

REVIEW

Mesenchymal stromal cells retrovirally transduced with prodrug-converting genes are suitable vehicles for cancer gene therapy

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Summary. – Mesenchymal stem/stromal cells (MSC) possess a set of several fairly unique properties which make them ideally suitable both for cellular therapies and regenerative medicine. These include: relative ease of isolation, the ability to differentiate along mesenchymal and non-mesenchymal lineages *in vitro* and the ability to be extensively expanded in culture without a loss of differentiative capacity. MSC are not only hypoinnogenic, but they mediate immunosuppression upon transplantation, and possess pronounced anti-inflammatory properties. They are able to home to damaged tissues, tumors, and metastases following systemic administration. The ability of homing holds big promise for tumor-targeted delivery of therapeutic agents. Viruses are naturally evolved vehicles efficiently transferring their genes into host cells. This ability made them suitable for engineering vector systems for the delivery of genes of interest. MSC can be retrovirally transduced with genes encoding prodrug-converting genes (suicide genes), which are not toxic *per se*, but catalyze the formation of highly toxic metabolites following the application of a nontoxic prodrug. The homing ability of MSC holds advantages compared to virus vehicles which display many shortcomings in effective delivery of the therapeutic agents. Gene therapies mediated by viruses are limited by their restricted ability to track cancer cells infiltrating into the surrounding tissue, and by their low migratory capacity towards tumor. Thus combination of cellular therapy and gene delivery is an attractive option – it protects the vector from immune surveillance, and supports targeted delivery of a therapeutic gene/protein to the tumor site.

Keywords: mesenchymal stromal cells; retroviral vectors; cancer gene therapy; prodrug-converting genes

Contents:

1. Main characteristics of mesenchymal stromal/stem cells
2. MSC as suitable vehicles for gene therapy
3. Retroviral vectors for gene therapy
4. Genetically engineered MSC for gene directed enzyme prodrug therapy
5. Herpes simplex virus thymidine kinase/ganciclovir approach
6. Cytosine deaminase/5-fluorocytosine approach
7. Purine nucleoside phosphorylase/fludarabine approach
8. Conclusions

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Abbreviations: 5-FC = 5-fluorocytosine; 5-FU = 5-fluorouracil; AT-MSC = adipose tissue-derived mesenchymal stromal/stem cells; CD = cytosine deaminase; CD::UPRT-MSC = MSC expressing cytosine deaminase::uracil phosphoribosyl transferase; GCV = ganciclovir; GCV-TP = ganciclovir triphosphate; GDEPT = gene directed enzyme prodrug therapy; HSVtk = Herpes simplex virus thymidine kinase; MLV = murine leukemia virus; MSC = mesenchymal stromal cells/mesenchymal stem cells; PNP = purine nucleoside phosphorylase; RCRV = replication competent retroviral vector; UPRT = uracil phosphoribosyl transferase

1. Main characteristics of mesenchymal stromal/stem cells

Population of rare, non-hematopoietic progenitor cells was originally isolated from bone marrow based on their ability to adhere to plastic culture dishes. Friedenstein *et al.* (1970) found them to be clonogenic in culture and multipotent for stromal precursors. Years later, scientists began to

explore the full potential of these cells, and they discovered that this population had properties of true stem cells (Caplan, 1991). A stem cell has the capacity for self-renewal and the ability to give rise to one or more types of differentiated progeny (Prockop, 1997; Morrison *et al.*, 1997). Within that context and based on its *in vitro* behavior, it seems that MSC might be qualified as stem cells thanks to their vast proliferative potential, clonal regeneration and production of differentiated cells (Pittenger *et al.*, 1999).

Extensive research revealed that cells obtained from bone marrow, adipose tissue, umbilical cord or other sources by plastic adherence are composed of heterogenic population of progenitor cells in various state of development, out of which “true stem cells” form only a subpopulation. We prefer designation “stromal”, however we would like to respect the opinion of other researchers cited in this review, therefore we use both terms as they occur in literature. The abbreviation “MSC” is used for both “stem” and “stromal” cells and means population of adherent progenitor cells with defined panel of surface markers and ability to differentiate under specific culture conditions.

MSC are currently defined by a combination of phenotypic, morphologic, physical and functional properties (Javazon *et al.*, 2004). The classical methodology identifies MSC by the colony forming unit (CFU) assay, which recognizes adherent spindle-shaped cells proliferating to form colonies, and which are able to differentiate into adipocytes, osteocytes and chondrocytes *in vitro* (Galderisi *et al.*, 2010).

The ISCT (The International Society for Cellular Therapy) has provided the minimum criteria for defining these multipotent cells. As stated above they adhere to plastic under standard culture conditions; differentiate to adipogenic, chondrogenic, and osteogenic lineages; express CD73, CD90, CD105, and do not express CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (Dominici *et al.*, 2006). In addition to standard methods of cell characterization, the relative benefits of more advanced molecular tools including assessments of the cell transcriptome, proteome, and secretome should be evaluated in creating new definition (Keating, 2012; Ranganath, 2012).

