MASARYK UNIVERSITY

FACULTY OF SCIENCE

DEPARTMENT OF EXPERIMENTAL BIOLOGY

IDENTIFICATION AND CHARACTERIZATION OF CANCER STEM CELLS IN SOFT TISSUE SARCOMAS

Ph.D. Dissertation

Alena Ňuňuková

Supervisor:
prof. RNDr. Renata Veselská, Ph.D., M.Sc.

Brno 2017

Bibliographic Entry

Author	Mgr. Alena Ňuňuková Faculty of Science, Masaryk University Department of Experimental Biology
Title of Dissertation:	Identification and characterization of cancer stem cells in soft tissue sarcomas
Degree Programme:	Biology
Field of Study:	Molecular and Cellular Biology
Supervisor:	prof. RNDr. Renata Veselská, Ph.D., M.Sc. Faculty of Science, Masaryk University Department of Experimental Biology
Academic Year:	2017/2018
Number of Pages:	99
Keywords:	Rhabdomyosarcoma, cancer stem cells, SOX-2, CD133, nestin, ABCG2, Oct4, NANOG

Bibliografický záznam

Autor:	Mgr. Alena Ňuňuková Přírodovědecká fakulta, Masarykova univerzita Ústav experimentální biologie
Název práce:	Identifikace a charakterizace nádorových kmenových buněk měkkých tkání
Studijní program:	Biologie
Studijní obor:	Molekulární a buněčná biologie
Školitel:	prof. RNDr. Renata Veselská, Ph.D., M.Sc. Přírodovědecká fakulta, Masarykova univerzita Ústav experimentální biologie
Akademický rok:	2017/2018
Počet stran:	99
Klíčová slova:	Rabdomyosarkom, nádorové kmenové buňky, ABCG2, CD133, nestin, Sox2, Oct3/4, Nanog

Abstract

Rhabdomyosarcoma (RMS) belongs to the most diagnosed soft tissue sarcomas in children and adolescents. This tumor characterised with a disruption of muscle development and its localisation could vary with a RMS subtype. Despite of intensive treatment, RMS patients often suffer for recurrence and metastases. This situation could be explained by a presence of the cancer stem cell (CSC) subpopulation within the tumor, which is responsible for tumor initiation, recurrence of the disease and resistance to therapy.

This dissertation thesis is focused on identification and characterization of CSCs in cell lines derived from RMS. Putative markers associated with CSC phenotype, as previously identified in various types of sarcomas, were chosen for this study. In all analysed RMS lines was detected an expression of ABCG2, CD133 and nestin with various frequency and intensity. Nevertheless, any relationship between expression of these markers and tumorigenicity of cells corresponding with a CSC phenotype was not confirmed. During analysis of the cell surface marker CD133, an unusual nuclear localization of this protein was identified; however with no influence on tumorigenicity of RMS cells.

Following experiments focused on a detection of pluripotency transcription factors revealed a relationship between tumorigenicity of RMS cells and increased levels of Sox2; only the cell line with increased level of Sox2 showed the capability to initiate tumors in the immunodeficient mice. This finding was verified using during this tumorigenicity assay, xenograft RMS cell lines were derived, in which possible changes in expression of CSC markers were studied during repeated injections into immunodeficient mice. Relatively stable levels of ABCG2, CD133 and ALDH were found during serial injections into mice. In contrast, a change in growth parameters towards the more aggressive phenotype was identified together with a change in levels of transcription factors Nanog, Oct3/4 and Sox2 which could suggest some relationship these two phenomenons.

Results of this dissertation thesis indicated that the previous putative CSC markers ABCG2, CD133 and nestin are not suitable for identification of CSC in RMS. Sox2 signalling pathway seems to be the most promising target of therapy against CSCs.

Abstrakt

Rabdomyosarkom (RMS) je k nejčastěji diagnostikovaným sarkomem ze skupiny nádorů měkkých tkání u dětí a dospívajících. Pro RMS je charakteristické narušení vývoje svalové tkáně a lokalizace RMS se může měnit v závislosti na subtypu nádoru. Navzdory intenzívní léčbě u pacientů velmi často dochází k relapsu a vzniku metastáz. Jedním z možných vysvětlení je přítomnost subpopulace nádorových kmenových buněk (CSC), které jsou zodpovědné za vznik nádoru, relaps onemocnění i rezistenci vůči léčbě.

Předmětem předkládané disertační práce byla identifikace a charakterizace CSC v buněčných liniích derivovaných z RMS. Pro potřeby této práce byly vybrány markery spojené s fenotypem CSC, jejichž přítomnost byla dříve prokázána u sarkomů. U všech studovaných RMS linií se potvrdila exprese ABCG2, CD133 a nestinu s rozdílnou četností a intenzitou. Souvislost mezi expresí těchto markerů a tumorigenicitou buněk odpovídající fenotypu CSC však nebyla potvrzena. Při analýze povrchového markeru CD133 byla identifikována neobvyklá jaderná lokalizace tohoto proteinu, která však neměla vliv na tumorigenicitu buněk.

