REVIEW

Athymic Nude Mice as an Experimental Model for Cancer Treatment

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Summary

Athymic nude mice, a murine strain bearing spontaneous deletion in the *Foxn1* gene that causes deteriorated or absent thymus (which results in inhibited immune system with reduction of number of T cells), represent a widely used model in cancer research having long lasting history as a tool for preclinical testing of drugs. The review describes three models of athymic mice that utilize cancer cell lines to induce tumors. In addition, various methods that can be applied in order to evaluate activity of anticancer agents in these models are shown and discussed. Although each model has certain disadvantages, they are still considered as inevitable instruments in many fields of cancer research, particularly in finding new drugs that would more effectively combat the cancer disease or enhance the use of current chemotherapy. Finally, the review summarizes strengths and weaknesses as well as future perspectives of the athymic nude mice model in cancer research.

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Cancer diseases and their treatment

Cancer is one of the most serious diseases and still one of the leading causes of both mortality and morbidity in a population. It represents a potential source of severe health complications that may be permanent, thus bringing burden not only to the afflicted individual, but also to the family and society as well. Incidence and mortality of different types of cancer can be influenced by several factors, e.g. socioeconomic status, different already well-defined cancer risk factors (e.g. diet, smoking, alcohol, exogenous hormonal use), but also other largely unknown factors playing role in these relationships (Weiderpass and Pukkala 2006). Up to now, more than 200 different types of cancer were described. Malignant tumors can be classified according to various parameters, for example their biological behavior (e.g. rate of growth, hypoxic status, aggressiveness, readiness to metastasing), tissue of origin (solid tumors and those derived from blood tissue), histological type (carcinomas, adenocarcinomas, sarcomas, etc.) or responsiveness to a chemotherapy. Some tumors tend to be chemosensitive, others might exhibit more or less resistance due to several factors or combinations of these factors, such as drug inactivation, drug target alteration, drug efflux, autophagy, etc. (Housman *et al.* 2014, van der Wekken *et al.* 2016, Farrow *et al.* 2014).

Various tumors can also differ in hypoxic status and prognosis. Hypoxia increased ability of malignant cells to survive, and also to migrate, invade and metastasize. Moreover, hypoxic cells exhibit therapy-resistance. Thus, tumor cells growing in the hypoxic conditions might survive and account for more invasive and metastatic behavior of the tumor. One of the components that can contribute to occurrence of these

phenomena is the carbonic anhydrase IX (CA IX) that is expressed only in hypoxic tumor cells. Presence of the CA IX worsens the overall prognosis, but on the other hand makes a promising target for an effective therapeutic agent (Pastorek and Pastorekova 2015, Kumari et al. 2016). Prognosis of a certain type of cancer is dependent on many other factors, like its histological type, location, certain specific traits, chemosensitivity, age of the patient, comorbidities. The best prognosis with a highest 5-year relative survival (5-y-RSC) exhibit testicular cancer (88.6 %), lip cancer (88.1 %) and thyroid cancer (86.5 %), as determined by population based study of cancer survival EUROCARE-5 (De Angelis et al. 2014). However, there are some tumors that have propensity for being clinically silent, or they have symptoms similar to other diseases up to the point, where they will invade neighboring tissues and organs or form distant metastasis. Prognosis of such tumors is generally poor. Rapidly fatal cancers like lung, oesophagus, liver, pleura and pancreas cancer can serve as examples (De Angelis et al. 2014, Seicean et al. 2015). Consequently, it is necessary to further develop new biomarkers for screening as a secondary prevention of cancer, such as micro-RNAs, DNA, RNA, DNA methylation patterns or new protein markers and introduce them to clinical practice (Dhaliwal et al. 2015, Majumder et al. 2015, Oh et al. 2015).