Because MSC populations are heterogeneous between species and within cultures, variable expression of surface markers is often observed. These discrepancies arise due to differences in isolation method, tissue and species of origin, and culture conditions (Javazon *et al.*, 2004). MSC have the ability to give rise not only to cells of mesenchymal origin, but to cells of all three germinal layers, which means that they have the ability to cross developmental boundaries (Porada and Almeida-Porada, 2010). It is assumed that the differentiation toward a particular tissue lineage is primarily driven by the tissue-specific microenvironment (Breitbach *et al.*, 2007).

No expression of costimulatory molecules makes them regarded as non-immunogenic and suggests that these cells

might be effective in inducing tolerance. MHC class I may activate alloreactive T cells, but a secondary signal would not engage with the absence of costimulatory molecules, leaving T cells anergic (Tse *et al.*, 2003; Le Blanc *et al.*, 2003; Kidd *et al.*, 2008). Recent studies have provided evidence that MSC are not only relatively non-immunogenic, but they also appear to have immunosuppressive and anti-inflammatory properties both *in vitro* and *in vivo* (Porada and Almeida-Porada, 2010). It has also been shown that MSC impair maturation and function of dendritic cells and inhibit human B cell proliferation, differentiation, and chemotaxis *in vitro* (Aggarwal and Pittenger, 2005). Due to their suppression of the lymphocyte proliferative response to allogeneic and xenogenic antigens (Le Blanc *et al.*, 2003), transplantation into an allogeneic host may not require immunosuppression (Javazon *et al.*, 2004).

2. MSC as suitable vehicles for gene therapy

The poor prognosis for patients with aggressive or metastatic tumors and the toxic side effects of currently available treatments necessitate the development of more effective tumor-selective therapies. Thanks to tumor-tropic properties, stem cells can be exploited for targeted delivery of anticancer genes (Aboody *et al.*, 2008). The ideal candidates of cellular therapy for clinical use are cells harvested without difficulty, which can be processed *ex vivo* very efficiently, and afterwards transplanted. MSC possess all these properties and thus could be suitable for use in human clinical trials (Giordano *et al.*, 2007b).

Despite significant advances in the field of cancer gene therapy, two major obstacles which limit the clinical potential of this approach remain: lack of tumor tropism of vectors and stimulation of immune response. Thus combination of cellular therapy and gene delivery is an attractive option – it would protect the vector from immune surveillance, and support targeted delivery of a gene or therapeutic protein to the tumor site (Dwyer *et al.*, 2010).

In addition to differentiative, trophic, and immunomodulatory properties, a large number of preclinical animal studies also highlighted another interesting and clinically valuable characteristic of MSC – their ability to selectively navigate to sites of injury or inflammation within the body (Jiang *et al.*, 2006). Tumors and their surroundings can be considered as “wounds that never heal” (Dvorak, 1986) and contain high numbers of inflammatory cells and cytokines that attract MSC (Dwyer *et al.*, 2007). The mechanism, by which MSC home to tissues and migrate across endothelium, is not yet fully understood, but it is likely that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSC, as in the case of recruitment of leukocytes to sites of inflammation (Chamberlain *et al.*, 2007).

It means that integration into the tumor stroma is thought to be mediated by high local concentrations of inflammatory chemokines and growth factors, such as epidermal growth factor-A, fibroblast growth factor, platelet-derived growth factor, stromal-derived growth factor 1 α (SDF-1 α /CXCL12), IL-8, IL-6, granulocyte-macrophage colony-stimulating factor, etc. (Kucerova *et al.*, 2010; Dwyer *et al.*, 2010). On the other hand, MSC have been reported to express a number of adhesion molecules including integrins, CD44, CD54/ICAM-1, CD106/VCAM-1, CD166/ALCAM (Minguell *et al.*, 2001). The functional chemokine receptors on MSC include CCR1, CCR7, CCR9, CXCR3, CXCR4, CXCR5, CX3CR1, and c-met (Myers *et al.*, 2010; Dwyer *et al.*, 2010). Variability of receptors reflects the heterogeneity of cultured MSC. This expression variability suggests that they show the potential to home to different tissues (Chamberlain *et al.*, 2007). MSC possess the ability to migrate to tumor foci regardless of tumor size, anatomic location or tissue of origin (Najbauer *et al.*, 2007). The unique ability to cross the blood-brain barrier provides a very attractive advantage in treating central nervous system-associated tumors (Aboody *et al.*, 2006; Danks *et al.*, 2007).