Následné experimenty zaměřené na detekci transkripních faktorů pluripotence odhalily souvislost mezi tumorigenicitou buněk RMS a zvýšenou hladinou Sox2 - schopnost vytvořit nádor v immunodeficientních myších byla detekována pouze u buněčných linií, které měly zvýšenou hladinu Sox2. Při testech tumorigenicity *in vivo* byly derivovány xenograftové RMS linie, u kterých byly následně sledovány případné změny v expresi markerů CSC během opakovaných injikací do myší. Bylo zjištěno, že nedochází k výrazným změnám v zastoupení ABCG2, CD133 a ALDH, byla však detekována změna růstových parametrů směrem k agresivnějšímu fenotypu a změne hladin transkripčních faktorů Nanog, Oct3/4 a Sox2, což může naznačovat spojitost těchto dvou jevů.

Z výsledků předložené disertační práce vyplývá, že původní kandidátní markery ABCG2, CD133 a nestin nejsou vhodné pro identifikaci CSC u RMS. Jako slibný cíl v protinádorové léčbě se zaměřením na CSC se pak jeví signální dráha Sox2.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor prof. RNDr. Renata Veselská, Ph.D., M.Sc. for her patience and willingness to lead and teach me through my Ph.D. study.

I would also thank to my collegueas from the Laboratory of Tumor Biology for their support, insighthful comments and for their maintenance of good mood in the laboratory. Specially thanks belong to RNDr. Jakub Neradil, Ph.D. and RNDr. Jan Škoda, Ph.D., who were part of our cancer stem cell team and really important part of my science world.

Last but not the least I woud like to thank my family, who support me in my study and life decisions and beloved ones, who understand and helped me with my science road.

Declaration

I declare that I worked on dissertation thesis on my own with the use of information sources, which are cited in the text.

Brno 29. 9. 2017 ……

Alena Ňuňuková

© Alena Ňuňuková, Masaryk University, 2017

CONTENT

List of abbreviations **10**

1. Introduction **12**

1.1. Pediatric sarcomas 13

1.1.1. Bone sarcomas 13

1.1.2. Soft tissue sarcomas 15

1.2. Rhabdomyosarcoma 16

1.2.1. Rhabdomyosarcoma subtypes 17

1.2.2. Clinical aspects and therapeutic approaches 19

1.3. Cancer stem cells 22

1.3.1. Cancer stem cell theory 23

1.3.2. Specific markers of cancer stem cells in soft tissue sarcomas 26

1.3.3. Functional tests of cancer stem cell phenotype 32

2. Aims **36**

3. Material and methods **37**

3.1. Cell lines and cell cultures 37

3.1.1. Primary cultures 37

3.1.2. Cell culture conditions 38

3.2. PCR methods 39

3.3. Immunodetection methods 40

3.3.1. Immunofluorescence 40

3.3.2. Transmission electron microscopy 41

3.3.3. Immunoblotting 41

3.4. Measurement of aldehyde dehydrogenase activity 43

3.5. Functional assays for verification of CSC phenotype 44

3.5.1. Sphere formation assay 44

3.5.2. Colony forming assay 44

3.5.3. *In vivo* tumorigenicity assay 44

4. Results **46**

4.1. Detection of CSC markers in rhabdomyosarcoma cell lines 47

4.1.1. Expression of ABCG2, CD133 and nestin 47

4.1.2. Expression of AC133 epitope in comparison with CD133 molecule 50

4.1.3. Distribution of CD133 within the cells and analysis of its nuclear localization 52
4.1.4. Analysis of aldehyde dehydrogenase activity in rhabdomyosarcoma cell lines 57
4.1.5. Expression of core stem cell markers Oct4, Sox2 and Nanog 60
4.2. Functional tests on CSC phenotype in rhabdomyosarcoma cell lines 63
4.3. Detection of CSC markers in xenograft cell lines 65
4.3.1. Expression of ABCG2, CD133 and nestin 65
4.3.2. Analysis of aldehyde dehydrogenase activity in xenograft cell lines 68
4.3.3. Expression of core stem cell markers Oct4, Sox2 and Nanog 70
5. Discussion 76
5.1. Sox2 is associated with tumorigenicity of rhabdomyosarcoma cell lines 76
5.2. Nuclear localization of protein CD133 in rhabdomyosarcoma cells 78
5.3. Activity of ALDH in rhabdomyosarcoma cell lines 80
5.4. Influence of serial xenotransplantation on rhabdomyosarcoma cell lines 81
6. Conclusions 84
7. References 86