Table 1. Examples of some commonly used anticancer chemotherapeutics and their mechanism of action.

| Therapeutic agents	Mechanism of action	Reference
Bleomycin	- produces oxidative cleavage of DNA and possibly RNA - mediates degradation of DNA, mRNAs, and active chromatin - causes G2 arrest, inhibition of DNA and RNA synthesis and induces apoptosis	Bimonte et al. 2015, van der Kuyl et al. 2002
Capecitabine	- is a prodrug converted in both tumor and normal tissues to the active drug fluorouracil (5-FU) - higher concentration of the conversion enzyme is found in cancer cells and liver	Walko and Lindley 2005
Cyclophosphamide	- its cytotoxic effect lies in the cross-linking of DNA strands and subsequent inhibition of cell division - in addition, it possesses immunomodulatory anticancer effects	Le and Jaffee 2012, Mukhtar and Woodhouse 2010
Doxorubicin	- intercalation into DNA (causes inhibition of DNA and RNA polymerases) and inhibition of topoisomerase II mediated DNA repair in the process of removing DNA supercoiling - free radical generation which causes damage to DNA, cellular membranes and proteins - other possible mechanisms are involved	Thorn et al. 2011, Pommier 2013, Tacar et al. 2013
Fluorouracil	- is a pyrimidine analogue which interferes with DNA synthesis and repair *via* inhibition of thymidylate synthase (exerts so called thyminless death) - affects normal function of nucleic acids *via* misincorporation into DNA and RNA - other possible mechanisms have been proposed (e.g. cell cycle regulation interference, inhibition of angiogenesis, etc.)	Zhang et al. 2008, Wyatt and Wilson 2009
Gemcitabin	- is an analogue of deoxycytidine that induces apoptosis *via* inhibition of DNA base synthesis and by incorporation into DNA, thus, precluding DNA synthesis	Mini et al. 2006
Hydroxyurea	- inhibition of DNA synthesis *via* inhibition of ribonucleotid reductase (enzyme responsible for the synthesis of deoxyribonucleotides)	Torrents 2014, Yarbro 1992
Irinotecan	- inhibition of topoisomerase-1, and consequent DNA breaks occur - induction of G2 arrest/delay and cell death	Xu and Villalona-Calero 2002
Metothrexate (MTX)	- belongs to the category of drugs called antifolates - inhibits conversion of dihydrofolate to tetrahydrofolate which is necessary for the synthesis of nucleotides, particularly thymidine. Thus, DNA and RNA synthesis is inhibited	Wong and Choi 2015, Schweitzer et al. 1990, Bleyer 1978
Oxaliplatin	- mechanism of action similar to other platinum-based substances like cisplatin - predominantly causes DNA damage (by inter-/intra-strand crosslinking) - other effects include: DNA/mRNA synthesis arrest, and inducing cytotoxic immunologic mechanisms	Alcindor and Beauger 2011
Paclitaxel (Pac)	- interferes with microtubule dynamics by its stabilizing effect on the assembly of microtubules - leads to defective chromosome segregation and blocks cell cycle completion - exerts inhibitory effect on microtubule fragment formation at centrosomes	Ganguly et al. 2010
Vinblastine (Vinb)	- another tubulin targeting drug like Pac - interference with microtubule dynamics *via* inhibition of microtubule assembly - exerts positive effect on microtubule fragment formation at centrosomes	Ganguly et al. 2010

Although origin of tumor´s induction is not yet fully understood, several factors are known to be responsible for initiation of the malignant degeneration of a cell. Among these are sporadic mutations of the genome, radiation and various chemical mutagens responsible for tumorigenesis, etc. Also, biological factors such as Epstein-Barr virus or Helicobacter pylori are known to play a role in the initiation of cancerogenesis (Geng and Wang 2015, Testerman and Morris 2014). In some cases, an individual can inherit familial predisposition to cancer. Such tumors then occur generally in an early phase of life, e.g. in childhood. In addition, several risk factors have been identified that are associated with the multistep process of cancerogenesis, including obesity, smoking, alcohol, or stress. Therapy of cancer is based on the utilization of various modalities, and chemotherapy represents one of the basic approaches applied in the treatment. Targets of various chemotherapeutic agents differ and comprise different intracellular pathways, processes and components of malignant cells, which together participate in the expression of the cell´s carcinogenic potential. They can affect the cell cycle, or regulate apoptosis of cells with many structural defects in DNA that are responsible for angiogenesis and spread of the cancer to other tissues and organs (Table 1). Nevertheless, research of new potent anticancer drugs that might target novel cellular pathways is still crucial and proper *in vitro*, and *in vivo* models are inevitable requirements for preclinical studies.

