

# Virotherapy as An Approach Against Cancer Stem Cells

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**Abstract:** It has been hypothesized that cancers originate from a small population of cells with stem cell-like characteristics, including self-renewal and pluripotency. Such tumor-initiating cells, also referred to as cancer stem cells, are thought to account for relapses following seemingly successful treatments, because their slow turnover and capacity for expelling anti-tumor drugs leaves them untouched by conventional treatment regimens. Targeting of cancer stem cells might be key for improving survival and producing cures in patients with metastatic tumors. Viruses enter cells through infection and might therefore not be sensitive to stem cell resistance mechanisms. During the last decades, oncolytic adenoviruses have been shown to effectively kill cancer cells, by seizing control of their DNA replication machinery and utilizing it for the production of new virions, ultimately resulting in the rupture of the cell. Human safety data in cancer trials has been excellent even when the dose of administered adenovirus has been high. Future approaches include additional modifications of the adenoviral genome that prime them to attack cancer stem cells specifically, utilizing lineage-specific cell surface markers, dysfunctional stem cell signaling pathways or up-regulated oncogenic genes. However, already existing oncolytic adenoviruses have displayed potential to efficiently kill not only differentiated cancer cells, but also tumor-initiating stem cells. Here, we review the current literature that supports the existence of cancer stem cells and discuss the potential of virotherapy for killing tumor-initiating cells.

**Keywords:** Cancer stem cells, tumor-initiating cells, oncolytic adenovirus, virotherapy.

## INTRODUCTION

Every year approximately 10 million people worldwide are diagnosed with cancer and by the year 2020 the number is expected to rise to 15 million. If a cancer is detected at an early stage, conventional modes of treatment, i.e. surgery, chemotherapy, ionizing radiation, hormonal therapies and monoclonal antibodies, can often completely eradicate the tumor and cure the patient. In contrast, with few exceptions, almost no patients with solid tumors metastatic to distant organs can be completely cured. Even in completely responding patients, relapse eventually occurs.

Tumors arise from accumulated genetic and epigenetic cellular alterations, which render the cell unresponsive to signals that normally regulate growth and apoptosis. As a result, the cell proliferates in an unregulated manner without respecting normal tissue compartments, ultimately leading to tumor growth. Although much is known about the mechanisms and mutations that give rise to a cancerous cell, the identity of tumor-initiating cells continues to be a matter of controversy. A growing body of evidence suggests that the instigator of the tumor is a cancer cell with stem cell-like characteristics. However, this is not the only theory set forth as an explanation for some of the puzzling features of the refractory nature of advanced tumors. For example, mutator phenotypes and epidermal to mesenchymal transition have also been proposed [1, 2]. Of note, these three theories may not be mutually exclusive.

The tumor-initiating cells, or cancer stem cells, are believed to escape conventional therapeutic strategies and thus be the cause of refractory cell clones. Radiation therapy and many of the various chemotherapeutic agents on the market today target cells based on how rapidly they replicate. Hormonal therapies and small molecular inhibitors are mostly cytostatic, i.e. they stop tumor cells from cycling. Treatment modalities like these are however ineffective towards putative cancer stem cells, which due to their quiescent stem cell-like nature will be unaffected by agents that target dividing cells [3, 4]. In addition, cancer stem cells are expected to expel anti-tumor drugs efficiently, due to a high-level expression of drug-transporter proteins [5]. These findings suggest that novel strategies, targeting cancer stem cells, are needed in order to reduce the risk for recurrence of neoplasms. In our opinion, oncolytic virotherapy constitutes a promising approach to target and kill tumor-initiating cells. If effective, the strategy might reduce risk of tumor recurrence and/or development of metastasis, while leaving normal somatic stem cells unharmed. Below, we will summarize the latest findings in favor of the cancer stem cell hypothesis and discuss the possibility of using oncolytic adenoviruses for the killing of tumor-initiating cells.

## STEM CELLS AND CANCER

### Stem Cells in Normal Tissue Homeostasis

Stem cells, by definition, possess the ability to perpetuate themselves (self-renewal) and the ability to generate any mature cell type of a particular tissue (unlimited potency). These unique traits are maintained by symmetric and asymmetric cell division. Asymmetric cell division yields one daughter cell with self-renewal characteristics and another

