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Review article

Critical early events in hematopoietic cell seeding and engraftment

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Abstract: Durable hematopoietic stem cell engraftment requires efficient homing to and seeding in the recipient bone marrow. Dissection of cellular and molecular mechanisms by retrospective analysis of functional engraftment studies imposes severe limitations on the understanding of the early stages of this process. We have established an experimental approach for *in vivo* functional imaging of labeled cells at the level of recipient bone marrow in real time. The adhesive interaction of hematopoietic cells with the bone marrow stroma evolves as the most important early event. Adhesion to the marrow, rather than the vascular endothelium, determines the efficiency of both homing and seeding, and is absolutely essential to maintain cell viability in the marrow. Seeding and engraftment may be improved either by bypassing homing or by localized transplant of a large number of cells in a relatively small marrow space. There is functional redundancy in the molecular pathways that mediate the cell-stroma interaction, such that blockage of a single pathway has only minor effect on homing and seeding. We hypothesize that successfully seeding-engrafting cells undergo extensive phenotypic changes as a consequence of interaction with the stroma, without engaging in rapid proliferation. Surprisingly, Fas-ligand appears to promote hematopoietic cell engraftment by immunomodulatory and trophic effects.

Key words: Hematopoietic stem and progenitor cells - Seeding - Bone marrow stroma - Localized transplantation - Fas-ligand

Obligatory adhesion of transplanted cells to the marrow stroma

Following transplant, hematopoietic stem cells infused into the systemic circulation distribute in accordance with hydrostatic and chemotactic determinants. The cells respond to chemotactic gradients generated by local endothelia, adhere, roll and penetrate into the tissue by means of trans-endothelial migration [21]. Homing of hematopoietic stem and progenitor cells (HSPC) by extravasation into the marrow space is considered to be a pivotal process, because this is the only site that hosts durable and multilineage differentiation of HSPC in normal conditions. Homing has caught much attention because of the phenomenal capacity of cells to respond to chemotactic signals and successfully connect to their "natural" site of residence [reviewed in 12, 16, 18, 21, 24].

One of the intriguing aspects of HSPC homing is its temporality, over a period of many hours, in both syngeneic and allogenenic transplant settings. Detectable levels of labeled cells may be found in the peripheral blood of recipients 6-9 and 3-6 hours after bolus injection of syngeneic and allogeneic cells, respectively. The difference is somewhat expected due to the anticipated entrapment of allogeneic cells in the host reticuloendothelial system. Theoretically, the prolonged peripheral circulation of injected HSPC may be explained either by failure of the cells to recognize the chemotactic stimuli and adhere to the endothelium, or by a bidirectional dynamic entry into and exit from the marrow space. Using intravital microscopy of the femoral marrow, fluorochrome-labeled cells appear and disappear within the marrow space in the hours following intravenous injection [4], suggesting the contention that homing and mobilization are indeed similar processes in opposite directions [26]. This leads us to believe that those cells that possess the mechanisms to enter the marrow space can use the

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same molecular route to exit this space (mobilization↔ homing↔seeding).

The rate limiting determinant of homing is in fact the adhesive interaction with the stroma. By 3 days after intravenous injection, only ~50% of the cells that home to the marrow establish stromal adhesion (seeding) [3]. The fate of non-adherent cells is death by apoptosis, because interaction with the stroma is required to preserve viability (Fig. 1). At this early stage post-transplantation, the loss of marrow-homed cells occurs in a non-immunogenic manner, before alloimmunity is activated [6]. We have shown a strong relationship of stromal-adherence and donor cell viability, though we could not prove this relationship by simultaneous measurements of adhesion and viability. The mechanism of clearance of the dead cells is unknown, though we presume that a fraction of the dead cells leave the bone marrow, while others are being cleared by phagocytic cells that survived conditioning.

A quantitative analysis of early engraftment revealed an advantage in recognition of the bone marrow by more primitive heamatopoietic progenitors, manifested by differential homing and seeding efficiencies of lineage-negative versus lineage-positive bone marrow cells. Assuming that homing is mediated by pre-existing adhesion molecules expressed by the transplanted HSPC, the affinity for the marrow stroma may be an intrinsic property of bone marrow cells at various levels of differentiation. In general, 2-10% of the infused lineage-negative cells home and seed in the marrow stroma *in vivo*, more efficient by a factor of 20-30 than lineage-positive cells [3, 4]. This process occurs physiologically, with loss of stromal-adhesion during differentiation in anticipation of release of matured cells into the peripheral circulation. However, selection of marrow-repopulating cells by their repertoire of adhesion molecules failed to accurately predict homing and engraftability [20].