Athymic nude mice as the first experimental model in cancer research

Anticancer drugs tested on cell cultures *in vitro* are often not transferable to conditions, in which tumors grow in humans, due to the lack of appropriate tumor microenvironment, which is common to tumor´s development and interaction during growth (e.g. vascularization issues, human immune system, etc.) (Shultz *et al.* 2014, Boone *et al.* 2015). Preclinical phase of cancer research needs to include *in vivo* models that mimic at least to some extent the clinical situation in a human organism, and are essential as a part of preclinical anticancer drug testing (Stakleff and Von Gruenigen 2003).

The first description of athymic nude mice was presented by Flanagan in 1966 in UK after their discovery in the stock of albino mice in Virus Laboratory of Ruchill Hospital in Glasgow, UK in 1962. Flanagan

(1966) first described both their phenotypical and genetic traits, and assigned the effect of visible lack of hair to a new recessive gene (localized on the chromosome 11), which he called nude (the abbreviation of which is nu, often used to describe the gene or the allele combination *nu/nu*; Fig. 1). Later, research has shed more light into the molecular biology, morphological, and immunological characteristics of the nude mice (Mecklenburg *et al.* 2005).
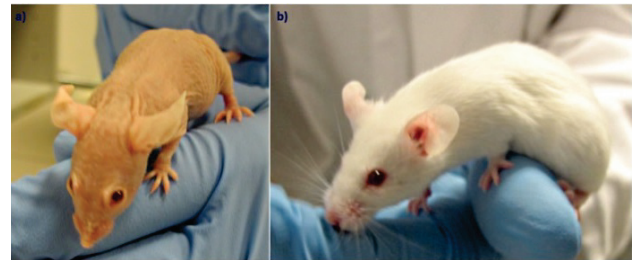
Fig. 1. Comparison of mice with homozygous and heterozygous mutation. (**a**) nude female homozygote (nu/nu), (**b**) nude female heterozygote (nu/+) (both around 7 weeks of age).

The most important trait of mice pertaining to the utilization in cancer research is their lack of thymus (Pantelouris 1973). Specific T-cell- mediated type of immunity is responsible for destroying infected cells (e.g. by viruses), or malignant cells. The T-lymphocytes are further responsible for the host versus graft reaction (so called HVGR), which occurs when a transplant (both allo- or xeno-) is being rejected by a host (Cadili and Kneteman 2008, Ruiz *et al.* 2013). Deficiency of T-lymphocytes considerably immunocompromises the mice and enables the engraftment, growth and eventually metastasizing of the tumor cells from the xenograft after the implantation (Stakleff and Von Gruenigen 2003, Sun *et al.* 2014).

Macroscopically, the skin of athymic nude mice seems to be hairless (Fig. 1). In reality, it contains normal hair follicles at birth, but later, as the hair shaft grows, it starts to coil within the infundibulum and fails to penetrate the epidermis (Flanagan 1966, Mecklenburg *et al.* 2005). The genetic background behind this phenomenon was revealed to be a spontaneous loss-of-function mutation resulting in a recessive homozygozity in the locus of FoxN1 (Forkhead box, previously called Whn or Hfh11) gene, encoding transcription factors (Mecklenburg *et al.* 2005). Heterozygotes do not exhibit any visible phenotypical changes (Fig. 1). It is known that there are some minor deviations regarding the

thymus size and immune response in comparison to healthy mice (Holub 1992).

Another phenotypical traits of the athymic nude mice are their retarded growth, reduced fertility, absence of vibrissae at birth, and limited life span of the majority of mice due to general body weakness (Flanagan 1966). Under normal housing conditions the athymic nude mice will live ranging from 6 months up to one year. Although both, viability and fertility are severely diminished, they can be enhanced when mice are bred under specific germ-free conditions, whereby their life span can be increased to that of a normal mouse (which is from 18 months to 2 years). Since athymic nude mice have underdeveloped mammary glands and are unable to effectively nurse their offsprings, athymic nude males are bred with heterozygous females (Holub 1992).