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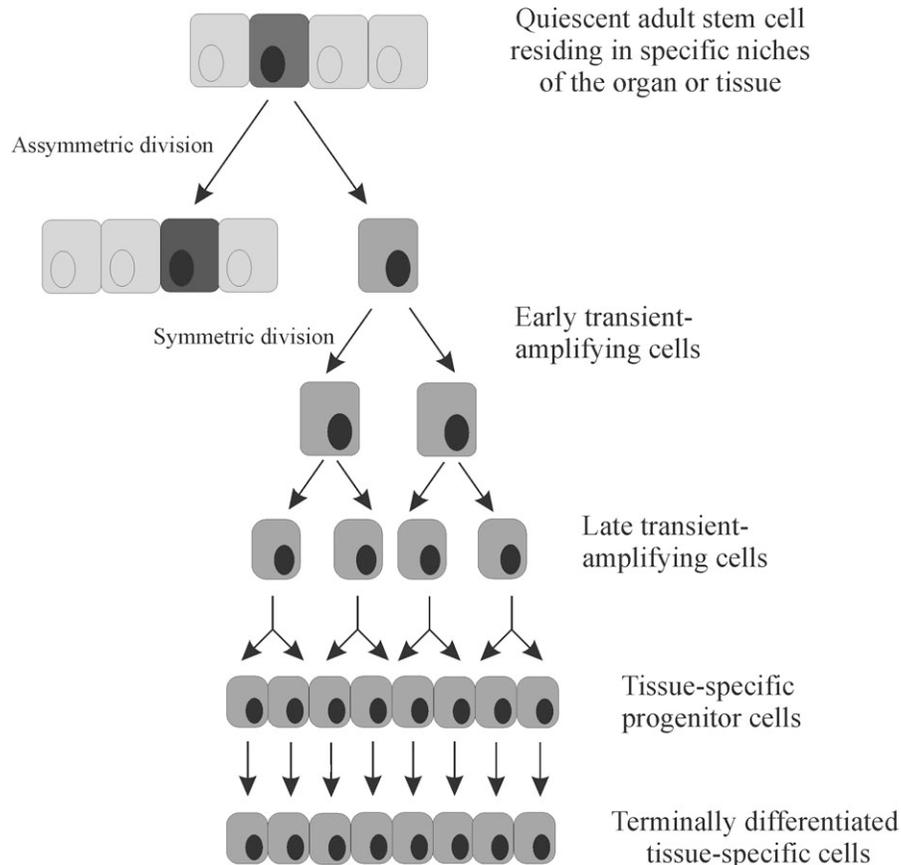
destined to differentiate. Asymmetric cell division leaves the number of stem cell intact and therefore does not account for the expansion of stem cell populations seen during development or in response to injury [6]. The expansion of the stem cell pool is achieved by symmetric cell division, in which the two daughter cells share identical characteristics, which may include self-renewal and multipotency.

The ability of normal, adult tissue to regenerate after trauma or disease, or to replace worn out cells is ensured by the presence of a small number of tissue-specific stem cells. Usually, adult stem cells remain in a dormant state [7], secluded into specific compartments of organs or tissues [8]. However, upon activation, stem cells can undergo asymmetric cell division, giving rise to one identical, undifferentiated daughter cell and one transient-amplifying (TA) progenitor cell (Fig. 1). What follows are multiple, symmetric cell divisions through which the TA progenitor cell generates tissue-specific progenitor cells and finally, the bulk of terminally differentiated cells with characteristics appropriate to the specific organ or tissue. The risky endeavor of multiple cell replications, each of which can result in genetic or epigenetic errors, is hence delegated to the expendable TA progenitor cells, which appear to have limited life-spans.

The TA progenitor cells are repeatedly replaced by new TA cells by asymmetric stem cell division. The actual stem cells are in this way largely protected from mutational dam-

age that may occur during cell division, which ensures their long-time survival. The stem cells lie imbedded in so-called stem cell niches surrounded by mature cells [8], which constitute a unique milieu that maintains stem cell homeostasis and regulates cell differentiation [9-11]. The importance of the microenvironment on stem cell differentiation can be observed when normal germinal cells from the gonadal ridges of a mouse embryo are transplanted into the unfamiliar environment of an adult mice testis. When faced with the foreign cellular setting, the germinal cells differentiate and form multicellular tumor masses known as teratocarcinoma [12]. Interestingly, injection of cultured teratocarcinoma cells back into their normal milieu, e.g. the inner mass of the blastocyst, leads to loss of their malignant characteristics and re-expression of normal phenotype [13].

Signaling pathways that are important during embryonic development have also been shown to play a key role in stem cell renewal, tissue repair and regeneration. These include pathways such as Wnt, Sonic hedgehog (Shh), Notch, Octamer-4 (Oct-4), bone morphogenetic protein (BMP) and Janus family kinase (JFK), which have been shown to tightly regulate the self-renewal process in normal stem cells [14-16]. The same signaling pathways appear to be the subject of dysfunctional activation in many cancer cells. An increased activity of Oct-4, Wnt and Shh is for instance noticed in breast cancer [17, 18]. Moreover, it has been speculated that



**Fig. (1).** Adult stem cells lie dormant in specific niches of the organ or tissue. Upon activation, asymmetric cell division can produce one identical stem cell and one early transient-amplifying cell, which can undergo symmetric cell divisions generating more early transient-amplifying cells, followed by late transient-amplifying cells. Additional symmetric cell divisions can generate tissue-specific progenitor cells and finally the terminally differentiated cells of the specific organ or tissue.

members of the Wnt family associated with the Wnt/ $\beta$ -catenin pathway are expressed in potential breast cancer stem cells and that up-regulation of the proto-oncogene Her-2/neu, member of the epidermal growth factor receptor family, is critically involved in breast cancer metastasis [19].