It is likely that HSPC change their repertoire of adhesion molecules during the first days post-transplantation. In a fraction of ~20% of the transplanted cells that are actively engaged in fast proliferation early after transplantation [3], this process is associated with phenotypic changes [22]. The majority of cells remains mitotically-quiescent or is slow cycling, as evident by the retention of cell surface fluorescent markers [3, 6]. The relatively quiescent state and slow cycling are associated with engraftment potential, and are accompanied by remarkable phenotypic changes [20, 22]. One example is the induced expression of very late antigen-4 (VLA-4) in transplanted cells following interaction with the marrow stroma [6]. Blockage of vascular cell adhesion molecule-1 (VCAM-1) resulted in a more pronounced decrease in seeding efficiency than inhibition of VLA-4. We think that the robust effect of VCAM-1 inhibition on seeding is mediated by efficient expression of VLA-4 following donor cell interaction with the stroma.

Functional redundancy of the adhesion molecules is evident from failure of inhibition of a single ligand/receptor interaction to abolish seeding. We believe that selection of engraftable HSPC by the repertoire of adhesion molecules that they express may be a conceptual error. Rather than selective seeding due to a single pre-expressed molecule, as hypothesized to occur in the case of homing, interaction with the marrow stroma is accompanied by rapid phenotypic changes in cell surface molecules. In the *in vivo* experimental setting we use marrow cellularity on days +1 and +3 post transplantation to determine the homing and seeding efficiencies of donor cells, respectively. Anti-VLA4 blocking antibodies reduced day +1 homing by a factor of 2, similar to their effect on acute adhesion of bone marrow cells to the stroma of femurs superfused *ex vivo* [6]. Because the inhibitory effect was more pronounced on day +3 post-transplantation (4.5-fold decrease), we assume that VLA-4 plays a more important role as mediator of seeding than of homing. In other words, the level of functional redundancy for VLA-4 is higher at the level of vascular endothelium than at the level of marrow microenvironment. In parallel, seeded cells also showed induced expression of putative stem cell markers including SCA-1 and c-kit (NA, unpublished data). Our interpretation of these data is that cells make use of the pre-existing repertoire of adhesion molecules only for the first steps of homing and early seeding. Soon thereafter, either synchronized by slow cycling or without division, some HSPC express additional molecules, a process that promotes engraftment.

As observed *in vivo*, donor cell seeding and engraftment in the host bone marrow is a highly organized process, which repeats itself with remarkable precision. According to the spatial and temporal patterns, we have categorized seeding and engraftment into three levels (Fig. 2). Donor bone marrow cells almost always adhere to the stroma in proximity to each other, forming a cluster of stem, progenitor and mature cells [9]. Primary clusters are located at the endosteal surface of the murine femoral epiphyses for the first 4-6 days post transplantation [5, 9]. The transition to secondary clusters is marked by a decrease in number of cells within the clusters, and onset of proliferation of some cells while others remain mitotically quiescent. We include in the secondary clusters two types of niches, one that maintains functional quiescence and will continue to do so for periods of many weeks, and a niche that promotes early proliferation of progenitors [5]. The site of clonogenic activity of HSPC shifts with time towards the more central regions of the epiphyseal marrow and the diaphyses, where tertiary niches host rapidly proliferating cells. Rare observations suggest that the progenitors crawl over the stromal surface towards the

