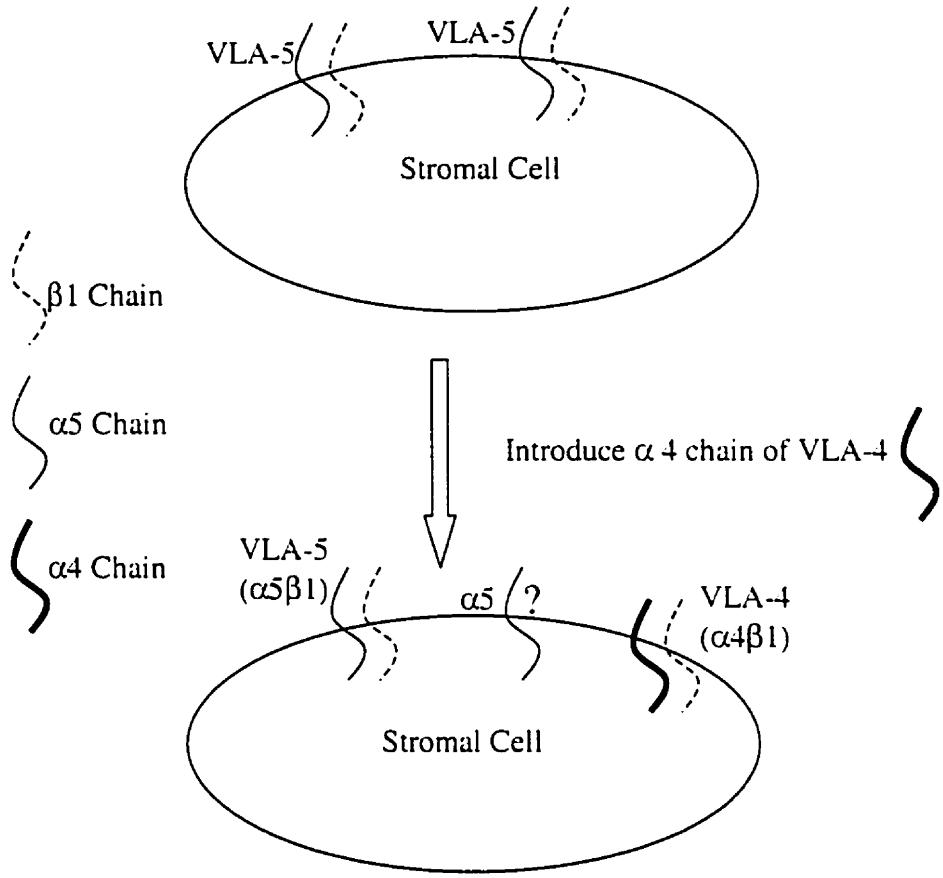
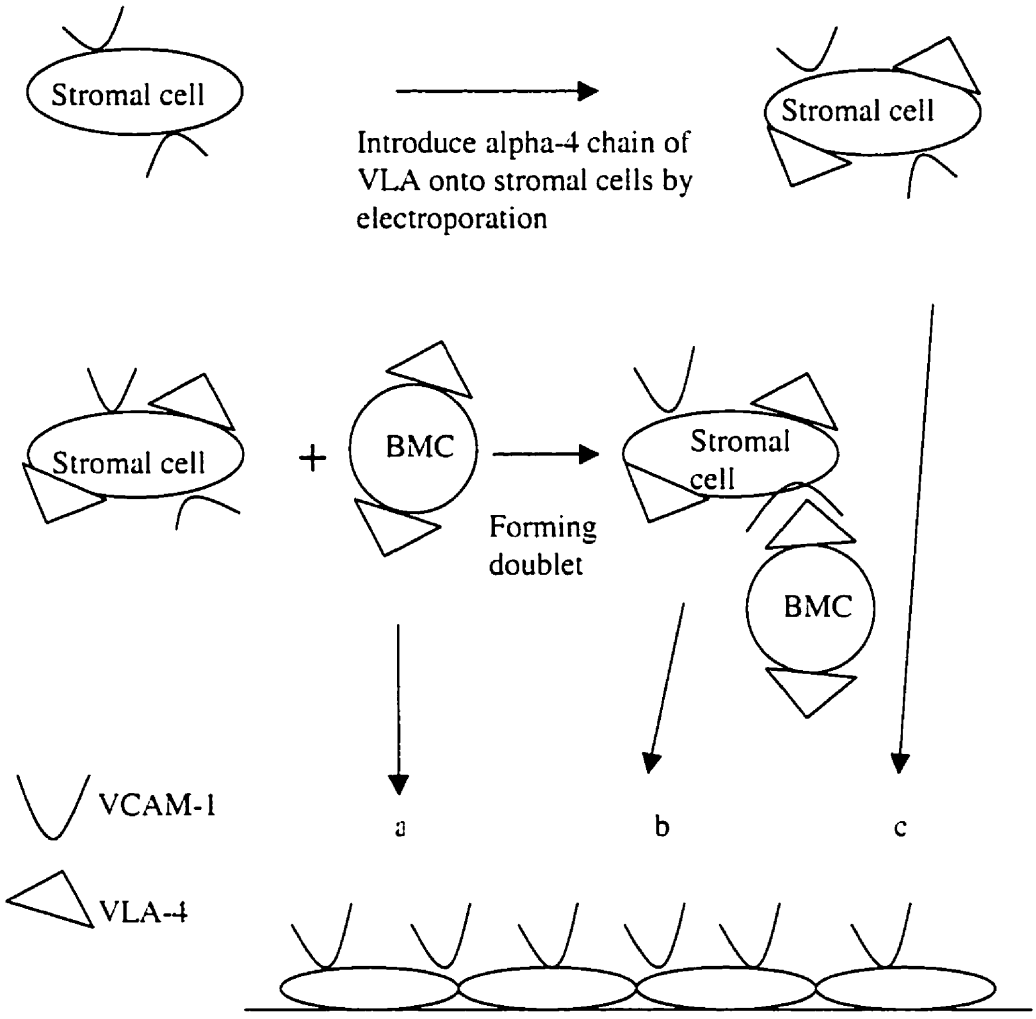


Fig 2. Stromal cell transfected with the VLA α4 chain is believed to express the heterodimer (α4β1)with the β1 chain to generate a functional cell surface VLA-4 molecule.



Endothelial cells with VCAM-1 expression in the recipient stroma

Fig 3. Cells with VLA-4 molecules bind to VCAM-1 expressing endothelial cells in recipient stroma: a. endogenous VLA-4 on bone marrow cells, b. increase of VLA-4 expression on doublets (present on both stromal cells and bone marrow cells), c. direct binding of VLA-4 transfected stromal cells to stroma via VLA-4/ VCAM-1 pathway. b and c are two possible mechanisms leading to enhanced engraftment /homing.

that murine VLA-4 can bind to both human and murine recombinant VCAM (Romanic and Madri, 1994).

Transfection of stromal cells with the alpha chain of VLA-4 is proposed as a means to generate a VCAM-1/VLA-4 interaction in this experimental model in order to enhance the interaction between infused stromal cells and the marrow microenvironment.

1.12 Hind limb irradiation

In vivo studies of murine models suggest that stromal cell engraftment in the bone marrow of unconditioned recipients occurs at a low level. Local irradiation may improve residence of donor cells in certain niches inside the bone marrow of transplant recipients.

The mechanism by which irradiated marrow stromal sites preferentially accepts donor stromal cells is not yet known. Possible explanations include the induction of cellular adhesion molecules, integrins, or extracellular matrix proteins on recipient stromal cell surfaces that stimulate attachment of circulating cells with complementary receptors (Greenberger, 1991). Alternatively, irradiation may simply clear a niche to provide space for the seeding of donor cells. There is evidence that transplanted marrow fibroblast progenitor cell lines can migrate through the circulation and seed into sites of irradiated marrow niches, where they can provide a supportive microenvironment for hematopoiesis (Anklesaria et al., 1987).

Conditioning regimes such as total body irradiation and chemotherapy before bone marrow transplantation serve to destroy the host immune system sufficiently to allow permanent engraftment and eliminate the abnormal tumor cells (Anklesaria et al, 1987). Local irradiation has fewer and less severe side effects and complications compared with those associated with total body irradiation. It is possible that local hind limb irradiation improves stromal engraftment by clearing and providing niches for infused stromal cells to reside in the marrow. To date, little is known about where within the bone marrow, and other tissues, transplanted stromal cells engraft even after local or total body irradiation.

1.13 Hypothesis

Modulation of adhesion molecules on stromal cells improves stromal engraftment by enhancing interactions between modified stromal cells and either endogenous stroma or co-infused marrow hematopoietic cells.

1.14 Objectives

i) Demonstrate the engraftment potential of bone marrow stromal cells transfected with the alpha chain gene of VLA-4 in both conditioned and unconditioned murine transplant recipients.

ii) Compare the engraftment level of bone marrow stromal cells in irradiated versus non-irradiated hind limbs.

iii) Compare the difference of engraftment potential between infusion of stromal cells and their syngeneic bone marrow cells versus stromal cells only.

iv) Identify location of infused stromal cells as a function of time after transplant including lung, liver, spleen and brain.

Chapter 2

MATERIAL AND METHODS

Modulation of stromal cell engraftment will be attempted in three ways: 1) Alpha4 chain plasmid transfection, 2) Total body irradiation of transplant recipients and 3) Co-infusion of bone marrow cells.

2.1 Mice

Male and female Balb/c mice (Jackson Laboratory, Bar Harbor HE) between 6 and 8 weeks of age were used for generating long term bone marrow cultures and as stromal cell transplantation recipients, respectively. They were maintained in the animal facility of Ontario Cancer Institute, Toronto, Canada.

2.2 Long term bone marrow culture (LTMC)

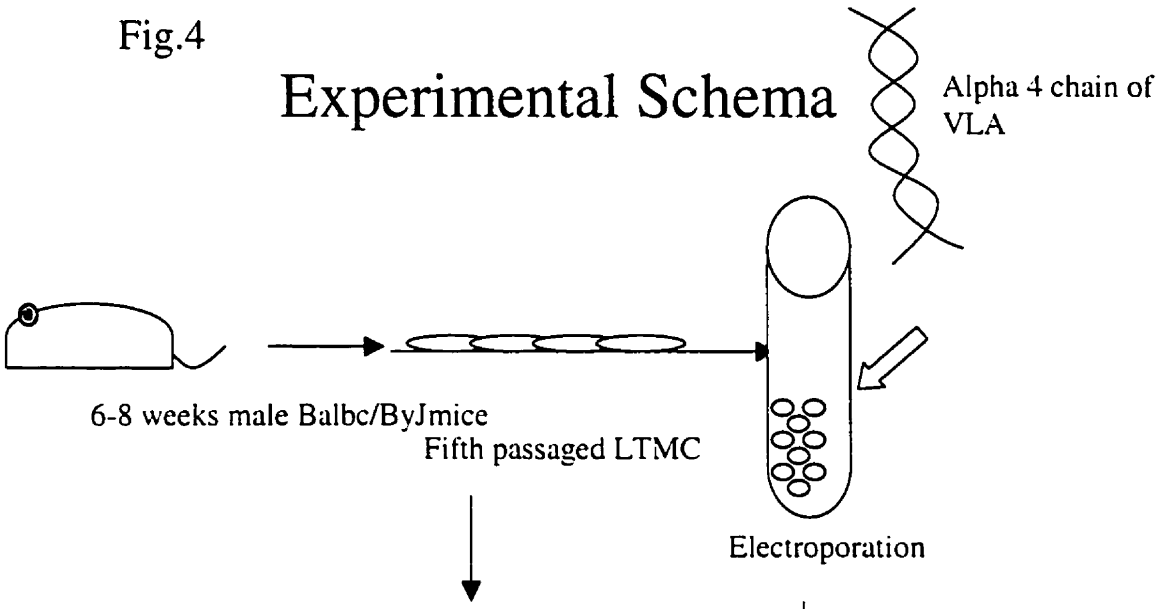
Fetal bovine serum (FBS) and horse serum (HS) were purchased from Life Technologies, Burlington, Ontario. Recombinant human FGF and recombinant human IL-1α were purchased from R & D Systems Inc. LTMC were initiated from Balb/c mice and were plated in flasks with media (McCoy medium supplemented with 12.5% fetal calf serum, 12.5% horse serum, rhFGF, rhIL-1, 0.8% essential amino acids, 0.4% non-essential amino acids, 1% glutamine, sodium pyruvate, sodium bicarbonate, vitamins, 10^{-7}M hydrocortisone which were ordered from GIBCO Canada Ltd., Burlington, Ontario.) changes every week. Adherent cells were serially passaged 4 to 6 times in LTMC medium until a homogenous stromal cell layer free of hematopoietic cells was obtained.

2.3 Characterization of stromal cells

Antibodies against the following antigens were used for immunohistochemical analysis: collagen IV and CD45. Alkaline phosphatase activity was demonstrated by cytochemical staining. Two cell lines, L929 and P388D1 (American Type Culture Collection, ATCC, Manassas, USA), were used as controls for the following stromal cell

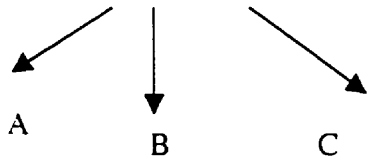
Fig.4

Experimental Schema



Phenotyping of stromal cells:
~Immunohistochemistry
~Cytochemistry

VLA-4 Expression by Flow Cytometry
~Detection the presence of alpha-4 chain with anti-alpha4 antibody
~Testing of functional VLA-4 molecule with VCAM-Ig binding assay



Presence of Y chromosome:
~PCR
~In situ hybridization

A. Infuse intravenously $3x10^6$ male stromal cells (transfected vs. untransfected) with $3x10^6$ female BMC into unconditioned female recipients.
B. Infuse intravenously $3x10^6$ male stromal cells (transfected vs. untransfected) into unconditioned female recipients.
C. Infuse intravenously $3x10^6$ male stromal cells (transfected vs. untransfected) with $3x10^6$ female BMC into conditioned female recipients.

phenotyping experiments carried out at room temperature unless otherwise specified. L929 (fibroblast cell line) and P388D1 (mouse monocyte-macrophage cell line) were characterized using specific immunohistochemical and cytochemical markers (table 1).