Although in athymic nude mice the thymic primordium that is formed by epithelial precursor cells normally develops, its maturation into the mature thymus is defective (Prowse et al. 1999). The interplay between different autocrine and paracrine regulatory signaling molecules, their receptors and corresponding intracellular pathways, is necessary for the normal thymic and T-lymphocyte development and function, such as tumor necrosis factor receptor (TNFR), transmembrane protein receptor CD40, etc. (Sun et al. 2014). With the absence of Foxn1 protein due to *nu/nu* recessive mutation, both differentiation and proliferation of thymic epithelial cells (TECs) and progenitors of T-lymphocytes are defective. The TECs play a crucial role in the T-lymphocyte maturation. Other functions of Foxn1 protein by which it influences T-cells, are vascularization of the thymus, its colonization by T-cell progenitors, and their selection. Mechanism of action lies in regulating series of genes that encode molecules involved in thymic development and function, like Pax1 (paired-box1), CCL25 (Chemokine (C-C Motif) Ligand 25), etc. (Sun et al. 2014).

The model of the athymic nude mice has certain limitations, since the immunodeficiency is severe, but not absolute. Despite the fact that there are only few T cells in the periphery, the intact innate immunity, particularly high NK cell´s activity can limit the engraftment take rate (i.e. percentage of successfully engrafted tumors), growth, and ability to form metastasis of the majority of primary solid tumors, and makes the engraftment of malignant hematopoietic cells impossible (Shultz et al. 2005, Shultz et al. 2014). These issues could be resolved by using other types of immunodeficient mice, where NK

cell activity is decreased or even absent (Shultz et al. 2005, Shultz et al. 2014). Since activity of T-cells tends to increase with the age, it is appropriate to use younger mice (usually 5-10 weeks of age) to enhance the engraftment rate, and thus, reproducibility of the studies (Giovanella and Fogh 1985, Liu et al. 2015, Pacak et al. 2012).

Despite these hindrances, the athymic nude mice have been successfully used as a host for tumor cells and represent a suitable model for studying the behavior of cancer *in vivo*, such as engraftment, growth, invasion and metastasis. The main advantage of this model lies in the natural immunosuppression of mice. It is relatively simple to achieve engraftment of a desired type of tumor by simply inoculating the cancer cells into the animal host. Variety of common established tumor cell lines is used for inoculation. Another advantage is that the tumor can be easily observed in athymic nude mice when injected subcutaneously due to the natural lack of hair. Recently, the patient derived models (PDX) are being discussed, since they more genuinely mimic the microenvironment and behavior of the primary tumor (i.e. response to chemotherapeutics) (Boone et al. 2015). Moreover, they allow determination and better prediction of efficacy of biomarkers that could be useful for cancer diagnostics and prognosis assessment (Boone et al. 2015, Hidalgo et al. 2014, Marangoni and Poupon 2014). However, PDX are cost-ineffective and it takes a long time until a visible tumor can be observed in the mice. Most patients cannot afford to wait until their tumor model is tested for the drug response. In addition, various immunomodulators cannot be tested on this model since athymic nude mice lack the proper immune response that take place at the site of the primary tumor in human organism (Boone et al. 2015, Gazdar et al. 2015, Tentler et al. 2012).

Since immunodeficiency of athymic nude mice allows engraftment of stem cells, these mice can also be used in the field of stem cell research (Jang et al. 2016, Rodriguez et al. 2015). There are several types of mutant variants of mice that carry the nude gene mutation available in various laboratories. These „nude mice variants" have been produced by crossing other strains of mice (such as Balb/c, CD-1 or NMRI) with athymic nude mice. As already mentioned, either stable cancer cell lines, or cancer cells taken directly from the patient (PDX model) could be used to introduce the tumor into the mice, with both methods having their strengths and weaknesses.

Up to now, several new murine strains of immunodeficient mice were developed. Spontaneous mutation in C.B17 mice, termed *scid* (*Prkdc*^{scid}, protein kinase DNA activated catalytic polypeptide), largely prevents the development of mature T and B lymphocytes of the adaptive immune system. The first example of severe combined immunodeficiency (SCID) not primarily related to a hematopoietic cell abnormality but rather to an intrinsic thymic epithelial cell defect is the Nude/SCID phenotype, whose identification contributed to unravel important issues of T-cell ontogeny (Romano *et al.* 2012). The term "SCID" has now been adapted to refer to all severely immunodeficient strains of mice, including those expressing the *Rag1*^{null} or *Rag2*^{null} mutations (Shultz *et al.* 2014).