The issue of spontaneous transformation of MSC is a matter of debate (Lazennec and Jorgensen, 2008). The risk of tumorigenicity is far lower in cells from adult tissues but increases if cells are expanded in culture. Many cells display a limited lifespan in culture and progress to senescence. However, attention must be paid to the culture conditions (Prockop *et al.*, 2010). It has been shown, by using genomic hybridization, karyotyping, subtelomeric fluorescent *in situ* hybridization and telomerase activity, that human bone marrow-derived MSC (BM-MS) do not undergo transformation in long-term culture under appropriate conditions and also do not express telomerase or exhibit telomere shortening (Bernardo *et al.*, 2007). Tumorigenicity of adult MSC has never been proven. Some studies demonstrated that MSC can undergo malignant transformation *in vivo*. However, investigators have now found that "malignant" MSC were contaminated with fibrosarcoma and/or osteosarcoma-derived cells (Klopp *et al.*, 2011). In order to evaluate if retroviral transduction and subsequent antibiotic selection change the properties of MSC or select rapidly proliferating subpopulations, we compared naive MSC with genetically engineered populations. We did not observe a general increase in MSC proliferation or chemosensitivity which could be attributed to retroviral transduction itself. Increased proliferation of CD::UPRT-MS (MSC expressing yeast cytosine deaminase fused with uracil phosphoribosyl transferase) was caused by the expression of enzymes involved in nucleotide metabolism. This proliferation advantage was lost in high-passage cultures. No substantial difference was observed in number of senescent cells between untransduced (naive) and retrovirally transduced cells (Kucerova *et al.*, 2012). It

appears that murine MSC are more susceptible to chromosomal aberration under *in vitro* cultivation than human cells (Tolar *et al.*, 2007).

MSC were shown to efficiently home to sites of injury and actively participate in tissue repair by secreting cytokines and growth factors that can restore tissue homeostasis, by reducing local inflammation and by differentiating in one or more cell types resident in the injured tissue (Spees *et al.*, 2003; Keating, 2006). There has been done a great deal of work, some of which is quite promising and can be therapeutically useful in repair of skeletal tissues, myocardia, CNS or spinal injury (Javazon *et al.*, 2004; Myers *et al.*, 2010). MSC are currently being investigated in clinical trials for a wide spectrum of indications, ranging from cell replacement to immunotherapy (Giordano *et al.*, 2007a; Ringden *et al.*, 2006).

Tumor-favoring potential of MSC is also a hot research topic. These cells are known to support tumor angiogenesis and metastasis formation, to create a microenvironment favorable for growth of cancer cells and to down-modulate the immune system capabilities in the host organism (Gallerisi *et al.*, 2010). Both protumorigenic and antitumorigenic effect of AT-MS has been demonstrated on malignant cell behavior dependent on the mutual interplay between stromal and malignant cells (Kucerova *et al.*, 2010, 2011a).

3. Retroviral vectors for gene therapy

Viruses have been selected as gene delivery vehicles because of their capability to deliver foreign genes efficiently, and sustain their expression. They have evolved mechanisms to enter the cells and use the cellular machinery to express their genes (Hu and Pathak, 2000). These are the major reasons why viral vectors derived from retroviruses, adenoviruses, adeno-associated viruses, herpesviruses and poxviruses are employed in many clinical gene therapy trials worldwide. Among these systems, retroviral vectors represent the prominent delivery system, since these vectors have high gene transfer efficiency and mediate high expression of genes. The advantages are also determined by their stable integration into the host genome, generation of viral titres sufficient for efficient gene transfer, infectivity of the recombinant viral particles for a broad variety of target cell types and ability to carry foreign genes of sizes <8 kb. These properties are essential prerequisite for persistence of the transgene in transduced cells and their progeny (Walther and Stein, 2000).

The retroviral vectors are predominantly derived from the moloney murine leukemia virus (MLV). The amphotropic virus is able to infect murine cells and variety of other cell species including human cells (Battini *et al.*, 1992).

Retroviruses were the first viruses to be modified for gene delivery (Anderson, 1996). An ideal vector must be efficient,

cell-specific, regulated, and safe (Hu and Pathak, 2000). The location of most *cis*-acting sequences in the terminal regions has enabled simple and effective retrovector design, making them the most widely used vector system (Rosenberg *et al.*, 2000). The host transcription machinery recognizes these *cis*-acting elements and thus mediates expression of the viral genes (Chang and Yee, 2012).