### The Cancer Stem Cell Hypothesis

The notion that tumors are derived from “embryonal cell rests”, i.e. cells with stem cell-like characteristics, was uttered for the first time in the second part of the 19<sup>th</sup> century by Julius Cohnheim [reviewed in 20]. However, the theory sunk into oblivion for many years, since a set of conflicting, and at that time, highly convincing papers were published proposing that cancer originates from differentiated epithelial structures [reviewed in 20]. The original idea by Cohnheim gained new interest some 40 years ago, when Till and McCulloch suggested that tissue-specific stem cells can induce cancer [21]. Shortly thereafter, it was proposed by Pierce that cancer is brought about by a maturation arrest of stem cells [22]. With advances made in stem cell biology, more and more evidence has accumulated, suggesting that tumors can originate from tissue-specific stem cells or their immediate progeny, which have lost their normal tight regulation of the self-renewal process. Alternatively, progenitor cells might re-acquire self-renewal capabilities through dysfunctional signaling pathways. It has been suggested that chronic myelogenous leukemia (CML) blast crisis, a feared escalation of disease progression, may be caused by hematopoietic progenitor cells that attained self-renewal ability by alterations in the Wnt signaling pathway [23].

A tumor consists of a heterogeneous mixture of cells at varying stages of differentiation. Cells with stem cell-like properties, including self-renewal and differentiation ability, usually constitute only a minor fraction of the whole tumor cell population. In 1997, Dick and co-workers reported that a small population of leukemic cells, recognized by CD34<sup>+</sup>CD38<sup>-</sup> cell surface markers, could transfer human leukemia into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, while thousands of other leukemic cells isolated from the same patient, but with different cell surface markers, could not [24]. The cancer induced by the CD34<sup>+</sup>CD38<sup>-</sup> cells displayed the same histological phenotype as the original leukemia from which the specific cancer cells were isolated, suggesting stem cell properties [24].

The first evidence that tumor-initiating cells may be present in solid tumors came when Al-Hajj *et al.* isolated a CD44<sup>+</sup>CD24<sup>-/low</sup> cell population from breast cancer patients. Interestingly, these cells also displayed a number of stem cell characteristics including extensive proliferative potential and the ability to generate diverse cell types with limited developmental and proliferative capacity [25]. A mere two-hundred CD44<sup>+</sup>CD24<sup>-/low</sup> cells, isolated from malignant breast tumors, were able to induce new tumors when implanted into immunodeficient NOD/SCID mice [25]. In contrast, 20 000 cells isolated from the same tumor, yet displaying differing cell surface markers were not tumorigenic [25]. In true stem cell manner, tumor-inducing CD44<sup>+</sup>CD24<sup>-/low</sup> cells constituted a maximal 10 % of the total cell population and grew *in vitro* as nonadherent spherical cell clusters simi-

lar to the so-called mammospheres found in normal mammary stem/progenitor cell cultures [25, 26].

During the following year putative tumor-initiating cells from human brain tumors were discovered [27]. Glioma stem cells exhibit characteristic neural stem cell markers CD133 and nestin, and grow *in vitro* as neurospheres similarly to normal neural stem cells [27, 28]. Subsequently, the presence of clonogenic, tumor-inducing cancer cells has been reported in several human tissues and organs. The characteristics and tumorigenicity of the putative cancer stem cells are summarized in (Table 1). However, it should be noted that the populations that have been proposed as tumor-initiating, probably contain also progenitors and TA progenitors. Further work is required to define characteristics of the actual stem cells.

Interestingly, many cell surface markers appear to be shared between putative stem cells from different tissues. For instance, CD44 is expressed on the surface of tumor-initiating cells from breast, bone, head and neck, colorectal, pancreatic and prostate cancer, whereas CD133 is expressed on the surface of cancer stem cells isolated from brain, colorectal and liver tumors. In addition, it is also likely that many cancer stem cells display improper function of one or several of the major signaling pathways Wnt, Hedgehog and Notch. Further, our data (see below) suggests that the p16/Rb pathway may also be dysfunctional in putative cancer stem cells.

### STEM CELL RESISTANCE TO RADIATION AND CHEMOTHERAPY

Quiescent stem cells from both normal and cancerous tissue are strikingly resistant to radiation and chemotherapy [3, 29-31]. Putative brain cancer stem cells will for instance activate the DNA damage checkpoint in response to radiation and are able to repair radiation-induced DNA damage more efficiently than the gross population of brain cancer cells, making these cells less inclined to undergo apoptosis [32]. In addition, stem cells express high levels of anti-apoptotic proteins [33, 34], which help avoid induction of apoptosis by radiation or chemotherapy. Moreover, stem cells express high levels of multidrug transporter molecules that exclude cytotoxic drugs from the cell [35,36]. Consequently, lipophilic fluorescent marker dyes like Hoechst 33342 will also be excluded from putative stem cells [5, 36], which may facilitate their identification from a population of tumor cells. Also, since chemotherapeutic agents can enter most cell types, and radiation hits all cells in its path, it is important to note that the selectivity of both approaches are chiefly based on the replication status of cells. Stem cells cycle very slowly, and since oxygen radical mediated DNA damage caused by radiation affects mainly replicating cells, non-cycling cells sustain much less damage. The same is true for most chemotherapeutics, which integrate into replicating DNA or inhibit molecules required in DNA replication.