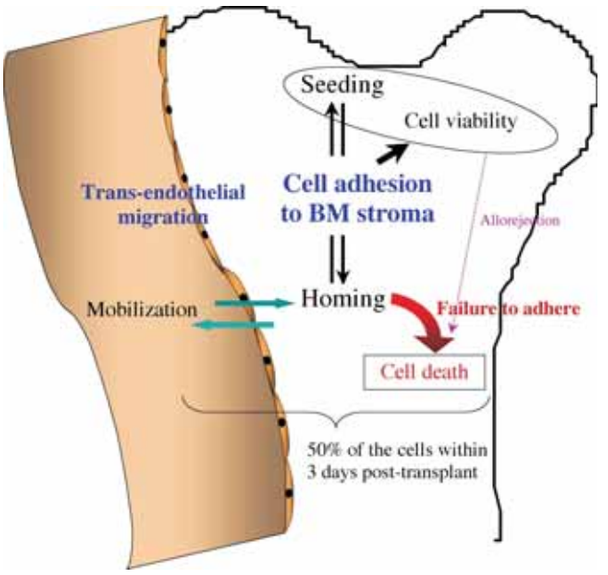

Fig. 1. Obligatory cell interaction with the marrow stroma. The dominant factor in homing and seeding is interaction with the marrow stroma. This interaction is tightly correlated to the survival of transplanted cells, and ~50% of the bone marrow-homed cells are lost in the early stage of seeding. A major cause of cell loss is failure to adhere to the marrow stroma, a non-immunogenic mechanism that culminates in cell death. On days 2-3 all marrow-residing donor cells are adherent and viable.

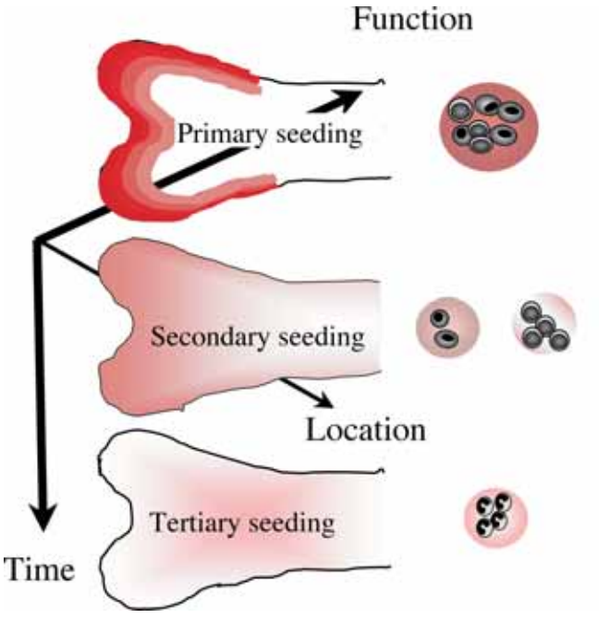

Fig. 2. Temporo-spatial organization of seeding in femoral marrow. Transition of cells to functionally-distinct niches is temporally and spatially organized in time and space within the marrow. Primary seeding occurs in niches located at the endosteal surface of the epiphyses that host clusters of stem, progenitor and mature cells for 4-5 days post-transplantation. Secondary seeding persists in the marrow periphery, and includes two types of niches: those that will host functionally- and mitotically-quiescent cells for long durations, and those that host early dividing cells. Mature transplanted cells are not present in the secondary niches. Differentiation and proliferation occurs in parallel to migration to the tertiary seeding niche located in the central marrow.

proliferation-supporting central regions of the marrow. This temporo-spatial organization of seeding and early engraftment illustrates the cooperativity of stromal regulation and both, adaptive and intrinsic HSPC responsiveness in the dynamic of early engraftment.

Localized engraftment

To dissociate between the homing and seeding, we have compared (Fig. 3): (1) intravenous transplantation, (2) intra bone-marrow injection, (3) isolated limb perfusion and (4) *ex vivo* bone perfusion. The conceptual advantages of directed HSPC injection into the bone marrow or an isolated limb preparation are: (a) avoids donor cell depletion by host immune system during circulation in the peripheral blood, (b) eliminates the homing process, (c) induces a mega-dose effect of hematopoietic stem cell engraftment, and (d) improves the competitiveness of donor cells for seeding niches.