A major leap forward in the engraftment of primary human cells, tissues, and tumors was the development of immunodeficient mice bearing a targeted mutation in the IL2-receptor common gamma chain gene (*IL2rg*^{null}). When combined with the *scid*, *Rag1*^{null}, or *Rag2*^{null} mutations, a mouse completely deficient in adaptive immunity and severely deficient in innate immunity is generated that is highly receptive to engraftment of human cells, tissues, and primary tumors (Shultz *et al.* 2007, Shultz *et al.* 2012).

Genetically modified organisms, especially genetically engineered mice (GEM), are also used in the study of genetic and molecular mechanisms of disease, often with the aim of developing treatments and interventions to improve health and longevity (Brayton *et al.* 2012).

Cell lines – a potent tool for tumor development in athymic nude mice

History of the cell lines used in cancer research dates back to the first stable cell line successfully isolated and distributed throughout the whole world. The HeLa cell line (as it was called after the patient Hentrietta Lacks from whom the tumor cells were harvested), prepared from the cells from cervical cancer (Lucey *et al.* 2009) and it was able to divide unlimited number of times when being grown under certain living conditions. This effect is due to the upregulation of telomerase activity in more than 90 % percent of cancer cells or by alternative pathways, all of which lead to maintaining the length of telomeres (Carnero *et al.* 2015). Immortalized cells form the basis of preclinical cancer research and studies represent one of the important sources of our current

knowledge of the biology of cancer. More than one hundred and twenty cultured human tumor cell lines produced tumors after subcutaneous inoculation of 1-20 million cells into nude mice (Fogh *et al.* 1977). The histopathology of tumors developed in mice correlated with the human tumor of origin in all cases.

Various cancer types of different organ, tissue, and histological origin can be isolated and maintained as a homogenous culture of the tumor cells with both genotypical and phenotypical expression profile of the primary tumor (Hwang *et al.* 2016). Homogeneity of the culture is of advantage since cells are uniform (both genotypically and phenotypically), and lack the cellular variability that exists in a primary tumor. Uniformity together with the immortality enables research of metabolic pathways participating in the cancerogenous process, identification of oncogenes and tumor suppressor genes, and various mutations that occur in the malignant cells, including diagnostic and therapeutical options in cancer treatment, without being limited by the durability of the cell culture (Gazdar *et al.* 2015). For example, effective novel drug screens can be performed on various tumors, or new biomarkers can be defined determining the prognosis or drug response according to adherence of patients to different subgroups (Hwang *et al.* 2016, Jeon *et al.* 2014, Collisson *et al.* 2011). Moreover, this method is relatively cost-effective, since the cell lines can be maintained by cryopreservation and passaging.

Certain disadvantages are connected with the utilization of established cancer cell lines, though. Cell lines alone might not always be suitable tool for cancer research or testing efficacy of anticancer agents (Boone *et al.* 2015, Gazdar *et al.* 2015). Even though genetic changes that occurred in the primary tumor may be maintained throughout the subsequent generations of the cell lines, there might be some genomical and phenotypical modification from the primary cells that have been harvested decades ago. This factor must be taken into the consideration since it might affect results of the preclinical anticancer drug testing (Boone *et al.* 2015). Moreover, cell cultures lack the heterogeneity of tumor cells (some cell lines may only represent a clone that was selected and preferentially grows in the artificial setting *in vitro*, but not in a real *in vivo* conditions) and also interactions with stromal microenvironment present in the primary tumor (Boone *et al.* 2015). This adds a barrier to the research of cancer therapy targeted against the immune cells and vasculature of the tumor *in vivo* (Gazdar *et al.* 2015).

Nevertheless, established cancer cell lines are still a useful and indispensable tool in cancer research, but the above-mentioned limitations must be taken into the consideration. As a result, *in vivo* models such as immunodeficient mice need to be included into preclinical research, where constitutional cancer cell lines can be implanted as xenografts into the mice (Gazdar *et al.* 2015, Stakleff and Von Gruenigen 2003). Further, we will focus on summarizing various procedures of inducing a tumor using stable cell lines as a xenograft, and ways of evaluating the tumor growth after treatment.