A tumor is, according to current belief, composed of a hierarchy of cells at different stages of differentiation, i.e. resting stem cells, proliferating transient-amplifying cells, differentiating cells and terminally differentiated cells, much like the cell populations of normal tissue. According to the cancer stem cell hypothesis, the differentiating and terminally differentiated cells may not actively contribute to malignant progression and development of resistance. Neverthe-

**Table 1. Cell Surface Markers Associated with Cancer Stem Cells. The Smallest Amount of Cells Reported Tumorigenic in Immunodeficient Mice is Denoted**

Cancer type	Cell Surface Markers	Tumorigenic cell number	References
Breast	CD44 <sup>+</sup> /CD24 <sup>-low</sup> /ESA <sup>+</sup>	200 cells	[25]
Brain (Glioma)	CD133 <sup>+</sup> /nestin <sup>+</sup>	100 cells	[27]
Bone (Sarcoma)	Stro-1 <sup>+</sup> /CD105 <sup>+</sup> /CD44 <sup>+</sup>	ND	[83]
Colorectal	a) CD133 <sup>+</sup>	1000 cells	[84]
	b) EpCAM <sup>high</sup> /CD44 <sup>+</sup> /CD166 <sup>+</sup>	200-500 cells	[85]
Head and neck	CD44 <sup>+</sup> /BMI-1 <sup>+</sup>	5000 cells	[86]
Leukemia	CD34 <sup>+</sup> /CD38 <sup>-</sup>	ND	[24]
Liver	CD133 <sup>+</sup>	1000 cells	[87]
Lung	Sca-1 <sup>+</sup> /CD45 <sup>-</sup> /PECAM <sup>-</sup> /CD34 <sup>+</sup>	ND	[88]
Melanoma	CD20 <sup>+</sup>	2*10 <sup>5</sup> cells	[89]
Myeloma	CD138 <sup>-</sup>	1-10*10 <sup>6</sup> cells <sup>#</sup>	[90]
Ovarian	CD31 <sup>-</sup> /CD45 <sup>-</sup>	5*10 <sup>5</sup> cells	[91]
Pancreatic	CD44 <sup>+</sup> /CD24 <sup>+</sup> /ESA <sup>+</sup>	100 cells	[92]
Prostate	CD133 <sup>+</sup> /CD44 <sup>+</sup> /α <sub>2</sub> β <sub>1</sub> <sup>hi</sup> /Sca-1 <sup>+</sup>	500 cells	[93,94]

**Abbreviations:** CD; cluster of differentiation, ESA; epithelial-specific antigen, also known as EpCAM, epithelial cell adhesion molecule; Stro-1, an antigen present on fibroblast colony-forming unit cells; PECAM; platelet endothelial cell adhesion molecule, also denoted CD31; BMI-1, an oncogene belonging to the Polycomb group family; Sca-1, stem cell antigen-1; ND, not determined

<sup>#</sup>The lowest amount of cells tested for tumor induction in immunodeficient mice.

less, all current therapeutical approaches target mainly these cell populations (see Fig. 2). Thus, there is an obvious need for novel strategies that target also tumor-inducing cancer stem cells. In the following section, we will discuss the potential of virotherapy in this regard.