Marker	L929	P388D1
Collagen IV	+	-
Alkaline phosphatase	+	-
CD45	-	+

Table 1 : Phenotyping of fibroblast cell line (L929) and mouse monocyte-macrophage cell line (P388D1) with immunohistochemical markers: Collagen IV and CD45 and cytochemical marker: Alkaline phosphatase.

2.3.1 Collagen IV expression

Passaged stromal cells, L929 and P388D1 were seeded onto chamber slides at least one day prior to staining. Cells were first washed in 1xPBS twice for 5 minutes each and then fixed with 3% paraformaldehyde in PBS for 15 minutes. Slides were permeabilized in 50% ethanol for 6 minutes and washed in PBS for three times and then incubated with primary antibody, BioDesign Rabbit anti-mouse collagen IV ordered from Pharmingen, Mississauga, Ontario, at a 1/40 dilution in 3% BSA in 1xPBS for 1 hour. Slides were washed in PBS for three times and incubated with secondary antibody, FITC-labeled Jackson Goat anti-rabbit IgG ordered from Pharmingen, Mississauga, Ontario, at a 1/100 dilution in 3% BSA in 1x PBS for 1 hour. Slides were washed in PBS three times and a mixture of 5μl DAPI, 0.5μl PI and 15μl antifade was added onto each slide.

2.3.2 Alkaline phosphatase activity

The entire staining procedure was provided by Sigma Diagnostics Alkaline Phosphatase kits for the histochemical semi-quantitative demonstration of alkaline phosphatase activity in leukocytes. Slides were fixed in ice cold acetone for 20 minutes prior to utilizing the diagnostic kit. Slides were then incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH9.5 with 2-amino-2-methyl-1,3-propanediol.

2.3.3 CD 45 expression

Slides seeded with cells were handled the same way as for collagen IV staining before the addition of antibody. Slides were incubated with FITC anti-CD45 Rat IgG2b monoclonal antibody ordered from Pharmingen, Mississauga, Ontario, at a 1:20 dilution in 3% BSA in 1xPBS for 1 hour. Slides were then washed in PBS three times and 10μl of antifade was added onto each slide.

2.4 Preparation of DNA plasmid

A plasmid containing the alpha 4 chain of VLA molecule was a gift from Dr. Martin Hemler, Dana Farber Cancer Institute, Boston, USA (figure 5). Construction of plasmids containing VLA α subunit cDNA, transcribed from the SFFV-LTR, and selection of stable transfectants have been described in his paper (Elices et al., 1990). DNA plasmid from ampicillin resistant colonies was prepared by small and then by large-scaled DNA preparation. Single colony was inoculated and grown at 37^{0}C overnight. Cells were then treated with lysozyme solution and sodium hydroxide as detergent. Supernatant was collected after centrifugation and precipitated with isopropanol. Pellet obtained was then suspended in TE with adding sodium acetate and ethanol. Plasmid was then digested with Sal 1 and Xba 1 purchased from MDC, University of Toronto, Toronto, Canada, to identify that the fragment of interest is the correct size. Fragment sizes here are 3.8 and 5.9 kb. Salmon sperm control DNA was prepared using a similar method by Dr. Xing Hua Wang in our lab.

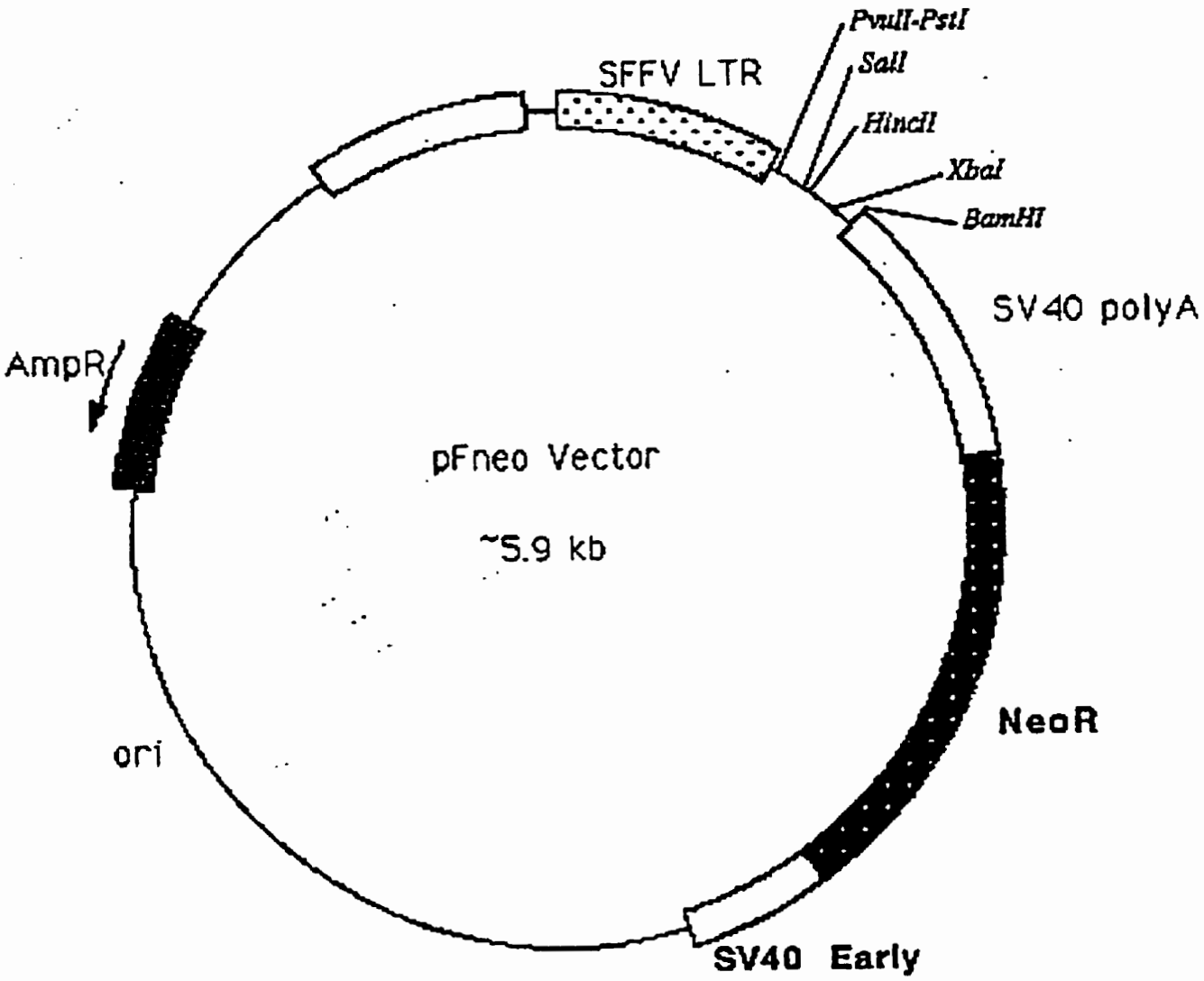


Fig 5. Map of bacterial plasmid carrying the VLA α4 chain transcribed from the SFFV-LTR

2.5 Electroporation of α4 chain (VLA-4) and salmon sperm DNA into stromal cells

Mouse stromal cells were transfected with the supercoiled alpha chain of the human integrin molecule VLA-4 plasmid by electroporation. The squarewave electroporator was used to electroporate stromal cells at the following conditions in which the optimal one was determined:

(1) 180V 70msec 1pulse

(2) Electroporation setting suggested for adherent fibroblasts (mouse B6 cells):
300V 99usec 4pulses

(3) Electroporation setting suggested for rat mammary epithelial adenocarinoma (Con8Hd6): 400V 99usec 5pulses

(4) Electroporation setting suggested for mouse fibroblasts (L929):
650V 99usec 5pulses

Stromal cells underwent electroporation with the BTX T820 electroporator following the above electroporation conditions. Supercoiled and purified DNA plasmid were used in this transient expression experiment at a concentration of 50μg/ml. For each transfection, two T150 flasks of stromal cells (approximately 5-6 x10^6 cells) were first detached with 0.25% trypsin and 1mM EDTA in 1xPBS and then 20% serum was added to inactivate trypsin. Cells were washed twice in PBS and DNA was added into stromal cells that had been suspended in 1ml 1xPBS. Cells were incubated on ice for 15 minutes, then were transferred to a 4mm gap cuvette and electroporated using the above settings. After being electroporated, cells were incubated on ice for 10 minutes before being put back into culture. Viability of transfected stromal cells was determined by manual cell count after staining with trypan blue post-transfection. Transfection of salmon sperm DNA into stromal cells for the control experiment was also performed as described above.

2.6 Coincubation of alpha 4 chain transfected stromal cells with its antibody

For group 2 of the control experiments, stromal cells, after being transfected with VLA-4 plasmid, were coincubated with 10μg/μl anti-α4 antibody for 30 minutes at room temperature. After the incubation, cells were washed in 1xPBS twice before injection into mice to remove excess antibody bound nonspecifically to the cell surface.

2.7 Flow cytometry

The transfected stromal cells were monitored for alpha chain expression in vitro before transplantating into mice. The presence of VLA-4 integrins on stromal cells before transfection was also assayed. Alpha4 chain expression on stromal cells after transfection was measured by flow cytometry using anti-alpha 4 antibody at timepoints: 3 days and 5 days post-transfection. FITC-conjuated rat IgG2a negative control, and FITC-conjugated rat anti-mouse CD49d (Integrin α4 chain) monoclonal antibody were purchased from Serotec Ltd. Oxford, England.

Flow cytometric analysis of cell surface markers was conducted to detect cells expressing the alpha4 chain of VLA-4. Transfected stromal cells were stained with FITC-conjugated rat anti-mouse CD49d (integrin α4 chain) monoclonal antibody. Transfected cells from the same sample were also labeled with FITC-conjugated rat IgG monoclonal immunogloblin isotype standard as control. Experiments done here showed that trypsin treatment could cause alteration of the expression level of any cell surface adhesion molecules. Therefore, cells were detached from tissue culture flasks with 1mM EDTA and then washed once in 1x PBS followed by two washes in PGB (PBS containing 20mM glucose, 1% bovine serum albumin). Cells after being resuspended in PGB were stained with FITC-conjugated anti-alpha4 antibody at a 1/25 dilution at room temperature. After 40 minutes of incubation, cells were washed twice in PGB and resuspended in the same buffer for flow cytometric analysis on a FACscan.

2.8 Immunofluorescence Staining of VCAM-Ig conjugates

A soluble ligand for α4β1 named recombinant VCAM-IgG fusion protein (VCAM-Ig) can be used as a means to directly investigate the ligand binding properties of VLA-4 molecules on transfected stromal cell surface in VLA-4/VCAM-1 interactions. The advantage of using VCAM-Ig is that the ligand binding properties of α4β1 can be directly demonstrated by fluorescence analysis and provides a means to directly assess the interaction of α4 integrins with their ligands.

Recombinant VCAM-Ig fusion protein, which consists of the two N-terminal Ig-like domains of human VCAM-1 (containing an α4β1 binding site) fused to part of the constant region of human IgG1, is a soluble ligand for α4β1 (Lobb et al, 1995). It has been shown previously that both human and mouse recombinant VCAM can bind to murine VLA-4 (Romanic and Madri, 1994). VCAM-1 is bivalent, and therefore shows increased affinity for α4β1.

Although α4 integrins are constitutively expressed on the surface of leukocytes, their interaction with ligands depends upon their activation state which is regulated by various stimuli. VLA-4 occurs in multiple activation states with different affinities to its ligand, VCAM-1 (Chen et al., 1999). T cell adhesion to immobilized VCAM or FN is augmented by antigen activation, phorbol esters and anti-TcR mAbs, not simply by increasing the density of VLA-4 (Chan et al., 1991 and Shimizu et al., 1990). The divalent cation manganese and certain β1-specific mAbs also increase α4β1-mediated adhesion to immobilized VCAM-1 and fibronectin. Mn^{2+}, in particular, induces optimal cell adhesion (Masumoto, and Hemler, 1993) since no binding was found in the absence of metal ions (Lobb et al., 1995).

After transfection with the alpha4 chain of VLA-4, stromal cells were cultured for 5 days, detached with 1mM EDTA in PBS and then washed thoroughly in 1xPBS. Human VCAM-Ig (1μg/ml, 2μg/ml, 5μg/ml and 10μg/ml), a gift from Dr. Roy Lobb, Biogen Inc, Cambridge, was added to four aliquots of $4x10^5$ transfected stromal cells suspended in 200μl TBS with 1mM $MnCl_2$ at room temperature. After one hour, cells were washed once in TBS-$MnCl_2$ and resuspended in the same buffer with FITC-conjugated goat anti-human IgG at a 1/100 dilution at room temperature. FITC-

conjugated goat anti-human IgG was purchased from Sigma, Missouri, USA. After 30 minutes of incubation, cells were washed twice and suspended in 0.5ml1xTBS. Fluorescence intensity correlating to binding between VLA-4 on stromal cells and VCAM-Ig was measured with a FAScan.

2.9 In vivo experiments

Female Balb/c mice served as transplant recipients, each receiving a single infusion of male stromal cells or a co-infusion with female bone marrow cells for (A) and (C). Three groups of mice were used in the in vivo experiments.

(A) $3x10^6$ male transfected vs. untransfected stromal cells were co-infused with syngeneic female bone marrow cells intravenously into two groups of four unconditioned syngeneic female Balb/c recipient mice via tail vein.

(B) $3x10^6$ male transfected vs. untransfected stromal cells were infused intravenously into two groups of four unconditioned syngeneic female Balb/c recipient mice via tail vein.

(C) $3x10^6$ male transfected vs. untransfected stromal cells were co-infused with syngeneic female bone marrow cells intravenously into two groups of four lethally-irradiated (9Gy total body irradiation) syngeneic female Balb/c recipient mice via tail vein.