For most gene therapy applications, it is not desirable to deliver a replication-competent virus into a patient because of possible spreading beyond the target tissue and possibility of adverse effects. Therefore, the viral components are divided into a vector and a helper construct to limit the ability of the virus to replicate freely (Miller, 1997). It is necessary to separate genes encoding for structural and enzymatic proteins (Gag/Pol), as well as the genes encoding envelope proteins (Env) from the retroviral genome, which can be achieved by the so-called split packaging design. The retroviral vector contains the packaging signal (Ψ), the primer binding site (PBS), the long terminal repeats (LTR), and harbors the transgene of interest (Maetzig *et al.*, 2011) (Fig. 1). Helper constructs are designed to express viral genes that are missing in the vectors and to support replication of the vectors. The helper function is most often provided in a helper cell format. When the viral particles infect target cells, the vector RNA is reverse transcribed to form a double-stranded DNA copy, which integrates into the host genome and forms a provirus encoding the gene(s) of interest. It is further expressed by the host cell machinery. However, because the vector does not express any viral proteins, it cannot produce infectious viral particles which could spread to other cells (Hu and Pathak, 2000). The removal of the structural genes does not interfere with the capability of the viral RNA to be packaged into infectious retroviral particles (Walther and Stein, 2000). Furthermore, the removal of the structural genes from the viral vector and of the packaging signal from the packaging

vector constructs gives the basis for safe and efficient production of recombinant virus particles for infection of the target cells (Markovitz *et al.*, 1988). Introduction of the transgene containing construct into a packaging cell line via transfection allows the establishment of stable producer cell lines for viral vector production. Since MLV infection does not cause any obvious pathogenic effect on cells, these producer cell lines continue to proliferate and secrete infectious virions into the culture media (Sheridan *et al.*, 2000).

The useful property of retroviral vectors is their ability to integrate efficiently into the chromatin of target cells. Although integration does not guarantee stable expression of the transduced gene, it is an effective way to maintain the genetic information in a proliferating or self-renewing tissue (Roe *et al.*, 1993). Gammaretroviral vectors are dependent on degeneration of the nuclear membrane during cell division to allow the virus to enter the nucleus. Lentiviral vectors actively enter the nucleus via the nuclear pore, thus enabling them to transduce non-dividing cells. Despite many years of investigation, precise mechanisms of target site selection and the interplay of viral integrase and host cell proteins are still unknown. Gammaretroviral vectors show a strong preference for integration close to transcription start sites (TSS) and CpG islands, which are enriched in gene regulatory elements. On the other hand, lentiviral vectors prefer integration inside of the transcription units of actively transcribed genes (Gabriol *et al.*, 2012).

The relative concentration of vectors is evaluated as a titer expressed as the concentration of viral particles and/or the number of virions that are capable of transduction. The producing particles usually represent only a small percentage of total particles (Kay *et al.*, 2001). The assay for vector titer and selection of transduced cells depend on the marker in the vector. The most popular used include the gene encoding fluorescence proteins like GFP or antibiotics selectable mark-

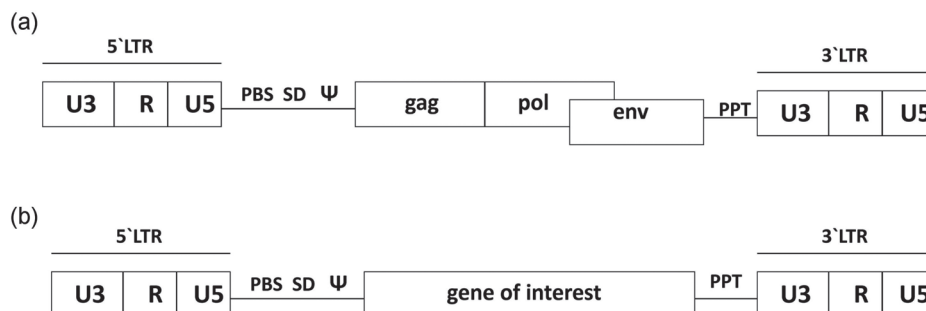


Fig. 1

Comparison of the genome structure of integrated retrovirus and retroviral vector

(a) Diagram of MLV provirus; (b) Diagram of integrated MLV-derived retroviral vector; LTR = long terminal repeat; U3 = unique sequence derived from 3' end of the viral RNA; R = repeated sequence; U5 = unique sequence derived from 5' end of the viral RNA; PBS = primer binding site; SD splice donor; Ψ = packaging signal; gag = genes for structural proteins; pol = region coding for genes needed for replication of retrovirus; env = genes for envelope proteins; PPT = polypurine tract (according to Maetzig *et al.*, 2011, adjusted).

ers like neo^R gene. Antibiotics selection takes approximately 2 weeks with repeated medium changes. This selection can detect even a few transduced cells in a population of 10⁶ untransduced cells (Chang and Yee, 2012).

Therapeutic viruses based on retroviral vectors generated by packaging cell lines are often used in different gene therapy approaches. Low transduction efficiency and low titer represent the main disadvantages for their usage. This issue can be solved by generation of highly retrovirus-producing cells. So-called “ping-pong amplification” between ecotropic and amphotropic packaging cell lines (with different host range) was found to be effective for an increase of virus production (Hlavaty *et al.*, 1999).