## VIROTHERAPY FOR KILLING CANCER STEM CELLS

### Oncolytic Virotherapy in Cancer Treatment

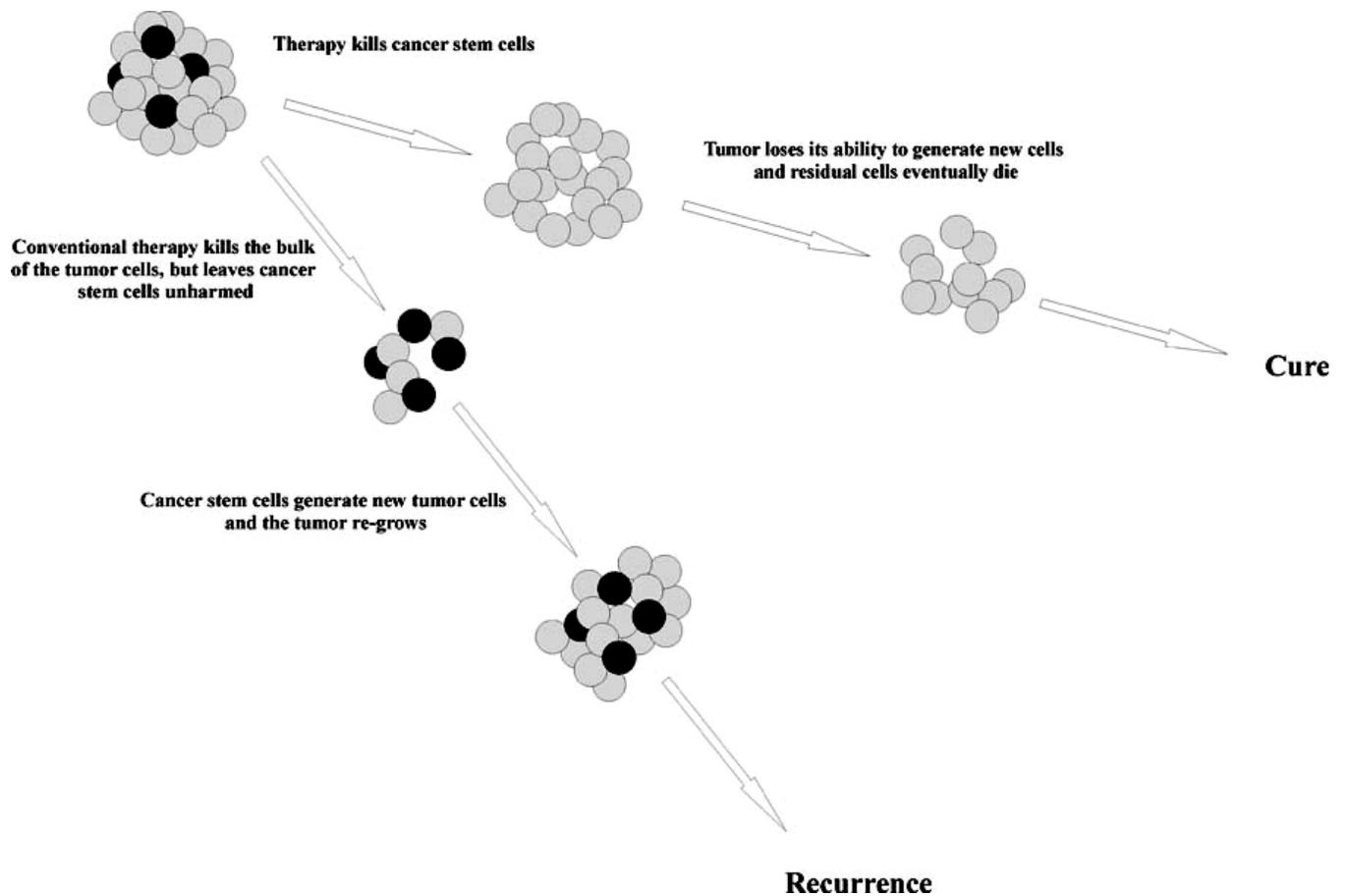
The potential of viruses for treating cancer has been known for more than a hundred years. The beginning of the 20<sup>th</sup> century brought with it the realization that tumors can regress in some cancer patients that contract viral infection. In the early part of the previous century, several trials were undertaken in which patients suffering from various types of cancer were treated by attenuated rabies virus, vaccinia virus and measles virus, to mention just a few [reviewed in 37]. Although many responses were seen, viruses were not understood very well and the nature of cancer even less. Progress in molecular biology and virology during the last decades has revealed stunning similarities in how viruses and carcinogenesis operate [38].

A virus particle consists of genetic material, which can be either DNA or RNA, protected by a protein coat called a capsid. Some viruses also feature lipid coating of the capsid. Viruses are incapable of replicating on their own. Instead, they infect suitable host cells and utilize their replication machinery for the production of new viral particles. Often,

the last step of replication is virus burst which results in cell death. These traits of viruses can be exploited by using them as oncolytic agents that target cancer cells. The pathways that are affected in the host cell upon viral infection are often identical to pathways mutated during tumor development. This allows removal of these properties from the virus, which reduces replication in normal but not tumor cells. For example, in tumor cells it is unnecessary to bind the retinoblastoma tumor suppressor/cell cycle regulator (Rb) protein for release of E2F, because the Rb/p16-pathway is aberrant in most if not all tumor cells [38] and abundant E2F is present. DNA viruses with strong oncolytic potential and reasonably well understood genomes, including adenovirus, herpes simplex and vaccinia virus, have been popular viruses for such genetic engineering. In addition, certain anti-viral measures of normal cells are lacking in tumor cells. For example, most tumors are defective in interferon/protein kinase R (PKR) signaling, because of the anti-tumoral effects of interferon. Lack of interferon, however, also renders tumor cells more susceptible to viruses. This may be one explanation which underlies the natural tumor selectivity of some human and non-human viruses including measles, vesicular stomatitis and Newcastle disease virus.

### Virotherapy Based on Adenoviral Vectors

Adenoviruses have several innate features which make them highly suitable as vectors for gene therapy [38-40]. The pathogenic traits of the members of the *Adenoviridae* family are well-documented and typically include mild infection of



**Fig. (2).** The difference between treatment strategies that affect cancer stem cells or differentiated tumor cells (which form the bulk of the tumor), respectively. The former kills cancer stem cells, after which the tumor regresses and eventually vanishes, since the residual cells eventually die if no new cells are produced. In contrast, the conventional therapy will at first decrease the tumor size by killing the bulk of tumor cells, but later the tumor will re-grow because of new tumor cells from the cancer stem cells.

the upper respiratory tract, eyes, or gastroenteritis. Adenovirus vectors have been shown to effectively transfer genes to both dividing and non-dividing cells *in vitro* and *in vivo*. An additional feature is very infrequent integration into the host genome, which reduces the risk for undesired mutations. The adenovirus vector is stable and can be produced at high titers according to good manufacturing practices. In addition, adenovirus gene therapy has an excellent safety record thus far when used in clinical trials against cancer [41].