2.10 Control experiment

Female Balb/c mice were used as transplant recipients, each receiving a single dose of male stromal cells and female bone marrow cells without irradiation.

(1) $3x10^6$ male stromal cells transfected with salmon sperm DNA were infused intravenously into three unconditioned syngeneic female Balb/c recipient mice via tail vein.

(2) $3x10^6$ male stromal cells transfected with alpha4 chain (VLA-4), then co-incubated with anti-α4 antibody were infused intravenously into three unconditioned syngeneic female Balb/c recipient mice via tail vein.

(3) $3x10^6$ untransfected male stromal cells were infused intravenously into three unconditioned syngeneic female Balb/c recipient mice via tail vein.

(4) $3x10^6$ male stromal cells transfected with alpha4 chain (VLA-4) were infused intravenously into three unconditioned syngeneic female Balb/c recipient mice via tail vein.

2.11 Hind limb irradiation experiment

To investigate the effect of local irradiation on the ability of stromal cells to engraft to the locally irradiated hind limbs, unmanipulated and non-transformed stromal cells were transplanted intravenously into mice whose left hind limbs were irradiated 4 hours previously, and into non-irradiated mice as controls.

$3x10^6$ male Balb/c stromal cells were infused through the tail vein into four female Balb/c recipient mice, with their left hind limb irradiated. Two female Balb/c recipient mice underwent hind limb irradiation but no transplantation while another two female recipient mice were transplanted with $3x10^6$ male Balb/c stromal cells without irradiation as controls. Conditioned mice received a single irradiation dosage of 9Gy on their left hind limb and then infused with stromal cells 4 hours later.

The irradiation were carried out using a double headed 100kV X-ray unit at a dose of 10 Gy. Each mouse was confined to a jig consisting of a lucite plate onto which fits a small lucite box. The mouse was placed inside the box with its right leg extended through an opening on the side. The tail passes through a hole in the back of the box and was held in position by taping to the platform. The jig was then placed into a lucite radiation chamber containing 2 lead collimators to define the radiation field.

The engraftment level of donor cells in the left and right limbs of recipient mice were then compared to determine if local irradiation enhances stromal engraftment.

2.12 Detection of presence of male donor stromal cells

Studies of transplantation biology rely on the detection of donor cells in transplant recipients. All recipients were sacrificed at 4 weeks post-transplant. Both femurs and tibias of each mouse were used for analysis and comparison of engraftment levels. A portion of harvested bone marrow cells were used to generate LTMC so that data on the percentages of donor stromal cells was also obtained. Portion of tissues such as liver, lung, brain and spleen was used to assay for engraftment levels. The engraftment level of donor stromal cells in the bone marrow and localization in other tissues were determined by the polymerase chain reaction (PCR) for Tdy (testis determining Y-region of the mouse chromosome) and fluorescence in situ hybridization (FISH) for male Y chromosome DNA sequence to identify the presence of male stromal cells. PCR was used to amplify Y chromosome-specific sequence of the sex-determining region of donor male stromal cells at a very low frequency. A murine Y chromosome-specific probe to identify in situ individual transplanted male cells by FISH in cell suspension provided a means to quantify levels of engraftment.

2.13 PCR

TDY1, TDY2, SCF1 and SCF2 were ordered from HSC Biotechnology Service Centre, Hospital of Sick Children, Toronto, Canada. dNTPs, Taq polymerase and its buffer were purchased from MDC, University of Toronto, Toronto, Canada. SCF1 and SCF2 are stem cell factor for PCR internal control. Y chromosome sequences lying within higher order repeats were amplified by PCR in DNA extracted from bone marrow, stromal cells and tissues. PCR analysis of stromal cells obtained from recipient marrow by serial passage of LTMC adherent layers (T4), was also performed. Genomic DNA was isolated from bone marrow cells, stromal cells and tissues by the proteinase K digestion and phenol-chloroform extraction methods (Lee et al., 1991). PCR sense primer, 5' GAC-TGG-TGA-CAA-TTG-TCT-AG 3'and anti-sense primer, 5'TAA-AAT-GCC-ACT-CCT-CTG-TG 3' were located at position 73-93 of the mouse Y-linked sequence and spanned position 365-345of the mouse Y-linked sequence respectively. It yielded a 292bp product (figure 6).

1 AGATCTTGATTTTTAGTGTTCAGCCCTACAGAAACATGATATCTTAAACT

51 CTGAAGAAGAGACAAGTTTTGGGACTGGTGACAATTGTCTAGAGAGCATG
TDY1
101 GAGGGCCATGTCAAGCGCCCCATGAATGCATTTATGGTGTGGTCCCGTGG

151TGAGAGGCACAAGTTGGCCCAGCAGAATCCCAGCATGCAAAATACAGAGA

201 TCAGCAAGCAGCTGGGATGCAGGTGGAAAAGCCTTACAGAAGCCGAAAAA

251 AGGCCCTTTTTCCAGGAGGCACAGAGATTGAAGATCCTACACAGAGAGAA

301 ATACCCAAACTATAAATATCAGCCTCATCGGAGGGCTAAAGTGTCACAGA

351 GGAGTGGCATTTTACAGCCTGCAGTTGCCTCAACAAAACTGTACAACCTT
TDY2
401 CTGCAGTGGGACAGGAACCCACATGCCATCACATACAGGCAAGACTGGAG

451 TAGAGCTGCACACCTGTACTC

Figure 6. Nucleotide sequence of part of the *Sry* gene (*Tdy* gene candidate) on the mouse Y chromosome. Primer sequences are underlined: product TDY1 and TDY2 of 292bp. Sequence derived from Gubbay et al., Nature 346(1990): 245-250.

2.14 PCR reaction mixture

The reactions were conducted in a final volume of 50μl consisting of 8μl of 1.25mM dNTPs, 5μl of NH_4Cl reaction buffer (160mM $(NH_4)_2SO_4$, 670mM Tris pH8 and 0.1% Tween 20), 1μl of $MgCl_2$, 50pM of sense and anti-sense primer, 0.25μl of DNA Taq polymerase, 20.4μl of dH_2O and 10μl of 0.1μg/μl sample. The thermal cycle profile consists of 40 cycles of 1 min of 94°C for denaturating double strand DNA, 1 min of 52°C for annealing, 1 min of 72°C for synthesizing and processing. Y chromosome sequences of PCR products were then identified on 3% ethidium-bromide-stained gels following electrophoretic separation at 90 volts on the basis of size. PCR reaction products were analyzed by ultraviolet visualization of ethidium-bromide-stained gel.

2.15 PCR sensitivity test

10 times dilution: 2μl of 0.18μg/ μl male bone marrow cell DNA (stock) was diluted in 18μl of distilled water to produce a 1:10 dilution. 2μl was taken from the 1:10 dilution and diluted in 18μl of distilled water separately to produce a 1:100 dilution. The same dilution process was performed until a 1:1000 dilution was obtained. 0.56μl of male DNA stock is 0.1μg of male DNA, 0.56μl of the 1:10 dilution is 0.01μg of male DNA, 0.56μl of the 1:100 dilution is 1ng of male DNA and so on. 2.89μl of 0.31μg/μl female bone marrow cell DNA (stock) were used to mix with different amount of male DNA for the PCR sensitivity test.

Double dilution: 10μl of 0.18μg/μl male bone marrow cell DNA (stock) was diluted in 10μl of distilled water to produce a 1:2 dilution. 10μl was taken from the 1:2 dilution and diluted in 10μl of distilled water separately to produce a 1:4 dilution. This was also performed until a 1:16 dilution was obtained. As 0.56μl of male DNA stock is 0.1μg of male DNA, 0.56μl of the 1:2 dilution is 0.05μg of male DNA and 0.56μl of the 1:4 dilution is 0.025μg of male DNA and so on. The same female DNA stock was used here (table 2).

10 x dilution						
Male DNA concentration	0.1μg	0.01μg	1ng	0.1ng	0.01ng	
Dilution	1x	10x	100x	1000x	1000x	
Volume of male DNA added	0.56μl of 0.18μg/μl male stock	0.56μl of the 1:10 dilution	0.56μl of the 1:100 dilution	0.56μl of the 1:1000 dilution	0.56μl of the 1:10000 dilution	
Volume of female DNA added	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	
Sensitivity level	10%	1.1%	0.11%	0.011%	0.0011%	
2x dilution						
Male DNA concentration	0.1μg	0.05μg	25ng	12.5ng	6.25ng	3.125ng
Dilution	1x	2x	4x	8x	16x	32x
Volume of male DNA added	0.56μl of 0.18μg/μl male stock	0.56μl of the 1:2 dilution	0.56μl of the 1:4 dilution	0.56μl of the 1:8 dilution	0.56μl of the 1:16 dilution	0.56μl of the 1:32 dilution
Volume of female DNA added	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock	2.89μl of 0.31μg/μl female stock
Sensitivity level	10%	5.26%	2.70%	1.37%	0.69%	0.35%

Table 2: 10x dilutions and double dilutions of male marrow DNA in female marrow DNA for PCR sensitivity test

2.16 Fluorescence in situ hybridization

The FISH technique utilized a murine Y chromosome-specific "painting" probe which is a biotinylated paint for male Y chromosome DNA sequences to identify in situ individual transplanted male stromal cells within female bone marrow.

2.17 Preparation of cell suspension for FISH

Fresh bone marrow cells and stromal cells were fixed in 3 additions of 3:1 methanol: acetic acid with flicking, spinning and removal of fixatives in between after being incubated in 0.075M warm KCl for 10 minutes. Fixed cells were then seeded on slides and air dried. The slides were treated with RNase purchased from Boehringer Mannheim (100μg/ml in 2 X SSC) at 37°C for 1 hour and then washed in 2X SSC three times for 3 minutes each. Slides were treated with formamide at 70°C for 2mins after being dehydrated by serial ethanol washings (70%, 90%, 100%) for 5 minutes each. The probe ordered from Cambio, United Kingdom was denatured at 75°C for 5 minutes. The slides and probe were then hybridized at 37°C overnight. Slides were washed in 50% formamide 3 times in 2X SSC at 45°C for 5 minutes, then 2X SSC 3 times at 45°C for 5 minutes the other day. Non-specific sites were blocked with Block 1(3% BSA + 0.5% Tween20 in 4 x SSC). Detection was performed by indirect immunofluoresence (Anklesaria et al., 1987). Slides were incubated with 40μl FITC-Avidin at 37°C for 30minutes and then washed in Tween 20 in 2X SSC at 45°C for 5 minutes. Counterstain (8μl DAPI/ Antifade + 3μl PI/ Antifade + 1μl Antifade) was added on each slide.

2.18 Statistics

All results are expressed as the mean $\pm$ SD of the indicated determinations. Since the design is not completely balanced, the statistical analysis was performed using the general linear model implemented in SAS. The effects were tested for significance using the type III sums of squares F tests at a level of significance of 0.05. The effects considered were: cond (conditioning of transplant recipients), BM (bone marrow cell co-infusion), transfec (transfection of stromal cells) and the interaction between cond and transfec, and BM and transfec. The same analysis was performed for nucleated marrow cells and stromal cells.

Chapter 3

RESULTS

3.1 Characterization of stromal cells

3.1.1 Collagen IV staining

The cytoplasm of stromal cells and L929 (positive control) was stained fluorescent green whereas cytoplasm of P388D1 look dull and yellowish (figure 7). The number of positively stained cells for each type is shown in table 3.

	Total no. of cells counted	No. of positive cells	Freq. Of positive cells
Stromal cells	967	967	100%
L929	797	797	100%
P388D1	859	0	0%

Table 3: Immunohistochemical staining of fifth passaged stromal cells, L929 and P388D1 cells with Collagen IV antibody.

3.1.2 Alkaline Phosphatase

Stromal cells and L929 cells (positive control) were found to have dark granules of alkaline phosphatase staining spreading all over their cytoplasm whereas such granules were not observed in P388D1 cells under the light microscope (figure 8). The number of cells with granules is shown in table 4.

	Total no. of cells counted	No. of positive cells	Freq. Of positive cells
Stromal cells	875	821	93.8%
L929	761	719	94.5%
P388D1	734	0	0%

Table 4: Cytochemical staining of fifth passaged stromal cells, L929 and P388D1 cells with Alkaline phosphatase.

3.1.3 CD 45 staining

Stromal cells and L929 cells (negative control) stained for CD45 looked dull and yellow whereas P388D1 cells looked bright green under fluorescence microscope (figure 9). The number of positively stained cells is shown in table 5.

	Total no. of cells counted	No. of positive cells	Freq. of positive cells
Stromal cells	813	0	0%
L929	953	0	0%
P388D1	874	874	100%

Table 5: Immunohistochemical staining of fifth passaged stromal cells, L929, P388D1 cells with anti-CD45 antibody.

3.2 PCR sensitivity

The sensitivity of all PCR tests was at least 0.625%, that is 0.625% of male DNA can be detected in female DNA but could be as high as 0.35% (figure10).