Models of tumor induction in athymic nude mice

Various murine models have been used to gain insight into the pathophysiology of cancer, and they serve as an *in vivo* instrument in preclinical testing of various treatment modalities and diagnostic strategies. The basic feature of all these models is their suppressed immunity. Because of their immunodeficiency and ability to tolerate cancer xenografts, athymic nude mice represent a widely used murine model for inducing different types of cancer. The nude mutation prevents development of functional T-cells and provided an early model for engraftment of human cell lines derived from solid tumors (Fogh *et al.* 1977). However, these mice bear some limitations due to presence of humoral adaptive immune system and also an intact innate immune system.

Since the nude mice are still a valid model utilized as a tool in the preclinical phase of testing new drugs (particularly as a second step to confirm the effect of drug's activity that was observed *in vitro*) (Burkhart *et al.* 2014, Rouleau *et al.* 2015), and also conventional therapy in various new forms, combinations, or for drug resistance (Guo *et al.* 2016, Yeon Kim *et al.* 2016, Burkhart *et al.* 2014), it will be useful to summarize various methods used to develop a tumor and describe different possible endpoints of the treatment. Three basic models of a tumor induction include orthotopic, heterotopic, and metastatic model (Fig. 2) (Schuh 2004, Shaw *et al.* 2004). In the orthotopic model, cells are either surgically implanted or injected into the site of the tumor´s origin. In the heterotopic model, cells are injected into a subcutaneous space (s.c.). Both these methods have their strengths and weaknesses, pertaining to simplicity, relevance, or tumor engraftment rate (Stakleff and Von Gruenigen 2003). In the metastatic model, the tumor cells are injected either into the lateral tail vein of the mouse or intra-cardially

(i.e. into the left ventricle) (Khanna and Hunter 2005). Cells will disseminate into the body forming lesions in various organs. In addition, the metastatic model can also be established by intraperitoneal (i.p.) injection, intraosseal injection (into tibia or femur), intrasplenic injection or electing other intravenous routes (for reviews see (Saxena and Christofori 2013, Simmons *et al.* 2015, Bollard *et al.* 2013).
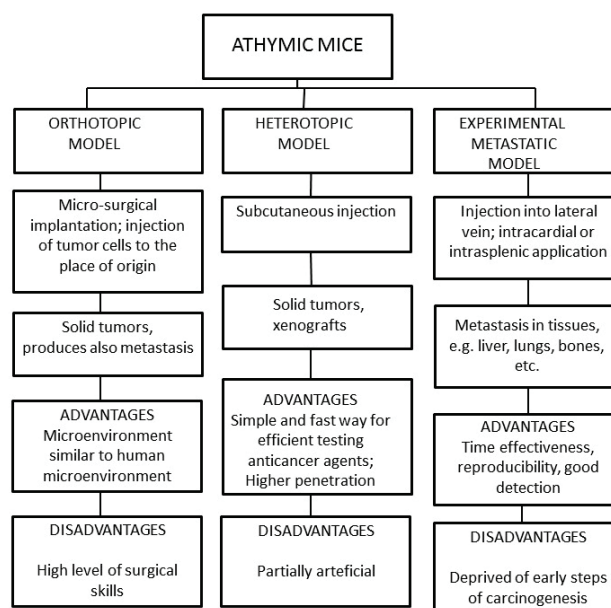
Fig. 2. Schematic diagram of three models of cell's injection into athymic nude mice.

Subcutaneous heterotopic model is the most common one due to its relative simplicity in design and evaluation (the hairlessnes of mice enables the growth of the tumor to be observed with the naked eye). It has been therefore utilized as a model enabling rapid screening of various new compounds (Killion *et al.* 1998). Suspension of tumor cells is standardly injected into the right or left flank of mice (or both at the same time), but other sites can be elected as well (Schuh 2004). The appropriate amount of cells should be used to establish a tumor, since small amounts of cells can result in unsuccessful engraftment and growth of the xenograft (Fogh *et al.* 1977). The usual number of cells required to produce a solid tumor is in the order of 10^6 (injected usually in 0,1 ml cell suspension), but the exact number depends on the specific cancer cell line that is utilized and viability. The tumorigenicity and metastasis formation of xenografted cell lines can be further enhanced by coinjection of a reconstituted basement membrane matrix (Matrigel) (Bao *et al.* 1994).