Isolated limb perfusion resulted in massive retention of donor progenitors (as much as 68% of the blasts in the femur of myeloablated hosts) corroborating the high affinity of primitive hematopoietic cells for the bone marrow [11]. With proximal occlusion of the femoral artery and vein, there were very few (0.3%) labeled cells

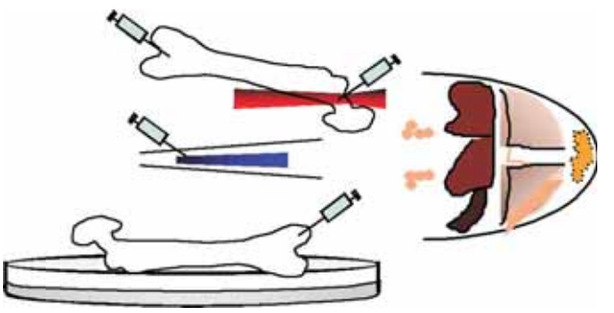

Fig. 3. Four approaches to study homing and seeding. Intravenous injection results in systemic dissipation of the cells and chemotaxis-driven homing to the marrow. In isolated limb perfusion preparations the cells still have to cross the endothelial barrier to enter the marrow space, and with proximal occlusion of blood vessels the transplant may be localized to one limb. Intra-bone marrow injection introduces the cells directly into the bone marrow, eliminating both homing and trans-endothelial migration. Intra-bone perfusion may be performed in isolated bones *ex vivo*, to analyze the primary cell-stroma interaction that does not involve *de-novo* expression of adhesion molecules and cytokine receptors.

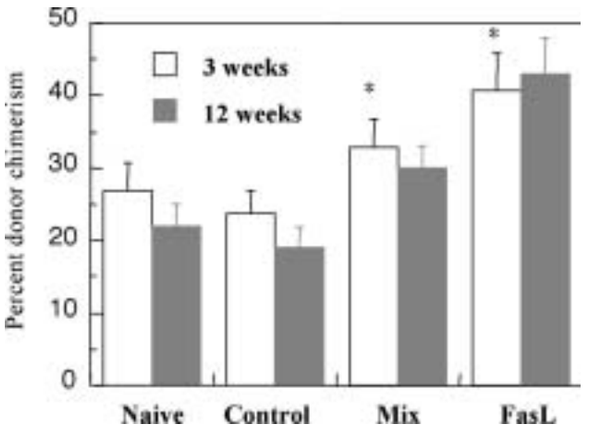

Fig. 4. Enhanced engraftment of bone marrow cells expressing Fas-ligand protein in non-myeloablated hosts. An allotransplant model of major and minor antigen-disparity, where recipients conditioned with busulfan (LD_{25}) were injected with unmanipulated bone marrow cells (Naïve), cells expressing a non-functional (Control) and functional Fas-ligand protein. One third (Mix) or all of the transplanted bone marrow cells (FasL) expressed a non-cleavable recombinant Fas-ligand protein. The levels of donor chimerism were assayed in the peripheral blood of hosts 3 and 12 weeks post-transplantation. *$p<0.05$

in the contralateral femur, as compared to ~9% observed with intra-bone marrow injection and intravenous injection [11]. Intra-arterial injection without proximal vascular occlusion is also an efficient approach to increase the number of donor cells in the bone marrow, though it did not result in detectably superior engraftment compared to intravenous injection [13]. In all localized transplants (intra-arterial, intra-bone marrow and isolated limb perfusion), the superior donor cellularity in the ipsilateral femur persisted for weeks, consistent with a slow rate of bone-to-bone migration of HSPC [1, 7, 13, 17]. Progenitors mobilized from the infused to the contralateral femurs showed immature phenotypes, but engaged in proliferative activity at high rates [7], supporting the ability of hematopoietic stem and progenitor cells to perform stochastic clonogenic activity.

Isolated limb perfusion resulted in comparable levels of peripheral blood donor chimerism at 3-4 weeks as compared to intravenous transplants, despite the smaller size of donor inoculum by one order of magnitude. We attributed this effect to a local "mega-dose" effect, created by the relatively large number of cells infused into a small marrow space [10]. Localized bone marrow transplantation showed superior engraftment of T-cell-depleted bone marrow cells and reduced incidence and intensity of graft versus host disease [1, 17]. Thus the first three conceptual advantages of localized transplants were supported by the experimental findings. It is unclear however, whether seeding is a competitive process, where donor cells are to replace some of the host cells in the bone marrow. Repeated injections of 10^5-10^8 cells both *in vivo* (NA, personal communication) and *ex vivo* [2] resulted in progressive retention of cells, without evident saturation of the marrow seeding niches. Considering that we could load more than 8×10^6 lin^- cells in one femur (which comprises ~7% of the total murine marrow space), for any practical purpose in the transplant setting the availability of seeding niches is not a limiting factor.