In self-inactivating vectors (SIN), the promoter/enhancer sequences within the U3 region of the viral 3′-LTR are deleted or mutated to prevent insertional activation of genes in the host genome for the safety of retroviral vector. After viral reverse transcription, both the 3′-LTR and 5′-LTR are transcriptionally inactive, without any interference with expression of the internally promoted therapeutic genes (Yu *et al.*, 1986).

To increase *in vivo* transduction efficiency, a new generation of replication-competent or conditionally replicative viral vectors have been successfully analyzed for their natural or genetically enhanced oncolytic potential (Wollmann *et al.*, 2005). Replication competent retroviral vectors (RCRV) were created to deliver the therapeutic gene to almost all tumor cells (Dalba *et al.*, 2007; Tai and Kasahara, 2008). RCRV based on MLV exhibit unique characteristics thanks to MLV intrinsic tumor selectivity due to its inability to infect quiescent cells. It can also achieve highly selective and stable gene transfer throughout entire solid tumors *in vivo*. RCRV with suicide gene mediate synchronized cell killing after prodrug administration, and due to their ability to undergo stable integration, residual cancer cells serve as a reservoir for long-term viral persistence even when they migrate to new sites. This enables multiple cycles of prodrug administration to achieve improved treatment efficacy (Tai and Kasahara, 2008). While neutralizing antibody responses to MLV do occur, it has been shown that it causes only minor inflammation, with little effect on viral titer, and no associated pathology (Ram *et al.*, 1993; Hein *et al.*, 1995). Very promising results using RCRV were achieved by Hlavaty *et al.* (2011) by injection of the vector into the orthotopic mouse tumor xenografts revealing substantial infection and virus spread. Significant bystander effect was observed in all human glioma cell lines tested.

4. Genetically engineered MSC for gene directed enzyme prodrug therapy

Many (pre)clinical studies targeting of solid tumors with anti-cancer gene therapy has been hindered by systemic

toxicity, low efficiency of delivery and transient expression of transgene. Cell-based targeting using MSC has been utilized to overcome these issues. The tumor-specific migratory ability makes MSC possible for delivery of toxic substances to the main tumor mass and its surrounding niche, as well as to invasive parts, without adverse effects on normal tissue (Bexell *et al.*, 2010; Myers *et al.*, 2010). Tumor-homing capability of MSC was demonstrated on many models. They are able to migrate to primary or metastatic tumors derived from glioma, breast, colon, ovarian, lung or prostatic carcinoma or melanoma upon systemic administration (Kucerova *et al.*, 2007, 2008; Cavarretta *et al.*, 2010; Reagan and Kaplan, 2011).

One of the cancer gene therapy approaches involves delivery and expression of prodrug-converting genes (suicide genes), which encode enzymes not toxic *per se*, but catalyzing the formation of highly toxic metabolites following the application of a much less toxic prodrug. The use of suicide gene/prodrug combinations is known as gene directed enzyme prodrug therapy (GDEPT). GDEPT describes a process whereby the expression of exogenous genetic information generates a product which increases the toxicity of an administered prodrug. There is a set of properties that the “perfect” suicide gene/prodrug combination should exhibit, but ideal combination of properties still does not exist. The suicide gene/gene product should be either absent in the human genome, or expressed at low levels, and should not be toxic; it should exhibit high catalytic activity upon expression in tumors; be sufficient for full activation of the prodrug without the necessity for further catalysis by endogenous enzymes; and it should be small – allowing the use of expression vectors. The prodrug should have high affinity for the enzyme encoded by the suicide gene; should be able to penetrate into a solid tumor and be efficiently taken up by tumor cells; have a half-life long enough to maximize the bystander effect within the tumor; exhibit no toxicity prior to activation; the toxic form(s) of the drug should be able to diffuse intercellularly – to allow killing of tumor cells via the bystander effect; the toxicity of the activated prodrug should be cell cycle-phase independent (Portsmouth *et al.*, 2007).

Therapeutic genes that have been inserted into stem cells and delivered to tumors with high selectivity include prodrug activating enzymes (cytosine deaminase, thymidine kinase, carboxyl esterase); interleukins (IL-2, IL-4, IL-12, IL-23); interferon β; apoptosis-promoting genes (tumor necrosis factor-related apoptosis-inducing ligand, TRAIL); metalloproteinases (PEX); chemokine (CX3CL1) or antagonist of hepatocyte growth factor (NK4) (Aboody *et al.*, 2008; Menon *et al.*, 2009).