Collagen IV Staining

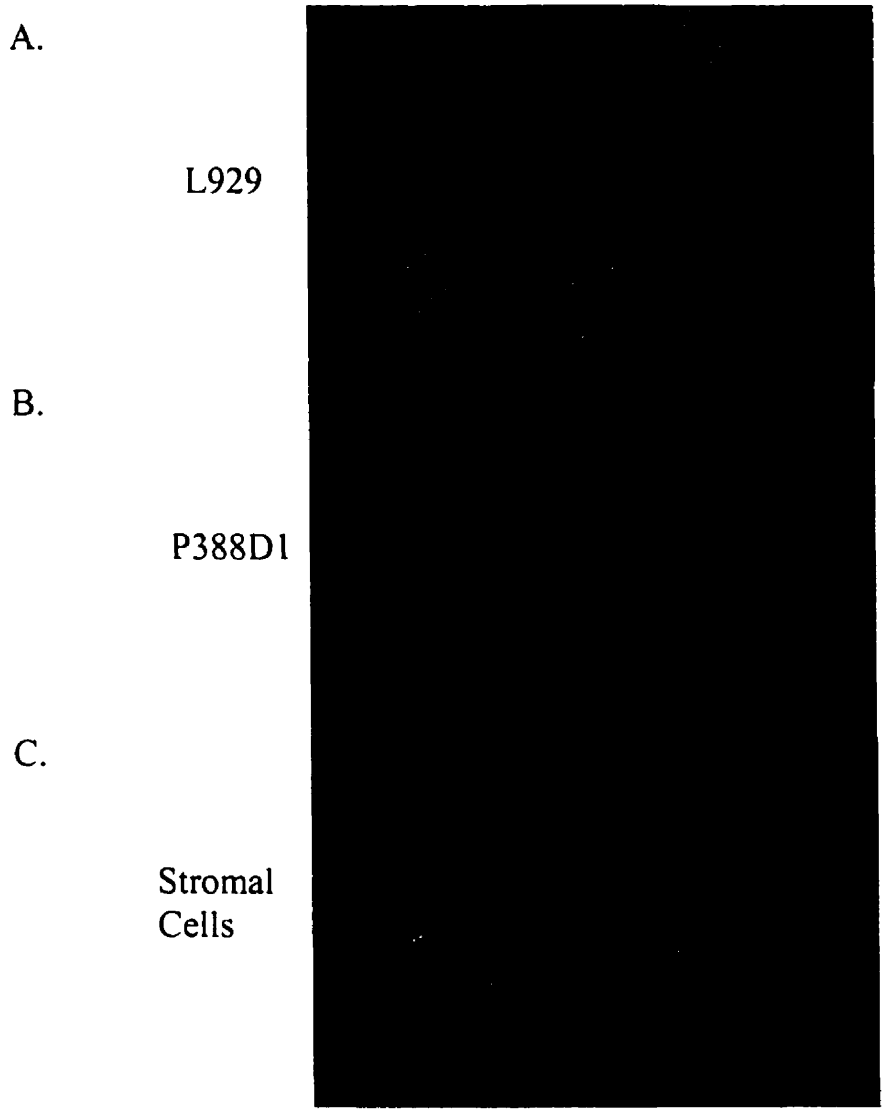


Fig 7. Indirect immunofluorescence staining with anti-collagen IV monoclonal antibody on A. L929 (positive control). B. P388D1 (negative control). C. Stromal cells (positively stained)

Alkaline Phosphatase Staining

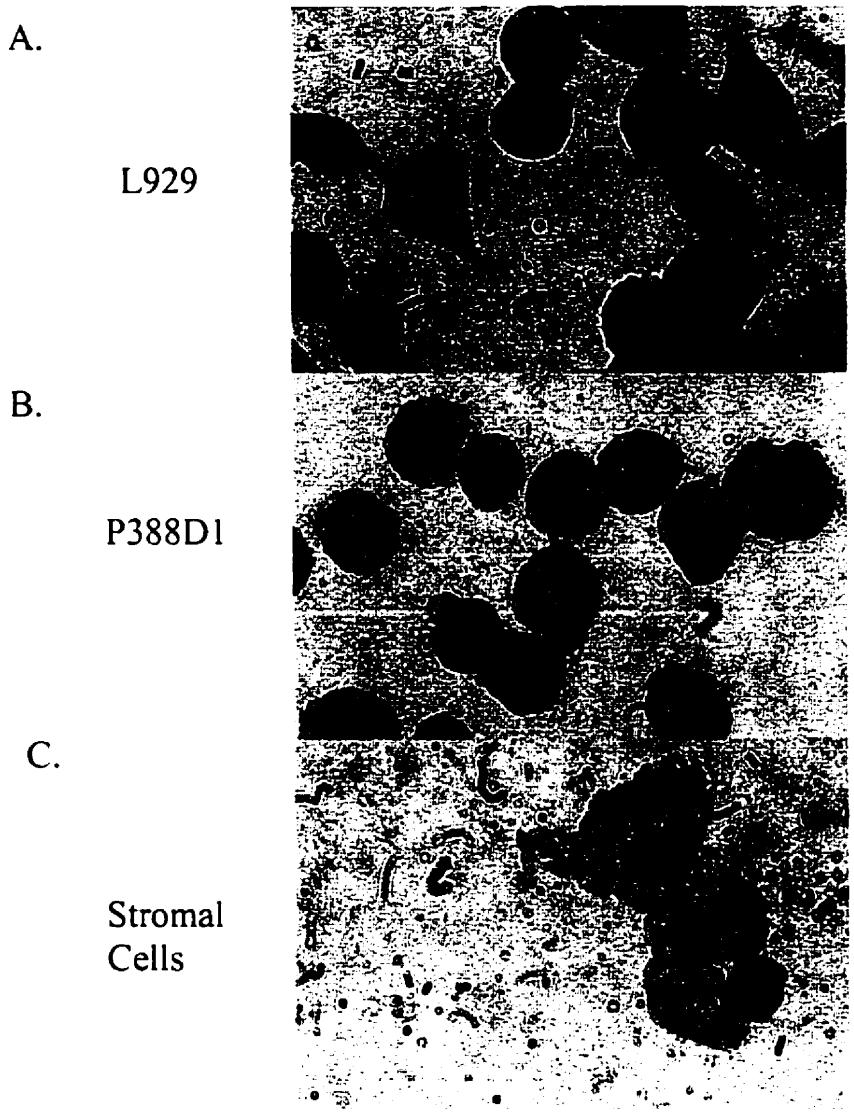


Fig 8. Cytochemical staining with alkaline phosphatase on A. L929(positive control). B. P388D1 (negative control). C. Stromal cells (positively stained)

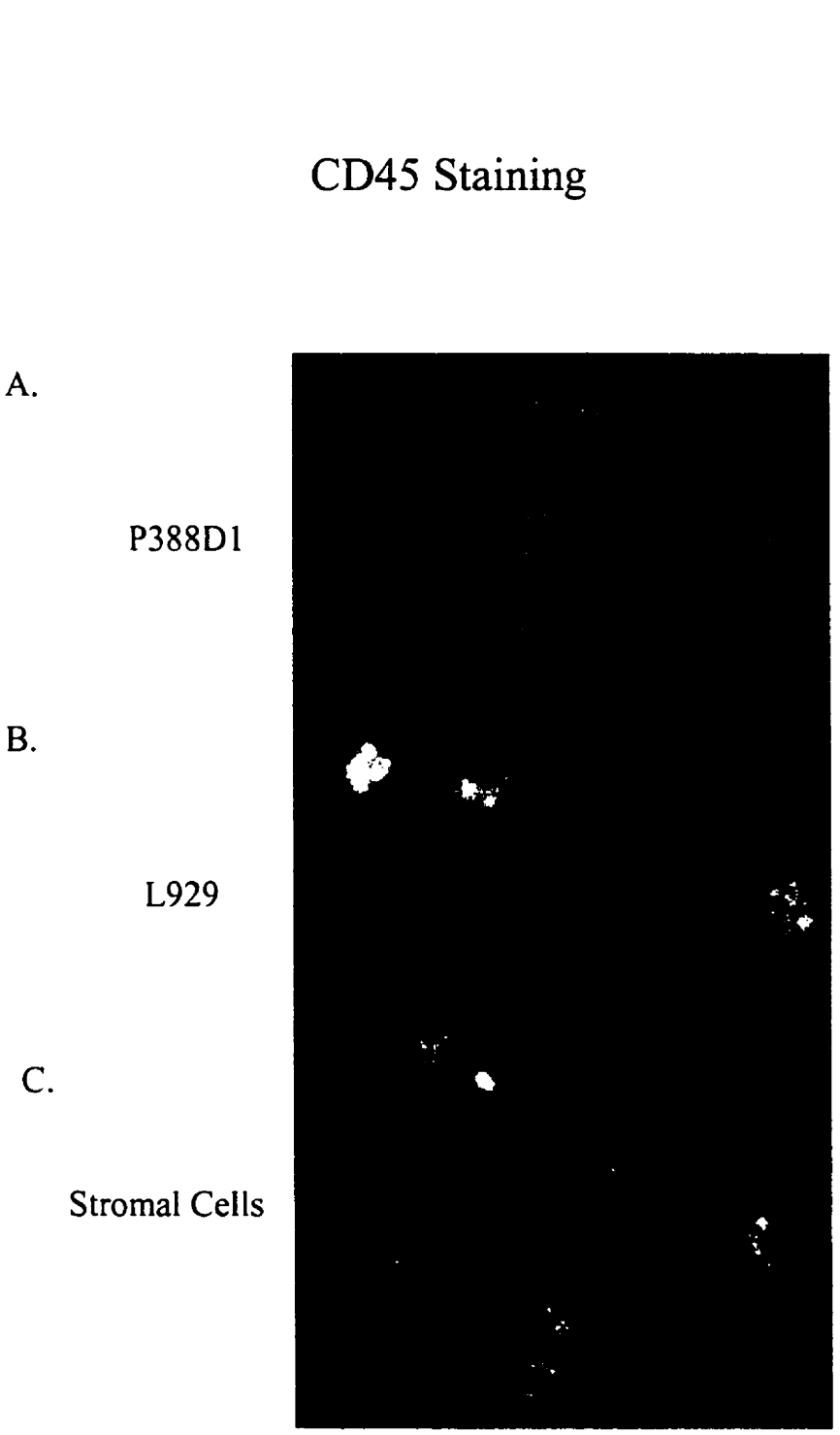
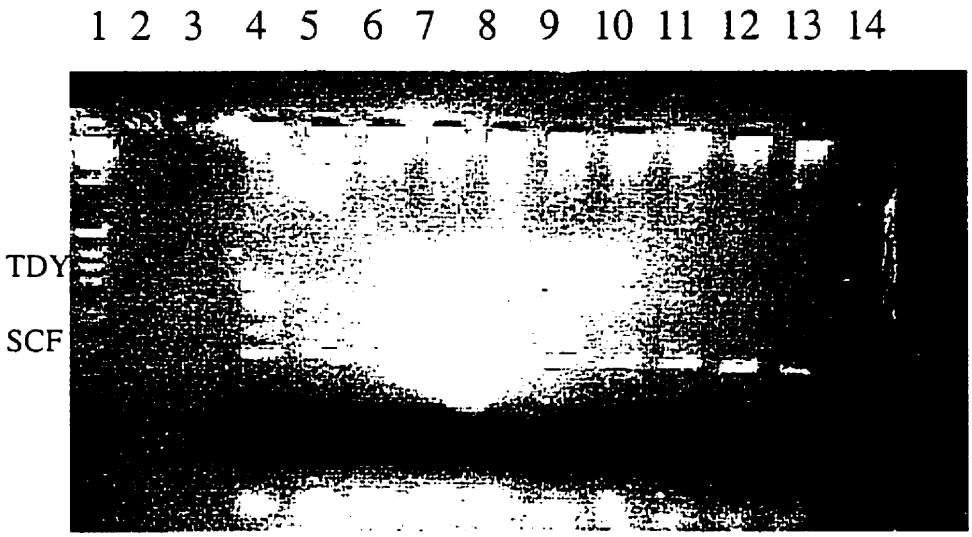


Fig 9. Immunofluorescence staining with anti-CD45-FTIC monoclonal antibody on A. P388D1 (positive control). B. L929 (negative control). C. Stromal cells (negatively stained)



1. 1kb ladder
2. Blank
3. Blank
4. 10% of male DNA in female DNA
5. 1.1% of male DNA in female DNA
6. 0.11% of male DNA in female DNA
7. 0.011% of male DNA in female DNA
8. 5.26% of male DNA in female DNA
9. 2.70% of male DNA in female DNA
10. 1.37% of male DNA in female DNA
11. 0.69% of male DNA in female DNA
12. 0.35% of male DNA in female DNA
13. Female DNA
14. Distilled water

Fig 10. Lanes 4 to 7 shows 10x dilution of male DNA from its stock, 0.18ug/ul, in female DNA and lanes 8 to 12 shows double dilution of male DNA from its stock, 0.18ug/ul, in female DNA which made up a total DNA concentration of 1µg in each reaction volume.

3.3 Interaction between exogenous α4 and endogenous β1

Stromal cells are devoid of VLA-4 surface expression and have no VCAM-1 binding capacity but have endogenous β1 subunits. The α4 subunit of VLA was transfected into stromal cells which then expressed VLA-4 as a result of association between exogenous α4 chain and endogenous β1 chain. To evaluate the expression of VLA-4 on the surface of stromal cells, we carried out flow cytometric analysis. Stromal cells transfected with α4 plasmid expressed substantial amounts of cell surface VLA-4 molecules, α4β1, as detected by FACS analysis.

Since characteristics of stromal cells are similar to that of adherent fibroblasts, rat mammary epithelial adenocarcinoma and mouse fibroblasts, electroporation settings suggested for these cell types were used. Flow cytometry diagrams of alpha 4 chain transfection under these four electroporation settings are shown on figure 11 to 14. Percentages from histograms were obtained by subtracting the untransfected population from the transfected one beyond the intersection of two curves. Interpretation of these data are hampered by the well-documented high level of autofluorescence of marrow stromal cells. Expression percentages of alpha 4 chain on stromal cells on day3 and day5 under four different electroporation settings are shown on figure 15. Percentages of alpha 4 chain expression shown above are higher on day 5 than on day 3 regardless of the setting used. It also shows that the percentages of alpha 4 chain expression using the conditions mimicking mouse fibroblasts (L929) was the highest. This setting was chosen as the optimal condition for stromal cells (figure 15). This may be due to the phenotypic resemblance between stromal cells and mouse fibroblasts.

The expression level of α4 integrin on day 1 was undetectable but increased from day 3 to day 5. Five days incubation after transfection is required to obtain the maximum level of VLA-4 molecule expression. Intervals beyond 5 days did not alter the intensity of the FITC-labeled α4 integrin.