Once the cells are inside the host, it usually takes from two to six weeks until the tumor becomes visible and measurable. Exact time depends on a cell line´s type, interaction with the host, and the site chosen for the inoculation (Schuh 2004, Dipersio 1981). It is likewise important to check or evaluate the cancer cell line´s take rate. As it was already mentioned, some cell lines do not produce tumors in athymic nude mice at all, and others might be difficult to engraft, e.g. CAKI1 clear cell carcinoma cell lines (Niu et al. 2012). The growth of the s.c. xenograft is usually measured 2 or 3-times a week by a caliper, preferably by the same person (Workman et al. 2010). The two distances (diameters) at right angles are measured, i.e. the width (w) – which is the greatest transverse diameter, and a length (l) – the greatest longitudinal diameter) (Pacak et al. 2012, Nölting et al. 2014). These parameters encode either the tumor surface area or volume (most commonly $l \ x \ w$ for tumor area and modified formula for elipsoid $1/2 \ (l \ x \ w^2)$ for tumor volume) (Tomayko and Reynolds 1989, Rahal et al. 2015).

Two basic approaches in verification of the efficacy of a chemotherapeutic agent can be studied. To prove that a certain drug exerts a chemopreventive effect on a tumor, the initiation of the treatment should start before the cell injection, or no later than the cell injection (Singh et al. 2004, Nölting et al. 2014). Secondly, either cytostatic (i.e. growth delay) or cytotoxic (tumor volume shrinkage) effect on an already growing tumor could be evaluated, where the treatment is given with a time delay (several days to weeks depending on how fast the xenografts grow) following the inoculation of the cells (Kelland 2004). Mostly, the therapeutical substance is applied as an oral gavage (Ellinghaus et al. 2013) or by i.p. injection (Yang et al. 2016). Other modes of applications are possible as well, such as subcutaneous (Kenmogne et al. 2015), intratumoral (Jiang et al. 2016), intravenous (tail vein, portal vein, etc.) or intraarterial (carotid artery) (Kim et al. 2016).

At the end of treatment, animals are sacrificed, tumors are extracted and weighed (Wang et al. 2016). To evaluate the potential anticancer activity of a substance, various endpoints can be set, such as a change in tumour growth rate, growth delay, cell survival (clonogenic assay), or levels of appropriate surrogate marker, e.g. proliferation marker KI-67 (Haddad and Yee 2008, Workman et al. 2010). Most commonly, the terminal weight of the tumor in the control and treated group can be compared. Also, curve of either the tumor volume or

area at different time points during experiment are commonly demonstrated (Wang et al. 2016, Ellinghaus et al. 2013), or the delay with which the treated group reaches a determined tumor size (Teicher 2006). The tumor growth can be also monitored using bioluminescence or fluorescence of the tumor cells (e.g. the cells that are stably transfected with a vector expressing light emitting enzyme, such as luciferase (Luc) or the green fluorescent protein (GFP), respectively) (Hoffman 2015, Simmons et al. 2015, Nölting et al. 2014). Various other modalities affected by the treatment can be studied in addition to the tumor growth, such as induction of apoptosis (Hui et al. 2015, Kim et al. 2012, Archana et al. 2013, Ma et al. 2014), or inhibition of angiogenesis (Yin et al. 2016, Ma et al. 2014). Finally, inhibition of the occurrence/growth of spontaneous metastasis can be evaluated, although the s.c. tumors are known to form metastasis less frequently, and there are more appropriate models available (Khanna and Hunter 2005).

Advantage of the experimental metastatic model lies in its time-effectiveness (rapid development of tumors), reproducibility and consistency (Khanna and Hunter 2005). In the case of peritoneal carcinomatosis model, the time period until the tumor becomes palpable may be longer (Yao et al. 2015). The tumor growth can be measured in vivo by either bioluminiscence or fluorescence. Other methods include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single photon emission computed tomography (SPECT) imaging, or radiography (Simmons et al. 2015, Khanna and Hunter 2005). The election of the specific method is directed by its availability, resolution, speed of imaging or throughput, and cost (Simmons et al. 2015).