Cancer gene therapy using suicide genes is limited by the impossibility to introduce the gene into all cells of the tumor mass. Therefore, the presence of so-called bystander effect provides an advantage in this type of treatment. Therapeu-

tic MSC release toxic metabolites that affect surrounding tumor cells (Hlavaty *et al.*, 1997) (Fig. 2). Numerous studies performed with suicide gene/prodrug combinations have demonstrated that complete tumor eradication is possible even when the suicide gene is expressed by less than 10% of cells (Cihova *et al.*, 2011; Kucerova *et al.*, 2013). Bystander effect can either be exerted by the free/facilitated diffusion of toxic metabolites or thanks to intercellular communication via gap junctions. It can be further improved by the immune system, whereby the release of foreign and/or tumor antigens from dying cells stimulate the immune system to eliminate tumor cells which do not express the suicide gene (Portsmouth *et al.*, 2007).

In order to address the safety of retrovirally transduced MSC many studies have been performed. Piccoli *et al.* (2008) demonstrated increased reactive oxygen species production in engineered BM-MSC due to regularly used selection markers. Expression of neomycin or puromycin resistance genes resulted in oxidative stress. As stated above, particular transgene can give a proliferative advantage but it does not preclude the entering of cells to senescence, and has no impact on safety of cancer gene therapy mediated by mesenchymal stromal cells (Kucerova *et al.*, 2012).

5. Herpes simplex virus thymidine kinase/ganciclovir approach

One of the most frequently studied therapeutic strategies is based on transfection with the Herpes simplex virus thymidine kinase gene (HSVtk) followed by ganciclovir (GCV) administration (Dillen *et al.*, 2002). One of about 70 proteins encoded by HSV-1, the 376 amino acid containing protein product of the HSVtk gene is needed by the virus for reactivation from ganglionic neurons during the latent stage of its life-cycle, since these cells express very low levels of endogenous thymidine kinase. The enzyme initiates the phosphorylation of deoxythymidine to deoxythymidine triphosphate for incorporation into nascent DNA. Expression of HSVtk to activate the acyclovir-derived prodrug 9-([2-hydroxy-1-(hydroxymethyl)ethoxy]methyl) guanine – ganciclovir was used for the first proof-of-principle of suicide gene therapy by Moolten in 1986. HSVtk is 1000-fold more efficient in monophosphorylating ganciclovir in comparison to mammalian thymidine kinases (Portsmouth *et al.*, 2007). The final product of consequent phosphorylation by cellular kinases, GCV-triphosphate (GCV-TP), competitively inhibits incorporation of the endogenous

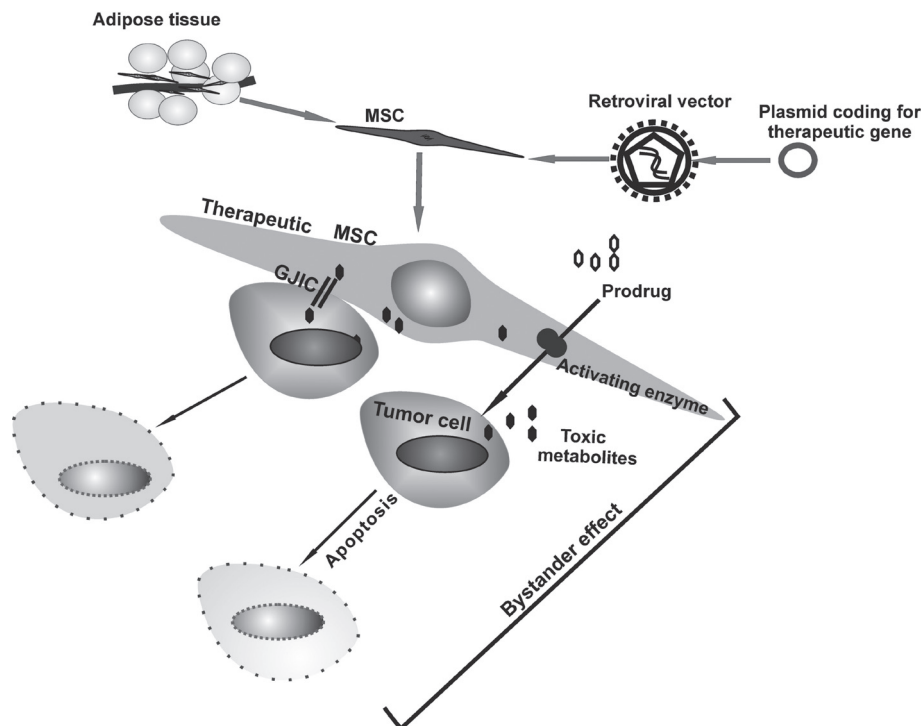


Fig. 2

GDEPT mediated by AT-MSC is based on bystander effect

AT-MSC are expanded and retrovirally transduced by prodrug-converting genes in order to express enzymes converting nontoxic prodrug to toxic metabolites. Engineered MSC release toxic metabolites by diffusion or via gap junctions. Uptake of toxic compounds by surrounding tumor cells induces apoptosis.