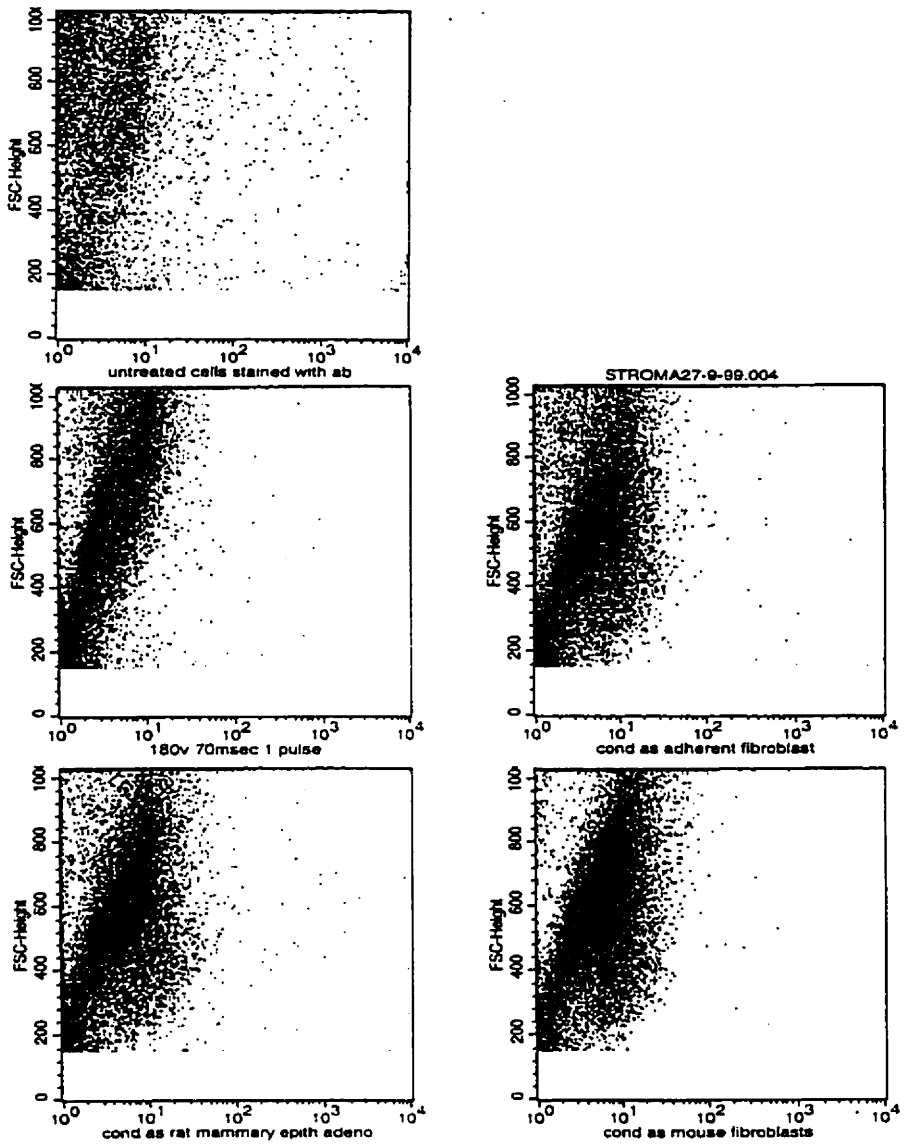


Fig 11. α4 chain of VLA expression under different electroporation settings detected by flow cytometry shown on dotplot 3 days after transfection. Horizontal axes represent fluorescence with FITC-conjugated anti-α4 monoclonal antibody.

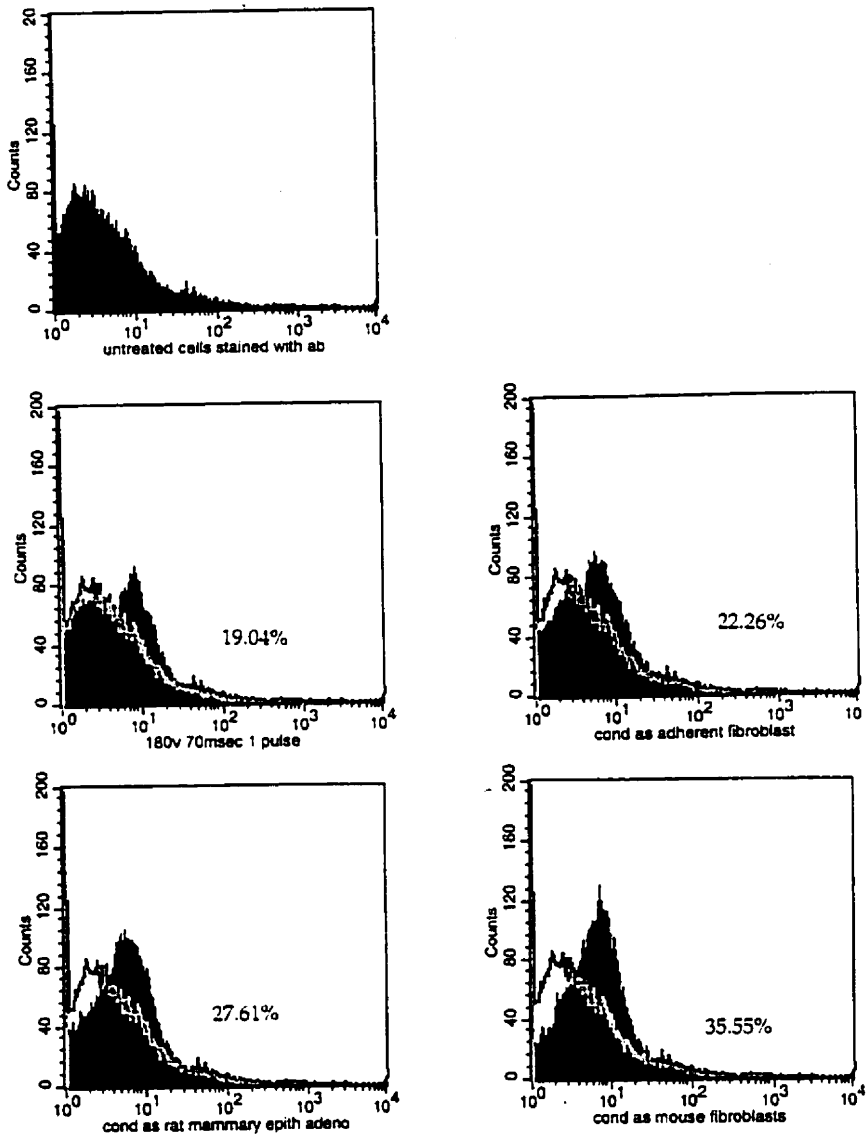


Fig12. α4 chain of VLA expression under different electroporation settings detected by flow cytometry shown on histogram 3 days after transfection. Horizontal axes represent fluorescence with FITC-conjugated anti-α4 monoclonal antibody.

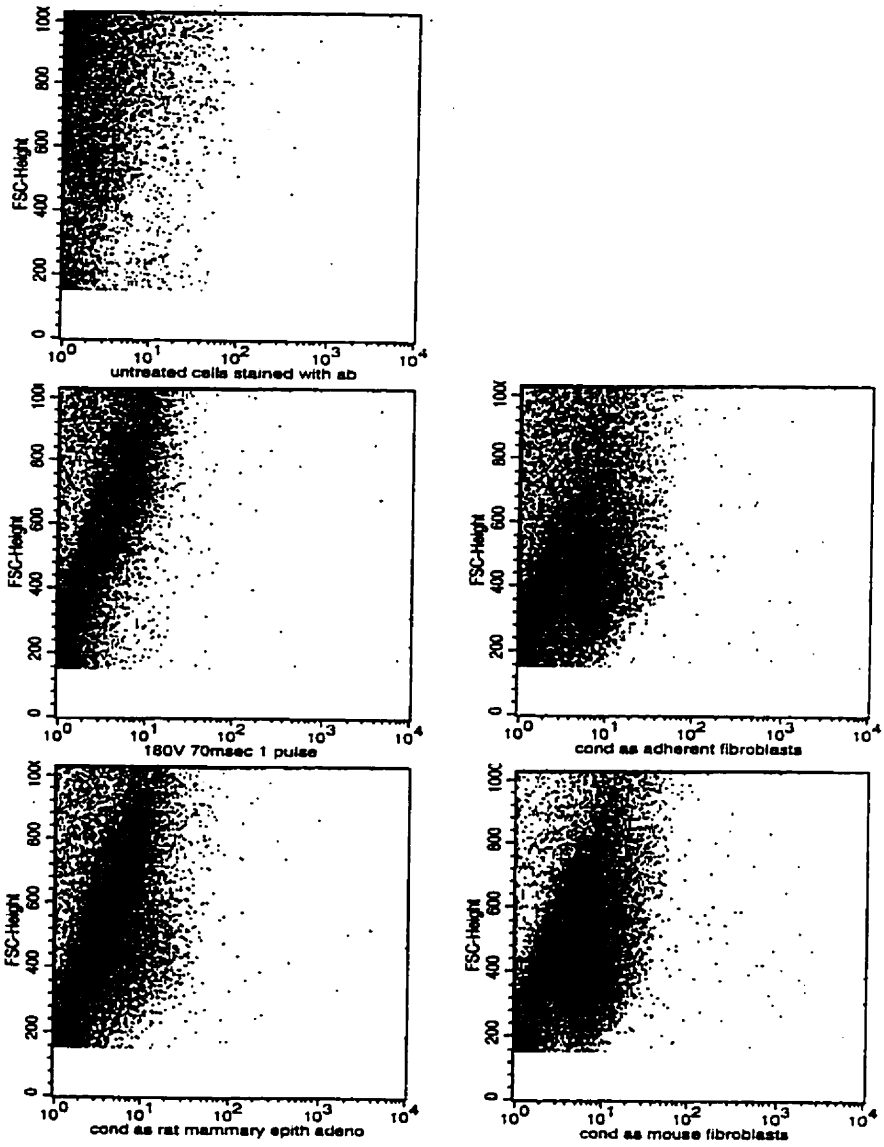


Fig 13. α4 chain of VLA expression under different electroporation settings detected by flow cytometry shown on dotplot 5 days after transfection. Horizontal axes represent fluorescence with FITC-conjugated anti-α4 monoclonal antibody.

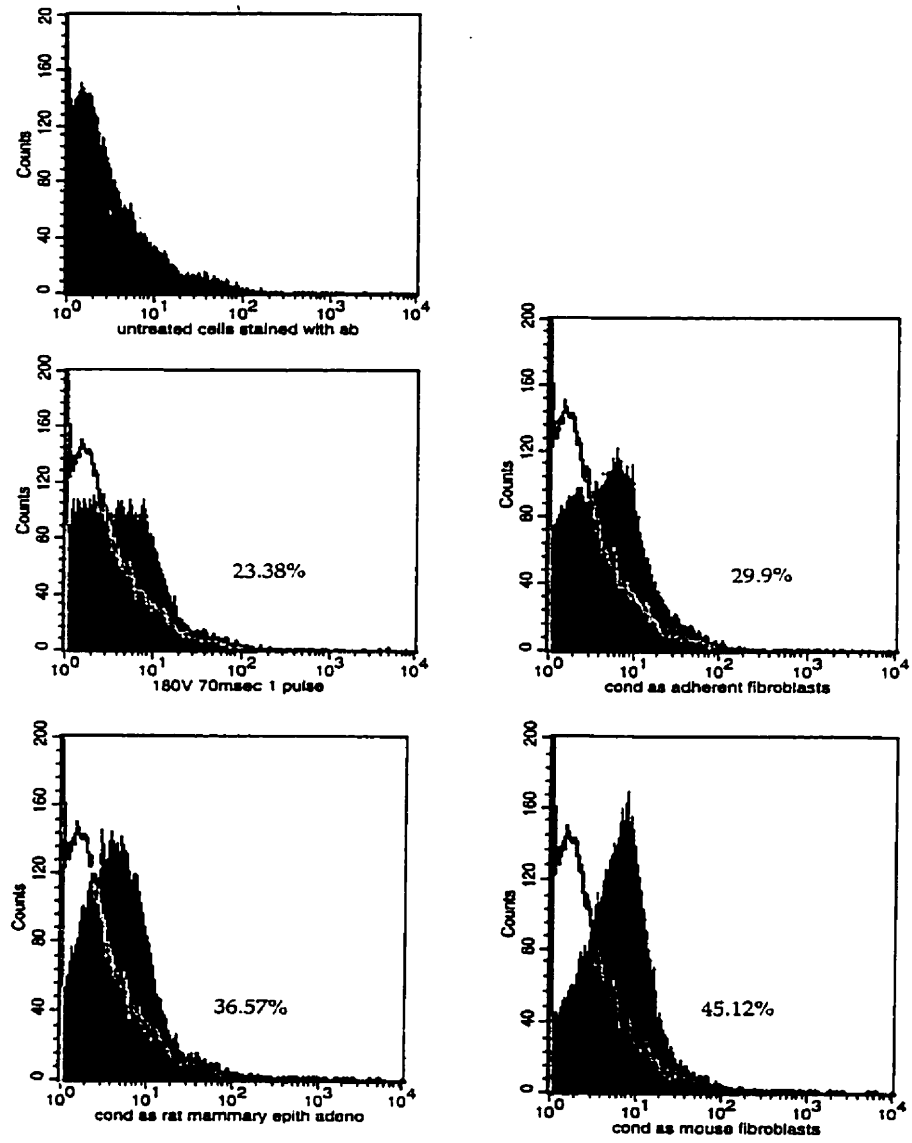


Fig 14. α4 chain of VLA expression under different electroporation settings detected by flow cytometry shown on histogram 5 days after transfection. Horizontal axes represent fluorescence with FITC-conjugated anti-α4 monoclonal antibody.