Surprise: toxic molecules in disguise

The stem cell compartment in the bone marrow presents surprising evidence of its responses to diverse physiological stimuli. A prominent example is the apparent engraftment-supporting role of Fas-ligand, a molecule that triggers apoptosis via interaction with its receptor Fas. In studies using Fas-ligand to induce peripheral tolerance in the bone marrow, an immune privileged site, we and others observed a remarkable enhancement of hematopoietic stem cell engraftment. One of the mechanisms of Fas-ligand is purely immunogenic, and involves antigen-selective depletion of alloreactive immune cells that would inhibit engraftment at the systemic level and in the bone marrow [8]. However, an additional trophic effect appears to support the activity of engrafting cells *per se*. Lentivirus-mediated infection of hematopoietic stem and progenitor cells with Fas-ligand resulted in improved short term engraftment [25], and addition of the soluble molecule to cultured cells improved viability [14] and enhanced clonogenic activity [11]. This behavior was initially attributed to a low expression of Fas by quiescent stem cells, rendering them resistant to Fas-ligand-mediated apoptosis [19] and the elevated expression of anti-apoptotic proteins in HSPC [15]. We tested the effect of Fas-ligand in a murine bone marrow transplant setting (Fig. 4). Although the engineered chimeric Fas-ligand protein persists for only 10 days *in vivo* [9], the short term superior engraftment was sustained for a period consistent with transition to durable engraftment. This molecule also blocked the modest decrease in levels of chimerism usually observed during transition to long term engraftment. This interesting phenomenon should be viewed in perspective to the elevated expression of Fas in hematopoietic stem and progenitor cells after transplantation [23], and the significant role of the Fas/Fas-ligand interaction as a mediator of allorejection [8]. It is yet unknown whether the trophic effect of Fas-ligand is mediated through Fas binding, and how it is distinguishable from the pro-apoptotic triggering pathway.

Concluding remarks

Engraftment potential is determined by the ability of hematopoietic progenitors to respond to the interaction with bone marrow stroma by expression of cell surface

molecules accompanied by slow cycling or divisional quiescence. Because the mechanisms of HSPC-stroma interaction are multiple, analysis of isolated pathways or molecules may miss the multidimensionality of this elegantly orchestrated process. *In situ* real time analysis of seeding in live subjects is a promising technique that will enhance our understanding of hematopoietic cell engraftment.

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References

[1] Askenasy N (2002) Localized bone marrow transplantation leads to skin allograft acceptance in non-myeloablated recipients: comparison of intra-bone marrow and isolated limb perfusion. Stem Cells 20: 86-93

[2] Askenasy N, Farkas DL (2002) Antigen barriers or available space do not restrict *in situ* adhesion of hemopoietic cells to bone marrow stroma. Stem Cells 20: 80-85

[3] Askenasy N, Farkas DL (2002) Optical imaging of PKH-labeled hematopoietic cells in recipient bone marrow *in vivo*. Stem Cells 20: 501-513

[4] Askenasy N, Farkas DL (2003) *In vivo* imaging studies of the effect of recipient conditioning, donor cell phenotype and antigen disparity on homing of haematopoietic cells to the bone marrow. Br J Haematol 120: 505-515

[5] Askenasy N, Stein J, Yaniv I, Farkas DL (2003) The topological and chronological patterns of hematopoietic cell seeding in host femoral bone marrow after transplantation. Biol Blood Marrow Transplant 9: 496-504

[6] Askenasy N, Yolcu ES, Shirwan H, Stein J, Yaniv I, Farkas DL (2003) Characterization of adhesion and viability of early seeding hematopoietic cells in the host bone marrow *in vivo* and *in situ*. Exp Hematol 31: 1292-1300

[7] Askenasy N, Yolcu ES, Shirwan H, Wang Z, Farkas DL (2003) Cardiac allograft acceptance after localized bone marrow transplantation by isolated limb perfusion in non-myeloablated recipients. Stem Cells 21: 200-207