Human adenoviruses consist of an up to 38 kb linear DNA genome that is protected by a non-enveloped icosahedral virus capsid [42]. Three major and several minor proteins constitute the scaffold of the virus capsid [43]. The greater part of the protein shell is made up by hexon, of which there is 720 copies per virion particle. Each of the adenovirus twelve icosahedral vertices houses a penton base fundamant, consisting of five penton monomers, from which a trimeric fiber polymer protrudes. Globular knobs form the end of the twelve fibers and constitute the major attachment and recognition point of the adenovirus to cellular receptors.

Most adenovirus serotypes, including the commonly used adenoviral gene therapy vector serotype 5 (Ad5), bind to the primary receptor, the coxsackie-adenovirus receptor (CAR), through high-affinity interactions with the fiber knob [44,

45]. Next, the virus particle is internalized into the host cell through formation of clathrin-coated vesicles, mediated by interactions of an arginine-glycine-aspartate (RGD) motif in the penton base with cellular  $\alpha_v\beta$  integrins [46]. The clathrin-coated vesicles bearing the adenovirus then fuse with cytoplasmic endosomes, whereafter the viral capsid is disassembled and the genome transported to the nucleus through a microtubule-mediated process. Expression of the viral DNA leads to the formation of numerous new virus particles, and eventually to the destruction of the host cell and the spreading of the newly synthesized viruses to the surroundings.

The entry of the adenoviral vector into cells is determined by the availability of receptors on the cell surface. CAR, which is recognized by most serotypes including the gene therapy standard serotype 5, is abundantly expressed on most normal epithelial cells, but is sparsely expressed, completely lacking or expressed in the incorrect locale on many tumor cells [38, 40, 47-56]. This may constitute an obstacle to adenoviral cancer therapy approaches that rely on infection of the majority of tumor cells, since a major factor determining the oncolytic potency of a replicating adenovirus is its ability of infect the target cells [57-59]. However, strategies that utilize secretory therapeutic factors will not necessarily require infection of all cells of the tumor and may therefore be more appealing than intracellular effectors.

The deficiency of CAR on many cancer cells can be overcome by changing the adenovirus tropism through modifications of the fiber knob of the viral capsid. An example of adenoviral pseudotyping is Ad5/3 where the knob part of the Ad5 fiber has been exchanged with the knob from serotype 3, which belongs to a different subgroup of *Adenoviridae* and thus uses different receptors for cellular entry [48, 49, 60, 61]. The resulting Ad5/3 vector demonstrates CAR independent transduction and enhanced infection of cells not expressing or with low expression of CAR. Other modifications that improve viral tropism with regard to tumor cells include addition of an integrin binding arginine-glycine-aspartate (RGD) motif into the HI-loop of the fiber knob (Ad5- $\Delta$ 24RGD) [50, 62] or insertion of seven positively charged lysine residues followed by a glycine-serine linker sequence into the C-terminus of the fiber on Ad5 (Ad5.pK7- $\Delta$ 24) [63]. Alternative approaches to modify adenoviral tropism have been recently reviewed [38-40].

With regard to adenoviral gene delivery to stem cells, it has been reported that CAR is expressed to a low degree on mesenchymal stem cells, suggesting that the phenomenon may also be of interest in the context of other types of stem cells [64, 65, Bauerschmitz *et al.*, unpublished]. Thus, it may be important that capsid-modified adenoviral vectors Ad5/3- $\Delta$ 24, Ad5- $\Delta$ 24RGD-4C, and Ad5.pK7- $\Delta$ 24 have been shown capable of overcoming lack of CAR for gene delivery to stem cells [65].

### Oncolytic Adenoviruses

Oncolytic adenoviruses are genetically modified to replicate preferentially in tumor cells. Their replication ultimately leads to oncolysis of the infected cells, and intratumoral spreading of newly formed virus particles for local amplification of effect. Oncolytic adenoviruses can also disseminate from tumors for infection of distant metastases. Of note, oncolytic cell death is an immunogenic phenomenon, which can facilitate immune recognition of tumors [42, 66].