VLA Alpha-4 chain Transfection Experiment

Electroporation Settings suggested for:		Expression % on day 3	Expression % on day 5
No Transfection	0V 0msec 0 pulse	0	0
Trial	180V 70msec 1pulse	19.04	23.38
Adherent Fibroblasts	300V 99usec 4pulses	22.26	29.9
Rat Mammary Epithelial Adenocarcinoma	400V 99usec 5pulses	27.61	36.57
Mouse Fibroblasts	650V 99usec 5pulses	35.55	45.12

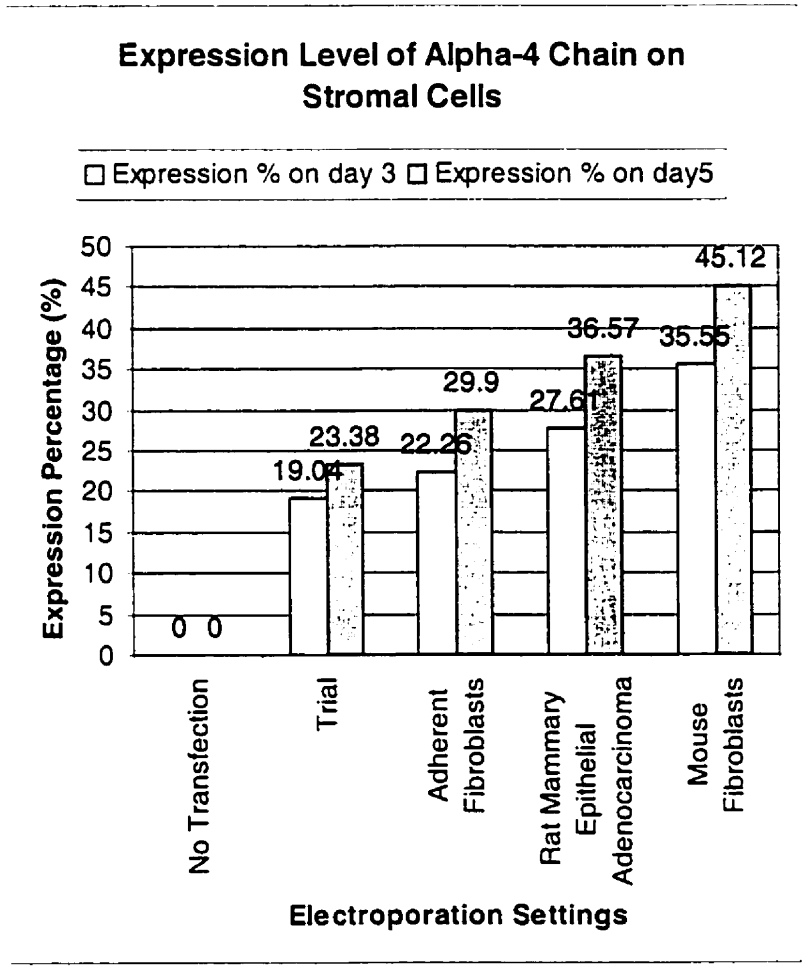


Fig 15. Summary of α 4 chain expression percentages under different electroporation settings.

3.4 VCAM-Ig binding assay

It was next necessary to determine whether the transfected α4 subunit associates with endogenous β1 subunit to form a functional VLA-4 molecule capable of binding to VCAM-1 on recipient stroma. α4 transfected stromal cells were tested for their binding ability with VCAM-Ig. The interaction between VLA-4 transfected stromal cells and soluble VCAM-Ig was assessed by FACS analysis. VCAM-Ig bound to 15.59%, 30.32%, 43.43% and 47.58% of transfected stromal cells when being used at 1μg/ml, 2μg/ml, 5μg/ml and10μg/ml, respectively (figure 16 and 17). Under the following conditions ($5x10^5$ cells in 200μl TBS, 1mM $MnCl_2$ for one hour at room temperature), increasing concentrations of VCAM-Ig bind to transfected stromal cells in a dose-response manner and reached a plateau at about 10μg/ml VCAM-Ig protein (figure 18). However, VLA-4 binding with VCAM-Ig was strongly induced in the presence of the divalent cation, Mn^{2+}, since VCAM-Ig /VLA-4 binding was not detectable in the absence of inducing agents even at 20μg/ml VCAM-Ig.

A soluble VCAM-Ig was employed to directly assess the interaction between VLA-4 and its ligand. It was shown that VCAM-Ig can be used for direct ligand binding measurements by FACS.

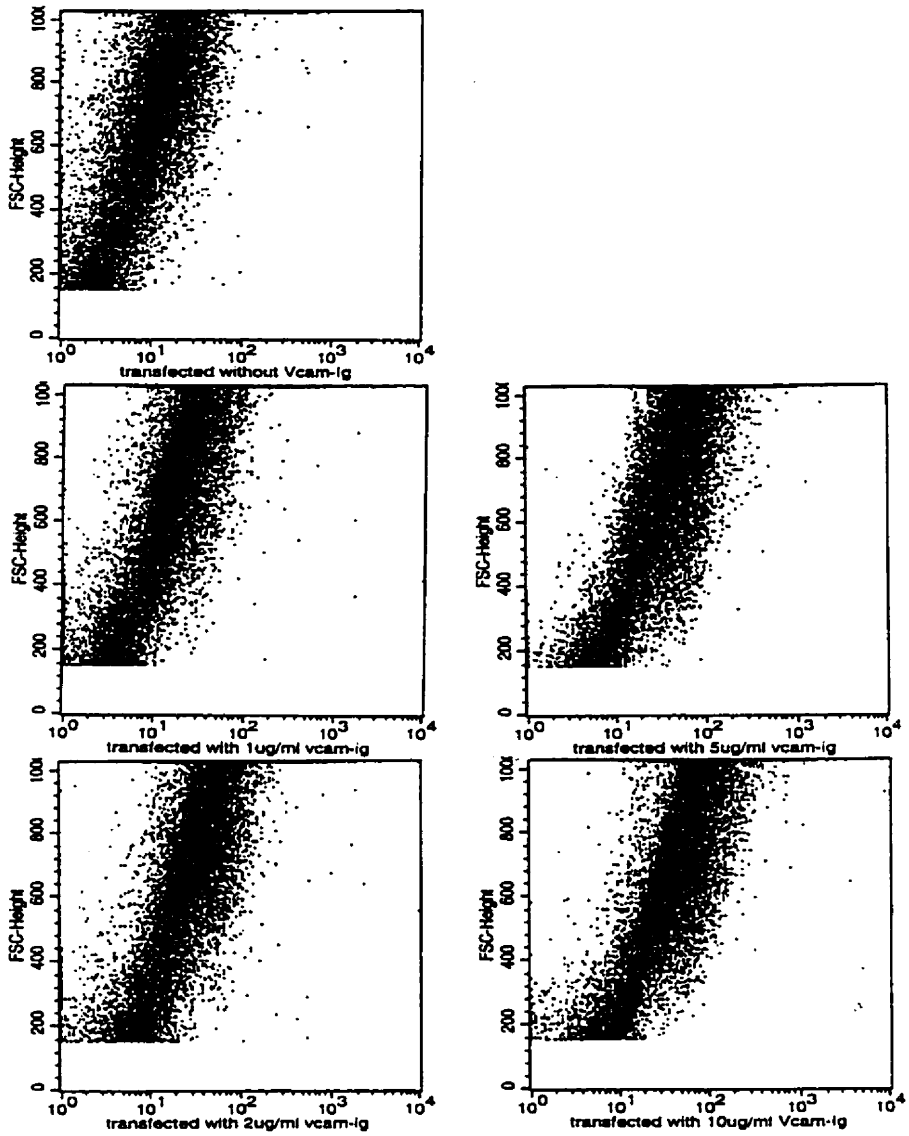


Fig. 16 Dotplot of binding assay between VLA-4 and VCAM-Ig with different protein concentrations: 1μl/ml, 2μl/ml, 5μl/ml and 10μl/ml in vitro by flow cytometry. Horizontal axes represent fluorescence with FITC-conjugated goat anti-human antibody.

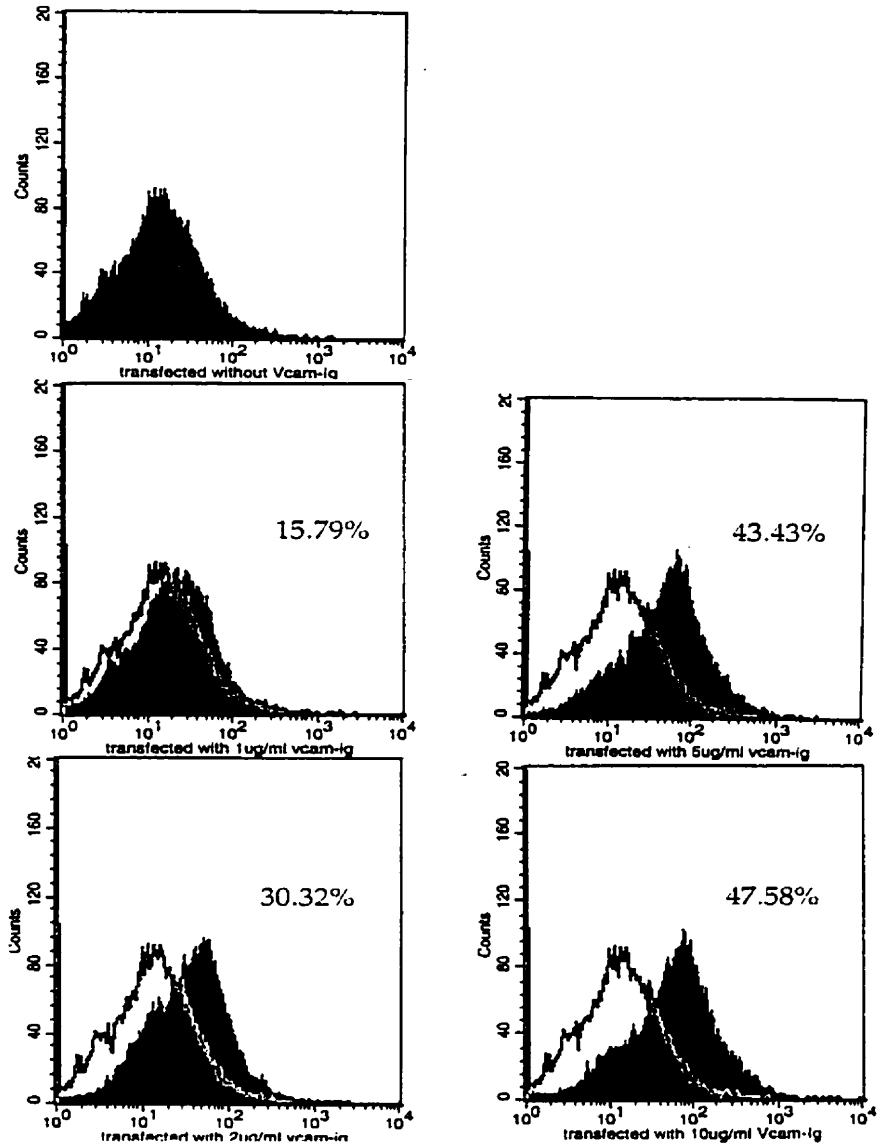


Fig 17. Histogram of binding assay between VLA-4 and VCAM-Ig with different protein concentrations: 1μl/ml, 2μl/ml, 5μl/ml and 10μl/ml in vitro by flow cytometry. Horizontal axes represent fluorescence with FITC-conjugated goat anti-human antibody.

VLA Alpha-4 Integrins Binding Assay with VCAM-Ig Fusion Protein

VCAM-Ig Concentration (ug/ml)	Binding Percentage of VLA-4 Integrins to VCAM-Ig
1	15.59
2	30.32
5	43.43
10	47.58

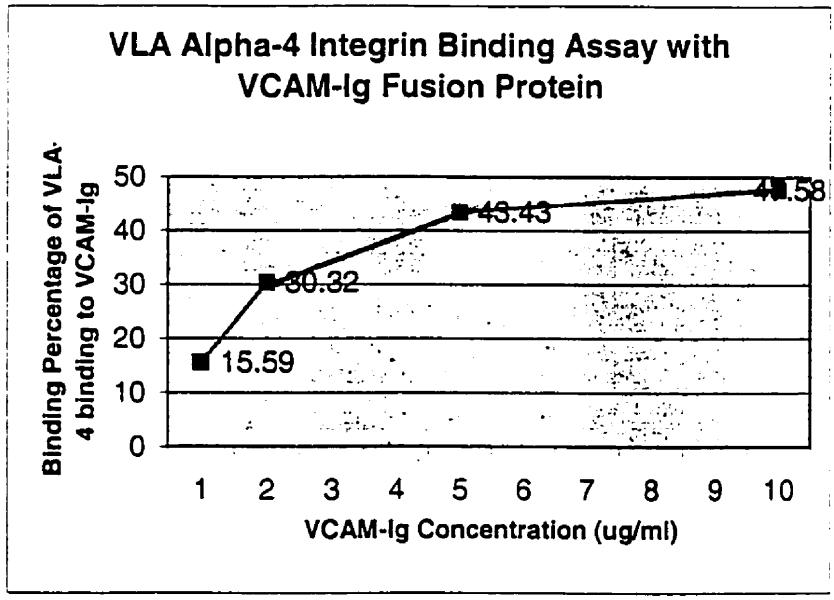


Fig 18. Summary of binding assay between VLA-4 and VCAM-Ig with different protein concentrations in vitro.