List of abbreviations

ABC	ATP binding cassette
ALDH	aldehyde dehydrogenase
ARMS	alveolar rhabdomyosarcoma
BCL-XL	antiapoptotic proteins
BSA	bovine serum albumin
CD	cluster of differentiation
CSC	cancer stem cells
CT	computer tomography
DEAB	diethylaminobenzaldehyde
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
ERMS	embryonal rhabdomoyosarcoma
FDG/PET	fludeoxyglucose/positron emission tomography
FGF	fibroblast growth factor
FKHR	the forkhead family of transcription factors
HRP	horseradish peroxidase
ICCC	International Classifiaction of Childhood Cancer
MRI	magnetic resonance imaging
MyoD1	regulator of myogenesis (myogenic differentiation 1)
NOD/SCID	non-obese diabetic/severe combined immunodeficiency
PAX	paired box transcription factor families
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride membrane
RMS	rhabdomyosarcoma
RT	radiation therapy
TEM	transmission electron microscopy
TIC	tumor initiating cells

VAC	combination of chemotherapeutics vincristine, dactinomycine (actinomycine D) and cyclophosphamide

1. Introduction

In the past years, cancer became one of the major public health issues. Although new possible targets and drugs have been developed, survival of patients with certain malignancies is still poor. One from the possible explanations of this problem is an existence a unique group of cells within tumor, called cancer stem cells (CSCs). CSCs were reported in 1997 by Bonnet and Dick in acute myeloid leukemia for the first time and they were described later in various types of human malignancies *(Bonnet and Dick 1997).* Their capability of self-renewal and their potential to differentiate into different cell types allowed them to create a heterogeneous tumor bulk. It is also assumed that CSCs are responsible for recurrence of cancer disease *(Komuro et al. 2007; Beck and Blanpain 2013).*

Cooperation of our Laboratory of Tumor Biology (Department of Experimental Biology) with the Department of Pediatric Oncology (University Hospital Brno) give us an opportunity to investigate the presence of CSCs in tumor cell lines derived from tumor samples taken from patients treated for different types of pediatric solid tumors. For the purpose of this Ph.D. thesis, rhabdomyosarcoma cell lines were chosen. Rhabdomyosarcoma (RMS) – the most frequent type of soft tissue sarcomas – are characterised by deregulation of myogenesis and muscle differentiation and they affect mostly children and adolescents *(Xia et al. 2002; Teicher 2012; Keller and Guttridge 2013).*

The main aim of this Ph.D. thesis was to identify and characterize CSCs in chosen RMS cell lines. An introduction to this thesis consists of the overview of soft tissue sarcomas with special regard to the RMS and of the summary of recent knowledge in CSC research, especially in human malignancies. The major part of this thesis is focused on the experimental analysis of selected putative CSC markers in RMS cell lines followed by functional assays on CSC phenotype (colony forming assay, sphere formation assay, test of tumorigenicity *in vivo*) and by characterisation of xenograft cell lines. Final part of this thesis is aimed at a discussion of our findings in the context of recently published relevant studies in the field of CSC research.

1.1. Pediatric sarcomas

Sarcomas are a hetegeneous group of malignancies of mesenchymal origin. These tumors appear in connective tissues and they are usually classified according their histologenetical origin into two main groups: bone sarcomas and soft tissue sarcomas. Another classification of sarcomas is based on their molecular characterisation: the first group, involves sarcomas with specific genetic alterations (chromosomal translocations or activating mutations) and these tumors are more typical for older patients; the second group of sarcomas show complex karyotypic abnormalities and are often diagnosed in children *(Anderson et al. 2012; Teicher 2012).*

Biological differences between adults and children are also noticeable in this group of malignancies: pediatric sarcomas have different incidence; etiology; growth parameters, and they have also higher metastatic potential. Children and adolescents better respond to the treatment, such as radiotherapy and chemotherapy and this is associated with their higher five-year survival rate (approximately 80%, however, significant proportion of children still suffer from recurrence of the disease and from metastases *(Friedman and Gillespie 2011; Anderson et al. 2012; Dela Cruz 2013).*

1.1.1. Bone sarcomas

Diagnosis of primary bone sarcomas is very difficult. These tumors affect cartilages and bones, i.e. parts of the organism, in which could metastasize other types of cancers, such as lung carcinoma, breast carcinoma or prostate carcinoma. Bone sarcomas are occasionally diagnosed in adults (less than 1%), but more often in children and adolescents (about 3%) usually in association with their fast bone growth. It exist more than 20 subtypes of bone sarcomas, which are classified into five groups in accordance with the International Classification of Childhood Cancer (ICCC):

a) **Osteosarcomas**
b) **Ewing sarcomas**
c) **Chondrosarcomas**
d) **Other specifies malignant bone tumors**
e) **Unspecified malignant bone tumors**