Oncolytic adenoviruses can be divided into subgroups based on the underlying strategy used for adenoviral modification. "Type 1" oncolytic adenoviruses contain loss-of-function mutations in their genome, which are compensated by mutations in the cancer cells. A deletion is typically manufactured into the immediate-early (*E1A*) or early (*E1B*) adenoviral genes, which results in mutant or absent proteins that cannot bind host cellular proteins required for induction of viral replication in non-tumorous cells. Nevertheless, in cancerous cells, the oncolytic adenovirus will be able to replicate due to pathway defects that make binding of those proteins unnecessary. For example, the  $\Delta$ 24 generation of viruses contain a 24-bp deletion in the constant region 2 (CR2) of *E1A* [67,68] and the protein is thus unable to bind to the Rb protein for induction of S-phase. Therefore, Ad5- $\Delta$ 24 (also known as *dl922-947*) has reduced ability to overcome the G1-S checkpoint in normal cells, and replicates preferentially in tumor cells where the p16/Rb-pathway is defective [67, 68]. It has been suggested that all human cancers are deficient in the Rb pathway and thus these viruses may be usable for many tumor types [69].

In "type 2" oncolytic adenoviruses, a tumor-specific promoter (TSP) is positioned into the adenoviral genome,

which restricts the viral replication to cancer cells expressing promoter-specific transcription factors [reviewed by [70]. The TSP is usually placed to control the *E1A* gene, but other early genes can also be regulated. A useful approach to improve specificity further is combination of type 1 and 2 transcriptional control [71]. Advanced generation viruses can feature multiple mechanisms for specificity and capsid modification for enhanced infection of target cells.

### Oncolytic Adenoviruses for Killing of Putative Cancer Stem Cells

Identification of alleged tumor-initiating cells allows testing the effect of antitumor approaches on them. We recently studied the efficacy of capsid-modified oncolytic adenoviruses for killing of putative breast cancer stem cells *in vitro* and *in vivo* [72]. Several reports advocate that breast tumor-initiating cells are found in the CD44<sup>+</sup>/CD24<sup>-low</sup> cell population [25, 26] and indeed, injection of CD44<sup>+</sup>/CD24<sup>-low</sup> cells into the mammary fat pads of NOD/SCID mice leads to rapid tumor formation [25, 72]. However, when the CD44<sup>+</sup>/CD24<sup>-low</sup> cells were infected with Ad5/3- $\Delta$ 24 prior to injection, no tumors appeared [72]. In addition, upon treatment of established CD44<sup>+</sup>/CD24<sup>-low</sup> derived tumors with Ad5/3- $\Delta$ 24 or Ad5.pK7- $\Delta$ 24, abrogation of tumor growth was seen [72]. Both Ad5/3- $\Delta$ 24 and Ad5.pK7- $\Delta$ 24 have a 24-bp deletion in the *E1A* gene, in addition to modifications to their capsid fiber knobs [59, 63]. Ad5/3- $\Delta$ 24 utilizes the serotype 3 receptor abundantly expressed on many tumor types for entry into the cell, whereas Ad5.pK7- $\Delta$ 24 can utilize the heparin sulfate proteoglycans for cellular entry. Importantly, both viruses are also effective in killing differentiated breast cancer cells [73]. The biodistribution of Ad5/3- $\Delta$ 24 and Ad5.pK7- $\Delta$ 24 is similar to Ad5 that has been very safe in cancer patients, which might predict good safety data. Because  $\Delta$ 24 generation viruses were able to replicate in putative breast cancer stem cells, our data suggests that p16/Rb pathway defects may arise early in carcinogenesis and are therefore present in cancer stem cells.

At present, no promoters active in cancer stem cells have been published. We hypothesized that possible candidates would include the promoters for multidrug resistance protein (*mdr*), telomerase (*hTERT*) and cyclo-oxygenase 2 (*Cox-2*) [Bauerschmitz *et al.*, unpublished]. Oncolytic adenoviruses featuring the Ad5/3 capsid modification and *mdr*, *hTERT* or *Cox-2* promoters controlling the *E1A* gene were therefore constructed [Bauerschmitz *et al.*, unpublished]. Further improvement in selectivity of the constructs towards cancer cells was achieved by deletion of the Rb binding site from the adenoviral *E1* region. The *in vitro* oncolytic potential of Ad5/3-*mdr*- $\Delta$ 24, Ad5/3-*Cox-2L*- $\Delta$ 24 and Ad5/3-*hTERT*- $\Delta$ 24 (featuring an additional deletion in the *E3A* gene) on CD44<sup>+</sup>/CD24<sup>-low</sup> breast cancer-inducing cells was equivalent to the highly efficient, but less selective Ad5/3- $\Delta$ 24. Of note, these viruses also mediated a reduction in the size of tumors resulting from injection of CD44<sup>+</sup>/CD24<sup>-low</sup> cell populations into mice [Bauerschmitz *et al.*, unpublished]. The results indicate that transcriptional targeting of adenoviral vectors using promoters active in cancer stem cells may be a useful way for eradication of those cells. Damage to normal stem cells can be avoided by transcriptional control features, in-

cluding deletion of the Rb binding site and tissue specific promoters.

Another plausible approach that might be usable for targeting of oncolytic adenovirus towards tumor-initiating cells is utilization of bispecific adapter molecules such as single chain monoclonal antibodies, through which the virus can attach to a specific cell surface protein [74]. For example, oncolytic adenoviruses capable of producing epidermal growth factor receptor (EGFR) targeting adaptor molecules have been constructed [58, 75, 76].