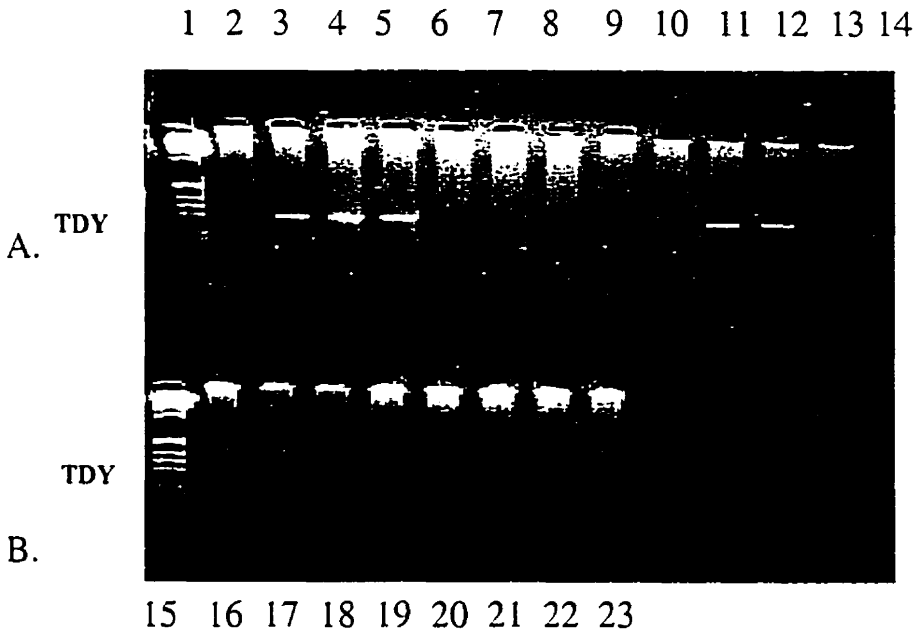
3.5 In vivo experiments: detection of donor cells by PCR

Group A. Unconditioned female recipients infused with male stromal cells (transfected vs. untransfected) and female bone marrow cells.

Group B. Unconditioned female recipients infused with male stromal cells (transfected vs. untransfected) only.

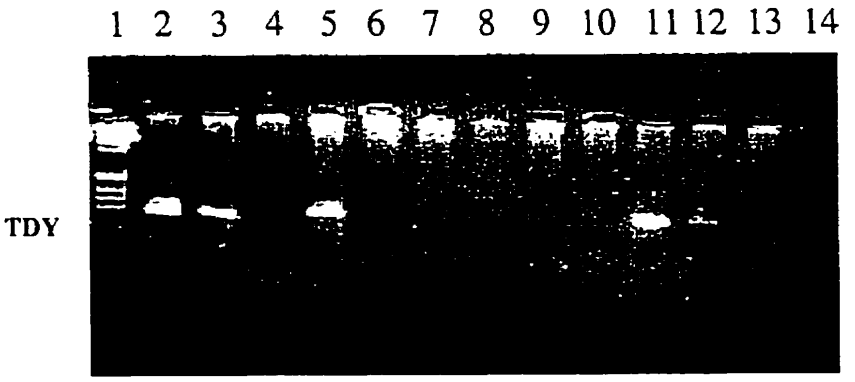
Group C. Conditioned female recipients infused with male stromal cells (transfected vs. untransfected) and female bone marrow cells.

PCR analysis of bone marrow and stromal cells from transplant recipients showed the presence of donor cells. The PCR data on bone marrow cells of recipients were investigated as follows. For group A, 3 of 4 unconditioned recipient mice infused with transfected stromal cells and bone marrow cells had PCR positive marrow and all 4 unconditioned recipient mice infused with untransfected stromal cells and bone marrow cells had a negative signal. For group B, all 4 unconditioned recipient mice infused with transfected stromal cells were negative as were the 4 unconditioned recipient mice infused with untransfected stromal cells. For group C, 3 of 4 conditioned recipient mice infused with transfected stromal cells and bone marrow cells had a positive PCR signal and all 4 conditioned recipient mice infused with untransfected stromal cells and bone marrow cells were negative (figure 19 and 20). However, DNA extracted from lung, liver, brain and spleen of recipient mice in all 3 groups were negative by PCR for donor signal, showing that no male donor cells were present in the tissue suspensions. The absence of donor cells in liver, spleen, lung and brain, 4 weeks post-transplant might be due to the presence of a certain barrier that hinders foreign cells entrance, for instance, the blood brain barrier in brain. Only bone marrow cell samples in transplant recipients giving a donor cell level of 0.69% or higher by FISH were positive with PCR, as this is the level of sensitivity of our assay.



1. 1kb
2-5: Bone marrow cell samples from unconditioned mice infused with transfected stromal cells and bone marrow cells in group A.
6-9: Bone marrow cell samples from unconditioned mice infused with untransfected stromal cells and bone marrow cells in group A.
10. Female DNA
11. 1% of male DNA in female DNA
12. 0.69% of male DNA in female DNA
13. 0.35% of male DNA in female DNA
14. Distilled water
15. 1kb
16-19: Bone marrow cell samples from unconditioned mice infused with transfected stromal cells in group B.
20-23: Bone marrow cell samples from unconditioned mice infused with untransfected stromal cells in group B.

Fig 19. PCR results of individual bone marrow cell samples in group A (transfected vs. untransfected) and group B (transfected vs. untransfected).



1. 1kb
2-5: Bone marrow cell samples from conditioned mice infused with transfected stromal cells and bone marrow cells in group C.
6-9: Bone marrow cell samples from conditioned mice infused with untransfected stromal cells and bone marrow cells in group C.
10. Female DNA
11. 1% of male DNA in female DNA
12. 0.69% of male DNA in female DNA
13. 0.35% of male DNA in female DNA
14. Distilled water

Fig 20. PCR results of individual bone marrow cell samples in group C (transfected vs. untransfected).

3.6 In vivo experiments: detection of donor cells by FISH

Transplantation of stromal cells in the murine model was examined by counting the number of positive cells in a total of 1000 cells by FISH for the donor cell level expressed in percentage (figure 21 and 22).

Fig 21. Positive control of fluorescence in situ hybridization (FISH) using male nucleated marrow cells under fluorescence microscope. Nuclei of male marrow cells showed a bright green fluorescent area in each cell denoted by the Y-chromosome paint. Magnification 1000X.

Fig 22. Presence of male donor cells in bone marrow aspirates of female transplant recipients by fluorescence in situ hybridization (FISH) under fluorescence microscope. Nuclei of male stromal cells showed a bright green fluorescent area denoted by the Y-chromosome paint whereas female nuclei lacked the positive signal. Magnification 1000X.

3.6.1 FISH data on nucleated marrow cells

Experimental Groups	Infused with transfected cells Freq. Of cells of donor origin (%)	Infused with untransfected cells Freq. Of cells of donor origin (%)
Group A Unconditioned group infused with stromal cells and bone marrow cells	mouse 1: 0.4 mouse 2: 2.3 mouse 3: 4.0 mouse 4: 4.5 **Mean**: 2.8 ± 1.86	mouse 1: 0.2 mouse 2: 0.0 mouse 3: 0.2 mouse 4: 0.2 **Mean**: 0.2± 0.11
Group B Unconditioned group infused with stromal cells only	mouse 1: 0.2 mouse 2: 0.2 mouse 3: 0.0 mouse 4: 0.1 **Mean**: 0.13 ± 0.09	mouse 1: 0.0 mouse 2: 0.1 mouse 3: 0.2 mouse 4: 0.0 **Mean**: 0.07 ± 0.08
Group C Conditioned group infused with stromal cells and bone marrow cells	mouse 1: 4.4 mouse 2: 5.0 mouse 3: 0.5 mouse 4: 4.7 **Mean**: 3.6± 2.13	mouse 1: 0.3 mouse 2: 0.3 mouse 3: 0.4 mouse 4: 0.3 **Mean**: 0.3 ± 0.04

Table 6. Frequency of male donor cells (%) in nucleated marrow cells of female recipients in experimental groups A, B and C.

In Situ Hybridization data on BMC
General Linear Models Procedure
Dependent Variable: PERC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	51.373	10.275	7.71	0.0005
Error	18	23.985	1.333		
Corrected Total	23	75.358			

R-Square	C.V.	Root MSE	PERC Mean
0.682	97.550	1.154	1.183

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cond	1	0.980	0.980	0.74	0.402
BMC	1	7.590	7.590	5.70	0.028
Transfec	1	11.526	11.526	8.65	0.009
Cond*Transfec	1	0.483	0.483	0.36	0.555
BMC*Transfec	1	6.708	6.708	5.03	0.038

Fig 23. F-test of engraftment percentage data on nucleated marrow cells in groups A, B and C.

We used F-test instead of ANOVA because it is not feasible to have a fourth group of conditioned recipients infused only with stromal cells because they would not survive the lethal irradiation.

Factors such as conditioning of recipient mice (Cond), transfection of stromal cells (Transfec) and co-infusion of bone marrow cells (BMC) are variables that may affect stromal cell engraftment. These data indicate that co-infusion of bone marrow cells and transfection to express VLA-4 strongly enhanced engraftment of stromal cells; P= 0.028 and 0.009, respectively. In contrast, the delivery of conditioning did not influence outcome (P= 0.402). It is of interest to note that the co-infusion of transfected stromal cells and bone marrow cells gave the highest engraftment levels (P= 0.038) [fig. 23].

Level of Cond	Level of Transfec	N	PERC Mean	PERC STD
n	n	8	0.114	0.098
n	y	8	1.464	1.876
y	n	4	0.303	0.039
y	y	4	3.643	2.126

Fig. 24 Mean level of engraftment (%) for conditioned versus unconditioned and transfected versus non-transfected groups.

It is noteworthy also, that even in unconditioned transplants of stromal cells in group B, the use of genetically modified stromal cells enhances stromal engraftment two fold. Examining unconditioned groups transplanted with transfected versus untransfected stromal cells shows a 12 fold increase in engraftment (1.46 versus 0.11%) [fig. 24].

Level of BM	Level of Transfec	N	PERC Mean	PERC STD
n	n	4	0.073	0.084
n	y	4	0.128	0.090
y	n	8	0.229	0.108
y	y	8	3.221	1.902

Fig 25. Mean level of engraftment (%) for BM versus no BM and transfected versus non-transfected groups.

When examining groups that received marrow cells and transfected versus untransfected stromal cells, transplants with transfected cells showed a 14 fold increase in stromal engraftment (3.22 versus 0.23%) [fig. 25].

These data suggest that the difference in stromal engraftment between conditioned versus unconditioned transplants with co-infusion of marrow and transfected stromal cells is not statistically significant (3.6 versus 2.8%; P=0.57). Moreover, the least effective conditions for engraftment, untransfected stromal cells infused into an unconditioned recipient can be enhanced almost 50 fold by using transfected stromal cells co-infused with marrow cells after conditioning. These data suggest that engraftment of VLA-4 expressing stromal cells is enhanced by the presence of bone marrow cells, possibly by the formation of cell complexes that exhibit increased adhesive properties as a result of increased VLA-4 expression on marrow as well as stromal cells.

As additional strategy to evaluate stromal engraftment employed the generation of stromal cells from the adherent layers derived from LTMC inoculated with recipient marrow cells. This served as a means of enriching for the presence of the stromal cell fraction in the marrow in order to enhance the detection of donor stromal cells.

3.6.2 FISH data on stromal cells

Experimental Groups	Infused with transfected cells Freq. Of cells of donor origin (%)	Infused with untransfected cells Freq. Of cells of donor origin (%)
Group A Unconditioned group infused with stromal cells and bone marrow cells	mouse 1: 7.0 mouse 2: 10.4 mouse 3: 10.1 mouse 4: 12.4 **Mean**: 10.0 ± 2.23	mouse 1: 7.1 mouse 2: 5.8 mouse 3: 5.5 mouse 4: 6.1 **Mean**: 6.1 ± 0.71
Group B Unconditioned group infused with stromal cells only	mouse 1: 2.6 mouse 2: 2.8 mouse 3: 1.4 mouse 4: 3.0 **Mean**: 2.5 ± 0.70	mouse 1: 2.0 mouse 2: 1.4 mouse 3: 2.4 mouse 4: 2.6 **Mean**: 2.1 ± 0.52
Group C Conditioned group infused with stromal cells and bone marrow cells	mouse 1: 24.5 mouse 2: 22.8 mouse 3: 17.1 mouse 4: 20.5 **Mean**: 21.2 ± 3.23	Mouse 1: 16.9 Mouse 2: 16.4 Mouse 3: 14.5 Mouse 4: 15.9 **Mean**: 15.9 ± 1.01

Table 7. Frequency of male donor cells (%) in stromal cells of experimental groups A, B and C.

In Situ Hybridization data on Stromal Cells
General Linear Models Procedure
Dependent Variable: PERC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1178.784	235.757	79.95	0.0001
Error	18	53.080	2.949		
Corrected Total	23	1231.864			

R-Square	C.V.	Root MSE	PERC Mean
0.957	17.837	1.717	9.628

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cond	1	444.472	444.472	150.73	0.0001
BMC	1	132.538	132.538	44.95	0.0001
Transfec	1	31.641	31.641	10.73	0.0042
Cond*Transfec	1	1.995	1.995	0.68	0.4215
BMC*Transfec	1	12.373	12.373	4.20	0.0554

Fig 26. F-test of engraftment percentage data on stromal cells in groups A, B and C.

These data parallel the results observed by examining the level of donor stromal cell engraftment with the nucleated marrow cells of transplant recipients (fig. 26). The results indicate that conditioning is a variable significantly enhancing engraftment (P= 0.0001) in addition to co-infusion with marrow cells and transfection of stromal cells (P=0.0001 and 0.0042, respectively).

	With transfected cells	With untransfected cells
Unconditioned group	9.97%	6.10%
Conditioned group	21.21%	15.94%

Table 8. Compares the effect of conditioning on groups that received marrow cells and transfected versus untransfected stromal cells.

Since it is not possible to have a conditioned stromal alone group, the comparison is then between unconditioned and conditioned recipients that received marrow cells and transfected versus untransfected stromal cells. These data show that regardless of the use of genetically modified stromal cells, conditioning of recipients enhances stromal engraftment 2 fold (21.21 versus 9.97% and 15.94 versus 6.10%) [table 8]. Unconditioned transplant groups with transfected cells shows a 1.6 fold increase in stromal engraftment (9.97 versus 6.10%) and conditioned transplant groups with transfected cells shows a 1.3 fold increase in stromal engraftment (21.21 versus 15.94%).

	With transfected cells	With untransfected cells
Group with BMC	9.96%	6.10%
Group without BMC	2.45%	2.10%

Table 9. Compares the effect of co-infusion of marrow cells and transfected versus untransfected stromal cells on groups with unconditioned recipients.

Since it is of interest to determine the effect of co-infusion of marrow cells and stromal cells on stromal engraftment, only unconditioned groups were analyzed to keep other variables constant. These data indicate the presence of marrow cells enhances stromal engraftment 4 fold (9.96 versus 2.45%) in the groups received transfected stromal cells and 3 fold (6.10 versus 2.10%)[table 9] in the groups received untransfected stromal cells.