DNA precursor deoxyguanosine triphosphate (dGTP) into DNA. The GCV-terminated strands of DNA are poor substrates for chain elongation, therefore the elongation of DNA is canceled and leads to cell death. Thus, replicating DNA is considered to be the major target in cancer cells for GCV activated by HSVtk (Mesnil and Yamasaki, 2000). It has also been indicated that GCV induced a morphological change in cells due to the reorganization of the cytoskeleton as well as an accumulation of cells in G2/M phase of cell cycle after 48–72 hr incubation (Halloran and Fenton, 1998). It has also been reported that treatment resulted in a Bcl-2 levels decline and activation of caspases, leading to apoptosis (Tomicic *et al.*, 2002). It has been demonstrated that GCV induced sister chromatid exchanges, chromosome breaks and translocations, suggesting an involvement of the homologous recombination repair in responding to GCV-induced DNA damage (Thust *et al.*, 2000a,b). It was suggested that phosphorylated GCV in the template blocked successful repair by homologous recombination, leading to cell death. These findings indicate that combining HSVtk/GCV with approaches that compromise homologous recombination repair could produce synergistic antitumor effects (Ladd *et al.*, 2011).

The cytotoxic GCV-TP is charged and therefore insoluble in lipid membranes, it cannot diffuse into neighboring cells and exert its toxic effects. However, a bystander effect is observed *in vitro* and *in vivo* when using this system. This is due to transfer of GCV-TP across gap junctions (GJs) which are formed between opposing plasma membranes. Gap junctions are composed of connexons (two juxtaposed transmembrane hemichannels consisting of six connexin (Cx) protein subunits). These intercellular channels with a central pore permit the transfer of molecules with less than Mr 1000 directly from one cell to another (Dillen *et al.*, 2002; Mesnil and Yamasaki, 2000).

It has been proposed that the bystander effect can be facilitated by apoptotic vesicles containing GCV-TP released from dying cells, which are phagocytosed by non-HSVtk-expressing cells (Freeman *et al.*, 1993). It was also hypothesized that gap junctional intercellular communication (GJIC) can protect the transduced cell by lowering its concentration of cytotoxic metabolites. This protection could permit the transduced “factory” to live longer, which could then kill more bystander cells, and has been called “Good Samaritan” effect (Wygoda *et al.*, 1997).

It was evaluated whether human AT-MSC could be exploited as cellular vehicles for HSVtk/GCV enzyme/prodrug combination to mediate cytotoxic effect on human tumor cells. Adipose-tissue derived human mesenchymal stromal cells were shown to form gap junctions with glioblastoma cells, thus rendering them suitable vehicle for the HSVtk/GCV therapy relying on transport of polar metabolites (Matuskova *et al.*, 2010).

6. Cytosine deaminase/5-fluorocytosine approach

The second extensively studied prodrug/converting gene system represents cytosine deaminase (CD) and 5-fluorocytosine (5-FC). This enzyme is present in fungi and bacteria but is absent in mammalian cells. It deaminates cytosine to uracil, and can also deaminate the nontoxic prodrug 5-fluorocytosine to its highly toxic derivative 5-fluorouracil (5-FU). This metabolite is then converted by cellular enzymes into 5-FUTP (5-fluorouridine triphosphate) and 5-FdUMP (5-fluorodeoxyuridine monophosphate). 5-FUTP can be incorporated into RNA in place of UTP, resulting in the inhibition of the nuclear processing of the ribosomal and messenger RNA. 5-FdUMP irreversibly inhibits thymidylate synthase, a key enzyme in the *de novo* synthesis of dTMP, a precursor for DNA synthesis (Altaner, 2008; Scartozzi *et al.*, 2011). 5-FU is a chemotherapeutic agent widely used for the treatment of several solid tumors. Many years after its first introduction, it still represents the backbone of many chemotherapy combinations (Scartozzi *et al.*, 2011). However, certain cancer cells may become relatively resistant to 5-FU because of its poorly efficient conversion to toxic metabolites (Harris *et al.*, 1994). *Escherichia coli* and *Saccharomyces cerevisiae* encode genes *upp* and *FUR1*. Their product is an enzyme called UPRT (uracil phosphoribosyl transferase) that can actively convert uracil to uridine monophosphate (UMP). Transfer of these genes to cells in GDEPT should induce a more efficient conversion of 5-FU to 5-fluorouridine monophosphate (5-FUMP), thus restoring the sensitivity of cells to 5-FU (Erbs *et al.*, 2000).

As in contrast to GCV-P derivatives, 5-FC and 5-FU can penetrate tumor cells by diffusion and exert the local toxic effect on neighboring cells, regardless of the presence of GJIC, which means that direct cell-to-cell contact is not necessary (Altaner, 2008). 5-FC is able to cross the blood-brain barrier, making this enzyme/prodrug approach well suited for the treatment of CNS tumors (Aboody *et al.*, 2008; Altaner and Altanero, 2012; Cihova *et al.*, 2011).