After treatment, the tumors can be evaluated in the organs or tissues selected by the frequency of the occurrence of the metastasis (e.g. in tail vein model – it is often the liver, because of the first capillary bed), but other sites, such as lungs are targeted as well (Khanna and Hunter 2005). In the intracardial model, the most frequent metastatic sites are determined by the tumor type; e.g. bone and lung in prostatic tumors (Bubendorf et al. 2000) or lung, bone, and liver in breast tumors (Weigelt et al. 2005). Consequently, in vivo or ex vivo imaging (the intensity of signal or number of lesions), gross anatomy (the number of macroscopic lesions), histopathology or immunohistochemistry (i.e. number of the tumors in selected sections, inhibition of

angiogenesis, detection of apoptosis, or proliferation index) of the tumors can be performed (Pacak *et al.* 2012, Nölting *et al.* 2014, Wang *et al.* 2016). In addition, the terminal weight of the tumors or survival rate of both groups can be compared (Khan *et al.* 2015, Shaw *et al.* 2004). Pertaining to the intraperitoneal model, difference in the number and weight of the intraperitoneally growing tumorous nodules may be assessed (Yao *et al.* 2015).

Although the subcutaneous and experimental metastatic model represents relatively a simple and fast way to determine the efficacy of anticancer agents, both of these models have their drawbacks, since they do not exactly reflect clinical scenario of the primary tumor (Khanna and Hunter 2005). The reason is particularly the lack of the microenvironment, where the primary tumors develop allowing the proliferation of specific tumor subclones, and thus modifying the behavior of the tumor as a whole. Moreover, experimental metastatic models are deprived of the early steps of carcinogenesis, and the structure of metastatic emboli formed in the blood stream differs from the one that we observe in in the tumors metastasizing spontaneously (Khanna and Hunter 2005, Stakleff and Von Gruenigen 2003, Killion *et al.* 1998). On the contrary, the microenvironment of the murine organ contributes to the development of the tumor similarly as the human microenvironment. In addition, orthotopic models more frequently produce metastasis spontaneously than the subcutaneous. Khanna and Hunter (2005) include specific examples of cell lines that are useful in spontaneous metastasis essays. Also, it has been estimated that responses of anticancer drugs in the orthotopic model correlate more with the activity seen in the clinical practice (Hoffman 1999). The orthotopic model is therefore generally considered to be more appropriate for studying tumor behavior, performing pharmacologic studies, and developing diagnostical procedures (Hoffman 1999).

To establish the orthotopic model, the cells can be introduced either by direct injection or by micro-surgical implantation (SOI) to the organ of the tumor´s origin. It has been shown that tumors formed by the direct injection exhibit lower rates of metastasis and differ in tissue architecture from the primary tumor in comparison to SOI implantation of the tumor fragments. In SOI, usually 1 mm^3 of tumor tissue is used for implantation, and the cells are taken either directly from the patient or the subcutaneous xenograft grown in athymic nude mice (He *et al.* 2015, Scott *et al.* 2014, Hoffman 1999). When using direct injection, the approximate number of cells does not differ from other models (Greco *et al.* 2016, Xiong *et al.* 2015, He *et al.* 2015). Various organ specific orthotopic models have been already developed, and new drugs have been successfully tested (Killion *et al.* 1998, Hoffman 1999). The endpoints used for evaluation of treatment efficacy are essentially the same, and they include inhibition of primary tumor growth, inhibition of metastatic events, extension of survival, or decrease in cachexia. The model is seen as a bridge from preclinical testing to the clinical practice, though it has certain limitation requiring high level of surgical skills for tumors to be correctly implanted (Hoffman 1999).

Conclusions

Athymic nude mice are a widely used murine model due to its relative simplicity and cost-effectivity, and has a long history as a tool for preclinical testing of drugs (Croy *et al.* 2001, Meyerrose *et al.* 2003). Here we have described three models that utilize cancer cell lines to induce tumors in athymic nude mice, various methods that can be applied in order to evaluate the activity of anticancer agents as well as their strengths and weaknesses. Although each of the models might have certain disadvantages, they are still considered as inevitable instruments in many fields of cancer research, particularly in finding new drugs that would more effectively combat the cancer disease or enhance the use of current chemotherapy (Killion *et al.* 1998). Novel mice strains with highly suppressed immune system (SCID, GEM, etc.) will enhance the effectivity of anticancer drug testing.

Conflict of Interest
There is no conflict of interest.

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