In addition to infecting putative breast cancer stem cells, adenovirus vectors are also capable of infecting tumor-inducing cells from other tissues. Ad serotype 16 and the chimpanzee Ad CV23 were recently shown to effectively infect low-passage brain tumor cell lines, including the CD133<sup>+</sup> and CD133<sup>-</sup> populations [77]. Brain tumor stem cells are thought to reside in the population bearing the neural stem cell marker, CD133<sup>+</sup> [27, 28]. Moreover, a high susceptibility of putative brain tumor stem cells towards Ad5-Δ24RGD was recently reported by Jiang *et al.* [78]. Hematopoietic and mesenchymal stem cells are also efficiently transduced by different types of modified adenoviruses [65, 79].

Since we now know that many putative cancer stem cell types can be transduced, this suggests that also the respective normal tissue stem cells may be transducible. This raises a safety concern and suggests the need for tight transcriptional control. However, since most humans have sustained infections with many wild type adenovirus strains (including serotype 3, which can enter stem cells) without stem cell eradication, normal tissue stem cells must have protective measures against viruses, and these measures seem to be lost when the cells become cancer stem cells. The first capsid modified oncolytic adenovirus Ad5-Δ24RGD has recently entered clinical testing. This virus can enter many types of stem cells [65, 72]. Nevertheless, Ad5-Δ24RGD has been safe in patients treated so far (4 treated at the time of manuscript submission), which supports the notion that normal tissue stem cells have additional protection against adenoviruses.

## FUTURE PROSPECTS

Virotherapy using adenoviral vectors constitutes a promising approach against tumors resistant to available treatment modalities. However, there are still many pitfalls that need to be overcome. One major obstacle is effective delivery to target tissue. Available clinical data implies that transduction of sufficiently large proportions of tumor cells continues to be the single most important factor limiting clinical benefit. Physical barriers, such as stromal cells and matrix, necrotic, hypoxic and hyperbaric tissue may constitute a problem to efficient intratumoral dissemination. Currently, several strategies are being explored to improve transduction of cancer cells. For adenoviruses, modifications of the capsid can improve viral tropism for tumor cells. Also, the bioavailability, i.e. proportion of adenovirus detected in systemic circulation after administration, needs to be improved substantially. In stark contrast to *in vitro* transduction, the intravascular biodistribution of adenoviral vectors is not influenced much by the expression of adenoviral cell receptors [80]. In mice, intravenously delivered adenovirus tends to accumu-

late into the liver, spleen, heart, lung and kidney, with the great majority ending up in the liver [53, 73]. In addition, tissue-specific macrophages, such as the Kupffer cells of the liver, are quite efficient in clearing adenovirus from blood [81]. Another potential obstacle for virotherapy is the induction of neutralizing antibodies towards proteins of the viral capsid, which can hinder systemic (but not local) re-administration. This obstacle can however be circumvented by using viruses based on another serotype for re-administration. Alternatively, even small changes in the adenoviral fiber knob can allow escape from pre-existing specific neutralizing antibodies [49, 59]. Further, anti-viral antibodies can be removed with e.g. columns featuring capsid proteins in an immunoapheresis approach. Alternatively, induction of neutralizing antibodies can be thwarted with anti-CD20 antibodies or cyclophosphamide [41].

Nevertheless, new antitumor concepts are desperately needed because chemotherapy and other conventional modalities can be expected to deliver only marginal additional improvements over current results. One possibility lies in agents that target cancer stem cells instead or in addition to differentiated tumor cells. Oncolytic viruses are the first approach shown effective against tumor-initiating cells. Moreover, oncolytic viruses have efficacy also against differentiated tumor cells. Further, virotherapy sensitizes tumor cells to radiation and chemotherapy [38]. Thus, oncolytic viruses hold significant promise for improving treatment options for patients with currently incurable cancer. The biggest obstacle in the field is the difficulty in translating approaches from the laboratory to patients and unfortunately no clear solutions to this problem are currently in sight [82].

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## ABBREVIATIONS

Ad	=	Adenovirus
BMP	=	Bone morphogenetic protein
CAR	=	Coxsackie-adenovirus receptor
CD	=	Cluster of differentiation
CSC	=	Cancer stem cells
ESC	=	Embryonic stem cell
EGFR	=	Epidermal growth factor receptor
Her-2/neu	=	A proto-oncogene encoding for EGFP
JFK	=	Janus family kinase
NOD/SCID	=	Non-obese diabetic/severe immunodeficient
Notch	=	A embryonic and tissue stem cell signaling molecule
Oct-4	=	Octamer-4, a transcription factor that is important for the self-renewal of undifferentiated embryonic stem cells

PKR	=	Protein kinase R
Rb protein	=	Retinoblastoma tumor suppressor/cell cycle regulator protein
Shh	=	Sonic hedgehog
TA cell	=	Transient-amplifying progenitor cell
TSP	=	Tumor-specific promoter
Wnt	=	A family of secreted proteins that regulate cell-cell interactions during development

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