Yeast cytosine deaminase was shown to produce a 15-fold higher amount of 5-FU compared to bacterial CD (Kievit *et al.*, 2000). The improvement in the efficiency of prodrug conversion was achieved by construction of bifunctional yeast fusion gene cytosine deaminase::uracil phosphoribosyl transferase (CD::UPRT). The gene product of a chimeric protein catalyzes the direct conversion of 5-FC into 5-FUMP and its enzymatic activity is increased at least 100-fold over native yeast CD. CD::UPRT as a prodrug-converting enzyme was reported to exhibit improved 5-FC conversion efficiency and higher bystander effect *in vitro* and *in vivo* (Graepler *et al.*, 2005; Altaner, 2008). This therapeutic regimen has proven efficacious in the treatment of experimental human colon carcinoma (Kucerova *et al.*, 2007), prostate tumor (Cavarretta *et al.*, 2010), melanoma (Kucerova *et al.*, 2008) and

medullary thyroid carcinoma (Kucerova *et al.*, 2011b). Many studies were performed on glioblastoma model (Altaner and Altanerova, 2012). Coadministration of rat glioblastoma and therapeutic mesenchymal stem cells with delayed 5-FC therapy improved the survival in a therapeutic stem cell dose-dependent manner, and induced tumor regression in a significant number of animals (Altanerova *et al.*, 2012). CD::UPRT-MSC were also capable of mediating increased cytotoxic effect of 5-FU on EGFP-MDA-MB-231 breast cancer cells. The bystander cytotoxicity of CD::UPRT/5-FU treatment was much more efficient when compared to treatment with 5-FU alone or CD::UPRT/5-FC (Kucerova *et al.*, 2012). We have demonstrated that particular tumor cell lines differ in response to CD::UPRT-MSC/5-FC system. Pharmacological inhibition of enzymes involved in the metabolism of 5-FC and 5-FU led to decreased sensitivity to treatment. Molecular inhibition of ABCB1 (MDR1) by RNA interference abrogated resistance to CD::UPRT-MSC/5-FC treatment caused by efflux of 5-FC metabolites (Matuskova *et al.*, 2012). Cihova *et al.* (2011) found that the high efficacy of CD::UPRT-MSC is caused by paracrine induction of proapoptotic genes in tumor cells. Significant increase of products of proapoptotic genes Bad, Bax, TRAIL R1/DR4, and FADD was detected in colorectal carcinoma cells. On the other hand, the amount of products of antiapoptotic genes Bcl-2 and Bcl-X was decreased.

7. Purine nucleoside phosphorylase/fludarabine approach

Purine nucleoside phosphorylase (PNP) catalyzes the cleavage of the glycosidic bond of purine nucleosides to generate ribose 1-phosphate and a free purine base (Zhang *et al.*, 2005). Although this reversible phosphorolysis of (2'-deoxy) purine ribonucleosides is a shared property of prokaryotic and eukaryotic PNP, the mammalian enzymes differ in sequence, structure, and function from their bacterial counterparts. An important difference between *E. coli* and mammalian enzyme is the ability of *E. coli* PNP to cleave adenosine analogs, thus its expression in cancer cells mediates the glycosidic cleavage of nucleoside prodrugs to highly toxic adenine analogs. This enzyme is being evaluated by numerous laboratories as a suicide gene therapy strategy for the treatment of solid tumors. The compounds based on three 2'-deoxyadenosine analogues: 6-methylpurine-2'-deoxyriboside (MePdR), 2'-Deoxy-2'-fluoroadenosine (2'-F-dAdo), and 9-(β -D-arabinofuranosyl)-2-fluoroadenine (F-araA) with excellent *in vitro* and *in vivo* activity diffuse across cell membranes and effectively kill transduced cells as well as the adjacent tumor cells that do not express *E. coli* PNP. Gap junctions or cell-to-cell contacts are not required to transport analogues across cell membranes. Excellent by-

stander activity has also been demonstrated *in vivo* (Zhang *et al.*, 2005; Hong *et al.*, 2004). According to Hong *et al.* (2004), this therapeutic approach is capable of destroying the quiescent (non-cycling) compartment of tumor cells *in vivo*. Mice given PNP-GDEPT showed a significant reduction both in prostate volume and in lung colony counts. Apoptosis was increased two-fold in treated prostates compared to controls (Martiniello-Wilks *et al.*, 2004). Thus preclinical studies using animal models have demonstrated that PNP anticancer gene therapy is a promising approach for the treatment of solid tumors.

8. Conclusions

Mesenchymal stromal cells retrovirally transduced to express therapeutic genes thanks to their ability to preferentially migrate toward tumor cells seem to be attractive candidates for clinical applications of stem cell-mediated cancer gene therapy. These engineered MSC have the "suicide" concept included within, therefore are safe for the therapeutic use regarding their indicated tumor-favoring properties.

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