These data suggest that the difference in donor cell frequency between conditioned versus unconditioned transplants with co-infusion of marrow and transfected stromal cells is also not statistically significant even though conditioning enhanced the stromal engraftment by 2 fold (21.2 versus 10.0%; P=0.42). The highest donor cell frequency was also with transfected stromal cells co-infused with marrow cells into lethally irradiated recipients while the lowest frequency was with untransfected stromal cells alone infused into unconditioned recipients (21.2 vs 2.1%; P=0.048).

3.7 Control Experiments: Detection of donor cells by FISH in nucleated marrow cells

	Freq. Of Cells of donor origin (%)
Group 1 (Unconditioned) Infused with salmon sperm DNA transfected stromal cells and bone marrow cells	mouse 1: 0.0 mouse 2: 0.1 mouse 3: 0.3 P-value of gp 4 vs. gp 1:0.0001 **Mean**: 0.1± 0.14
Group 2 (Unconditioned) Infused with α4 transfected stromal cells coincubating with anti-α4 antibody and bone marrow cells	mouse 1: 0.3 mouse 2: 0.3 mouse3: 0.2 P-value of gp 4 vs. gp 2:0.0001 **Mean**: 0.3± 0.05
Group 3 (Unconditioned) Infused with untransfected stromal cells and bone marrow cells	mouse 1: 0.3 mouse 2: 0.2 mouse 3: 0.1 P-value of gp 4 vs. gp 3:0.0001 **Mean**: 0.2± 0.08
Group 4 (Unconditioned) Infused with α4 transfected stromal cells and bone marrow cells	mouse 1: 3.7 mouse 2: 3.0 mouse 3: 2.6 P-value of gp 4 vs gp 1,2,3: 0.0001 **Mean**: 3.1± 0.58

Table 10. Frequency of male donor cells (%) in nucleated marrow cells of female recipients in groups 1, 2, 3 and 4 of control experiments.

In order to eliminate the possibility that improvements in engraftment were related to changes in stromal cell characteristics as a result of the electroporation procedure itself and not the specific expression of VLA-4, the following experiments were conducted. Group 3 represents the untransfected cohort. Group 1 mice received stromal cells transfected with irrelevant salmon sperm DNA and represent the electroporation control. Group 2 animals received transfected stromal cells co-incubated with an anti-α4 antibody to determine specificity of generating VLA-4 expressing stromal cells. Results were compared to the transplantation of VLA-4 expressing stromal cells.

The results show that engraftment was enhanced only in the mice transplanted with transfected stromal cells, indicating that the improvement was mediated by VLA-4 expression on the stromal population.

3.8 Hind limb irradiation experiment

PCR analysis of bone marrow cells from all transplant recipients did not show the presence of male donor cells in hind limbs nucleated marrow cells. Cell suspensions prepared from lung, liver, brain and spleen of recipient mice also gave negative results with PCR, showing that cells of donor origin were absent. Donor cells were not detected in the bone marrow aspirates of either irradiated and non-irradiated hind limbs. These data indicate that engraftment levels were below 0.69%, the level of sensitivity of our assay. We conclude that local hind limbs irradiation did not significantly enhance stromal cells engraftment.

Chapter 4

DISCUSSION

4.1 Overview

Previous studies from our and other laboratories suggest that bone marrow-derived stromal cells may serve as a useful vehicle for gene therapy. However, in vivo studies of murine models suggest that stromal cell engraftment in the bone marrow of recipients occurs at a very low level. Engraftment of stromal cells into unconditioned murine transplant recipients occurred at a frequency of approximately 1% using FISH in our lab.

One of the difficulties in this field is that there is little information on the homing capabilities of bone marrow stromal cells to the marrow cavity. A possible strategy to improve engraftment is to irradiate the hind limbs of mice prior to transplantation. Possible mechanisms that may enhance engraftment following limb irradiation include the induction of cellular adhesion molecules, integrins, or extracellular matrix proteins on recipient stromal cell surfaces that stimulate attachment of circulating cells with complementary receptors (Greenberger, 1991). However, lodgement of donor stromal cells in the marrow was no different in the marrow of irradiated hind limbs compared with unirradiated hind limbs. Although studies by Anklesaria et al., 1987 showed that pre-conditioning helps to create space in the marrow for donor cells to reside, Wert et al., 1980 showed that after irradiation, the bone marrow sinus epithelium is very severely damaged. It is possible therefore that the engraftment of stromal cells at irradiated sites is severely compromised as a result of damage to the sinusoidal microcirculation.

This experiment could be extended by including infusions with transfected stromal cells. It is possible that the irradiated bone marrow cavity lacks cells that are able to bind to the VCAM-1 adhesion molecule on normal stromal cells. In contrast, stromal cells expressing VLA-4 may more readily bind to the exposed endogenous stromal cells depleted of hematopoietic cells.

Another approach to improve engraftment of stromal cells is to enhance the interaction between the donor stroma and the host marrow microenvironment. This can

be done by modulating the adhesion molecule profile of donor stromal cells by introducing into the cells the α4 chain of VLA-4 to generate functional VLA-4 molecules. This integrin could then provide a means for circulating stromal cells to adhere to the microenvironmental cells in the marrow, as stem cells, which normally express VLA-4, are thought to do.

We have effectively optimized the conditions for engraftment within the limited parameters that we tested. The lowest engraftment levels obtained by infusing solely untransfected stromal cells into unconditioned recipients were enhanced almost 50 fold by using transfected stromal cells co-infused with marrow cells into conditioned recipients. Our data also suggest that the difference in stromal engraftment between conditioned versus unconditioned transplants with co-infusion of marrow and transfected stromal cells is not significant (3.6 versus 2.8%; P=0.57). The improvement in engraftment compared to the least effective conditions is 40 fold when using stromal and marrow cells in unconditioned recipients, the more appropriate model for human studies.

These data are encouraging as they indicate that this murine model may be applicable to human studies. The use of an unconditioned transplant protocol avoids the morbidity and possible mortality associated with the conditioning required for human stem cell transplant protocols. Intensive therapy prior to bone marrow transplant is important in treating malignancies, but less appropriate for other uses such as gene therapy.

The more direct comparison of transfected stromal cells with and without co-infused marrow cells into unconditioned recipients shows the significant superiority of including a marrow cell infusion: 2.8 vs 0.13%; P= 0.028, a 22 fold enhancement in engraftment. It is noteworthy that VLA-4 expression on stromal cells co-infused with bone marrow cells also confers enhancement to engraftment (2.8 vs 0.2%; P=0.035) in the unconditioned model as well as in lethally irradiated recipients (3.6 vs 0.3%; P=0.05).

These data strongly indicate that the engraftment of VLA-4 expressing stromal cells is enhanced by the presence of marrow cells. The results may be explained by the formation of cell complexes that exhibit increased adhesive properties due to VLA-4 expression not only on hematopoietic cells but also on stromal cells.

From the experiments conducted, it is not possible to determine the marrow cell type that facilitates the engraftment. However, it is possible to speculate that complexes involving stromal and stem cells may play a role. If this is the case, one can speculate that the VLA-4 expression on both stromal and stem cells may enhance homing to endogenous marrow stromal (via VCAM-1) cells in the microenvironment.

Studies with stromal cell layers rather than nucleated marrow cells were performed as an additional way to determine the frequency of donor derived stromal cells. It is estimated that the frequency of endogenous stromal cells in the nucleated marrow cell population is approximately 1%. A doubling of the population with infused stromal cells would only increase the total stromal population to 2%. Therefore, in an effort to increase the sensitivity of our detection methods, we looked at the stromal cell population only. Consequently, the proportion of donor cells is considerably higher. However, the same trends were observed as in studies using the detection of donor cells in marrow cells. The proportion of donor cells was 21% in conditioned mice co-infused with transfected stromal cells and marrow cells. In contrast, similar experiments with untransfected stromal cells give a log lower result (2%, P=0.048).

Similarly, when conditioned versus unconditioned transplants otherwise as indicated above were compared, donor levels were 21% versus 10%, respectively. Although this is a two fold increase with conditioned transplants, it is not statistically significant. This result is the same (although donor cell frequencies are different) as with assaying the nucleated marrow population [21.2% vs 10%; P=0.42 for stromal layers and 3.6vs 2.8; P=0.57]. The use of stromal layers for analysis validates our experiments with the nucleated cell population.

The apparently smaller difference between means for cultured cells versus nucleated marrow cells might be explained by the different proliferative capacity of donor stromal cells in vitro and in vivo. Donor stromal cells were selected from LTMC before infusion into recipient mice and may have additional capacity to proliferate to a greater extent and respond differently when returned to the in vitro conditions in which they were grown when compared with endogenous stromal cells obtained directly from the hosts.

It should be noted that the results with stromal layers obtained from the

conditioned recipients may not accurately reflect the proportion of endogenous stromal cells. This is because conditioned mice were treated with 9Gy and as a result, the proliferative ability of the stromal cells irradiated in vivo may have been reduced. These cells were expected to divide to the 4^{th} passage as effectively as the stromal cells infused after the irradiation. It is possible therefore that the proportion of donor cells is actually less that 21%.

This study demonstrates that electroporation is a simple and effective physical method to deliver the α4 chain gene of VLA plasmid to stromal cells. Other physical methods of DNA transfer have also been studied with stromal cells. For example, bead transfection involves the incubation of cells with glass beads in a DNA solution and gives a transient transfection efficiency of 5% in stromal cells (Matthews et al., 1993) whereas in our hands electroporation can give optimized transient levels of 45%. Retroviral transfer has not been studied in detail with stromal cells but is difficult to achieve in quiescent cells (Li et al., 1995) and the generation of new recombinant viruses is a potential risk (Moolten and Cupples, 1992). Nonetheless, further work comparing the gene transfer efficiency of different viral and physical methods in stromal cells would be useful.

This study shows that we achieved a moderately high transient expression efficiency of 45% in stromal cells up to 5 days after electroporation. The results compare favorably to other physical methods of DNA transfer. The approach we have taken is well suited to our goal of modifying the homing characteristics of stromal cells because the homing process probably takes only minutes before lodgement of the cells occurs in the marrow and there is no obvious advantage to confer long-term expression to the infused cells.

A drawback of the approach however is the low level of sustained transgene expression for genes to be used in therapy such as for Factor IX or VIII deficiencies in hemophilia. Other methods may be better for this purpose. For example, retroviral vector-mediated gene transfer offers the potential for long-term gene expression due to its stable chromosomal integration (Chuah et al., 1998).

4.2 Future direction

Although we have significantly enhanced the engraftment levels of stromal cells, our optimal levels may still be insufficient to provide a clinically useful model. Several strategies can be developed to increase the likelihood of making this a successful approach. First, it may be possible to further enhance stromal cell lodgement/ engraftment. In this context it may be interesting to explore other adhesive mechanisms such as $\alpha 5\beta 1$(VLA-5)/fibronectin that may also allow stromal cells to home specifically to marrow. Given that the VLA-5 molecule is found on stromal fibroblasts (Faid et al., 1996) and fibronectin is one of the components of the bone marrow extracellular matrix, the VLA-5/fibronectin pathway may be another mechanism to examine in enhancing stromal engraftment.

Another possibility would be to increase adhesion molecule expression by improving the plasmid vector. Previous studies from our lab indicate that the human immediate early CMV promoter is the most effective promoter in marrow stromal cells (Keating et al., 1990). A vector could be constructed in which the α 4 chain cDNA is driven by the hCMV promoter and results compared with the construct in which α 4 is driven by the SFFV-LTR (Elice et al., 1990). A recent study in fibroblasts and other cells shows that the SFFV-LTR is more effective than several cellular promoters and the SV40 promoter (Flasshove et al., 2000).

Given that marrow cells co-infused with stromal cells significantly enhance engraftment, it is possible that this effect could be optimized by enriching for the most responsible nucleated hematopoietic cell. This is most likely to be an early precursor cell that is equivalent to $CD34^+$ cell in humans. Studies could be conducted to extend our observations by using high concentrations of murine early hematopoietic cells with the characteristics $Thy\text{-}1^+$, $Sca\text{-}1^+$, Lin^- (Morrison et al., 1995).

A greater knowledge of the trafficking of stromal cells once infused may provide insights into how to enhance engraftment and also assess localization to tissues other than bone marrow. A sensitive imaging approach has been developed that detects small numbers of infused cells through the use of whole-body imaging (Sweeney et al., 1999). Cells labeled with a light emitting signal such as luciferase and the whole animal

(preferably a white mouse) is exposed to a low-light imaging system, comprised of an intensified charge-coupled device camera fitted with a 50-mm f1.2 Nikkor lens and a computer with imaging-analysis capabilities. A software program enables the signals to be quantified. This technology can be used to determine whether lodgement or proliferation in the marrow spaces is the critical issue and possibly which needs to be improved. It is not known whether the infused stromal cells are able to proliferate in vivo.

Since VLA-4 expressing stromal cells require the presence of manganese ion for optimal binding to VCAM-Ig in vitro, co-incubation of transfected stromal cells with manganese chloride to activate the VLA-4 receptors expressed prior to intravenous infusion might further enhance stromal engraftment.

Another approach to improve the model would be to increase the proliferative capacity of these cells without causing immortalization or transformation. One possibility would be to transfect with the telomerase gene, unlikely to be expressed in unmanipulated stromal cells. The high levels of transient expression and low levels of stable integration obtained with electroporation would also be an advantage here.

Another approach would be to improve the culturing of these cells. It takes 6 weeks to generate 4^{th} to 5^{th} passaged stromal cells from a long-term marrow culture adherent layer. The addition of fibroblast growth factor increases the growth of stromal cells, other cytokines should be explored to improve the growth characteristics further.

These extensions of our work would enhance the chance of this approach being useful in patients. It is possible that genetically modified stromal cells may be useful not only as gene therapy but in treating microenvironmental defects that are associated with chemotherapy (Clark and Keating, 1995).

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