[8] Askenasy N, Yolcu ES, Yaniv I, Shirwan H (2005) Fas-ligand as a double-edged immunomodulator to induce transplantation tolerance. Blood 105: 1396-1404

[9] Askenasy N, Zorina T, Farkas DL, Shalit I (2002) Transplanted hematopoietic cells seed in clusters in recipient bone marrow *in vivo*. Stem Cells 20: 301-310

[10] Bachar-Lustig B, Rachamim N, Li HW, Lan F, Reisner Y (1995) Megadose of T cell-depleted bone marrow overcomes MHC barriers in sublethally irradiated mice. Nat Med 1: 1268-1273

[11] Barcena A, Muench M, Song KS, Ohkubo T, Harrison MR (1999) Role of CD95/Fas and its ligand in the regulation of the growth of human CD34++ CD38- fetal liver cells. Exp Hematol 27: 1428-1439

[12] Berrios VM, Dooner GJ, Nowakowski G, Frimberger A, Valinski H, Quesenberry PJ, Becker PS (2001) The molecular basis for the cytokine-induced defect in homing and engraftment of hematopoietic stem cells. Exp Hematol 29: 1326-1335

[13] Chabner KT, Adams GB, Qiu J, Moskowitz M, Marsters ES, Topulos GP, Scadden DT (2004) Direct vascular delivery of primitive hematopoietic cells to bone marrow improves localization but not engraftment. Blood 103: 4685-4686

[14] Josefsen D, Myklebust JH, Lynch DH, Stokke T, Blomhoff HK, Smeland EB (1999) Fas ligand promotes cell survival of immature human bone marrow CD34+CD38- hematopoietic progenitor cells by suppressing apoptosis. Exp Hematol 27: 1451-1459

[15] Kim H, Whartenby KA, Georgantas RW 3rd, Wingard J, Civin CI (2002) Human CD34+ hematopoietic stem/progenitor cells express high levels of FLIP and are resistant to Fas-mediated apoptosis. Stem Cells 20: 174-182

[16] Kucia M, Reca R, Miekus K, Wanzeck J, Wojakowski W, Janowska-Wieczorek A, Ratajczak J, Ratajczak MZ (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells 23: 879-894

[17] Kushida T, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, Iida H, Ikehara S (2001) Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 97: 3292-3299

[18] Lapidot T, Petit I (2002) Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 30: 973-981

[19] Niho Y, Asano Y (1998) Fas/Fas ligand and hematopoietic progenitor cells. Curr Opin Hematol 5: 163-165

[20] Orschell-Traycoff CM, Hiatt K, Dagher RN, Rice S, Yoder MC, Srour EF (2000) Homing and engraftment potential of Sca-1(+)lin(-) cells fractionated on the basis of adhesion molecule expression and position in cell cycle. Blood 96: 1380-1387

[21] Quesenberry PJ, Becker PS (1998) Stem cell homing: rolling, crawling, and nesting. Proc Natl Acad Sci USA 95: 15155-15157

[22] Quesenberry PJ, Colvin GA, Abedi M, Lambert JF, Moore B, Demers D, Greer D, McAuliffe C, Dooner M, Lum LG, Badiavas E, Falanga V (2003) The marrow stem cell: the continuum. Bone Marrow Transplant 32, Suppl 1: S19-S22

[23] Saheki K, Fujimori Y, Takemoto Y, Kakishita E (2000) Increased expression of Fas (APO-1, CD95) on CD34+ haematopoietic progenitor cells after allogeneic bone marrow transplantation. Br J Haematol 109: 447-452

[24] Voermans C, Rood PM, Hordijk PL, Gerritsen WR, van der Schoot CE (2000) Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 18: 435-443

[25] Whartenby KA, Straley EE, Kim H, Racke F, Tanavde V, Gorski KS, Cheng L, Pardoll DM, Civin CI (2002) Transduction of donor hematopoietic stem-progenitor cells with Fas ligand enhanced short-term engraftment in a murine model of allogeneic bone marrow transplantation. Blood 100: 3147-3154

[26] Whetton AD, Graham GJ (1999) Homing and mobilization in the stem cell niche. Trends Cell Biol 9: 233-238

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