Osteosarcomas and Ewing sarcomas are the most frequent bone malignancies, which are diagnosed in children *(Bajciova et al. 2011; Friedman and Gillespie 2011).*

Osteosarcoma

Osteosarcoma is the most common, primary malignant tumor of bone that occurs predominantly in children and adolescents *(Teicher 2012)*. This tumor usually occurs between the ages of 10-16 years and a second peak of incidence is in older adults (30% of cases in patients over 40 years). Any bone in the body can be aggressed by osteosarcoma but the most affected areas are the distal femur, the proximal tibia and the proximal humerus, which represent sites containing the most proliferative growth plates (Fig. 1). The primary treatment for osteosarcoma consisting of a combination of surgery and chemotherapy allows remission in about 60% of patients; however, the prognosis remains poor due to chemoresistance and early metastases. In the most of patients treated for osteosarcoma, drug resistance is developed and these patients have a high risk of relapses and metastases, especially into lungs (*Dela Cruz 2013; Kushlinskii et al. 2016; Abarrategi et al. 2016; Serra and Hattinger 2016*).

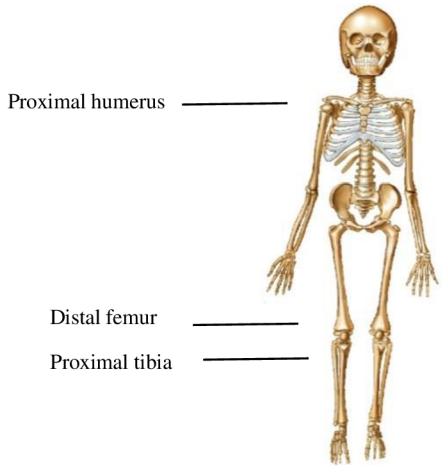

Fig. 1 The most common affected sites in osteosarcoma. The most common sites of osteosarcoma are presented (*Abarrategi et al., 2016*)**.** (image from: http://www.kidport.com/reflib/science/humanbody/skeletalsystem/HumanSkeleton.htm)

Ewing sarcoma

Ewing sarcoma is the second most common highly aggressive bone and soft tissue tumor in children and young adults. It's diagnosed by presence of fusion oncoprotein EWS-ETS, involving *EWS* gene, localized on chromosome 22, and the *ets*-type of gene of transcription factors on chromosome 11 (where belong FLI1, ERG, ETV1, ETV4 and FEV). Approximately 80% of cases of Ewing sarcoma have EWSR-FLI1 fusion, rarer translocation is EWS-ERG fusion in about 15% of cases *(Teicher 2012)*. The most

frequent areas affected by Ewing sarcoma are the pelvis, the femur, the humerus and the ribs. Five-year survival for localized tumors reaches 70%, but for patients with metastases and recurrent disease are chances of cure less than 20% *(Balamuth and Womer 2010).*

1.1.2. Soft tissue sarcomas

Soft tissue sarcomas represent a heterogeneous group of tumors developed in soft tissues of the body, such as muscles, tendons, fat, lymph and blood vessels, nerves and tissues surrounding joints. These tumors could develop in any part of the body, but the most frequently suffered parts are arms, legs and abdomen (Fig. 2). Soft tissues sarcomas are diagnosed in 6-8% of all malignancies in children and in 1% in adults. They metastasize at earlier stages of the disease, most often to lungs, lymph nodes and bone marrow *(Koutecky et al. 2002; Teicher 2012; Trucco and Loeb 2012).*

Soft tissue sarcomas are a group with a marked histological diversity, containing more than 50 subtypes. Classification of them is based on tumor morphology and three main subtypes can be distinguished:

Rhabdomyosarcomas and its subtypes

(embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, spindle cell/sclerosing rhabdomyosarcoma, pleiomorphic rhabdomyosarcoma)

Non-rhabdomyosarcomas and other types of soft tissue sarcomas

(leiomysarcoma, liposarcoma, synovial sarcoma, fibrosarcoma, malignant fibrous histiocytomas, alveolar soft – part sarcoma, malignant mesenchymoma, malignant peripheral nerve sheath tumor, hemangiopericytoma)

Nonspecific soft tissue sarcomas

All symptoms of soft tissue sarcomas depend on the localization of primary tumor in the body and they usually encompass edemas, later the pain and increased sensitivity in the affected part. There are no typical clinical symptoms for these tumors, and thus diagnostics is often very hard. Diagnostic procedure for the soft tissue sarcomas includes imaging methods (X-ray, CT, MRI and/or PET), histological and immunohistochemical examinations and cytogenetic or molecular genetic techniques

for detection of chromosomal translocations *(Koutecky et al. 2002; Bajciova et al. 2011).*

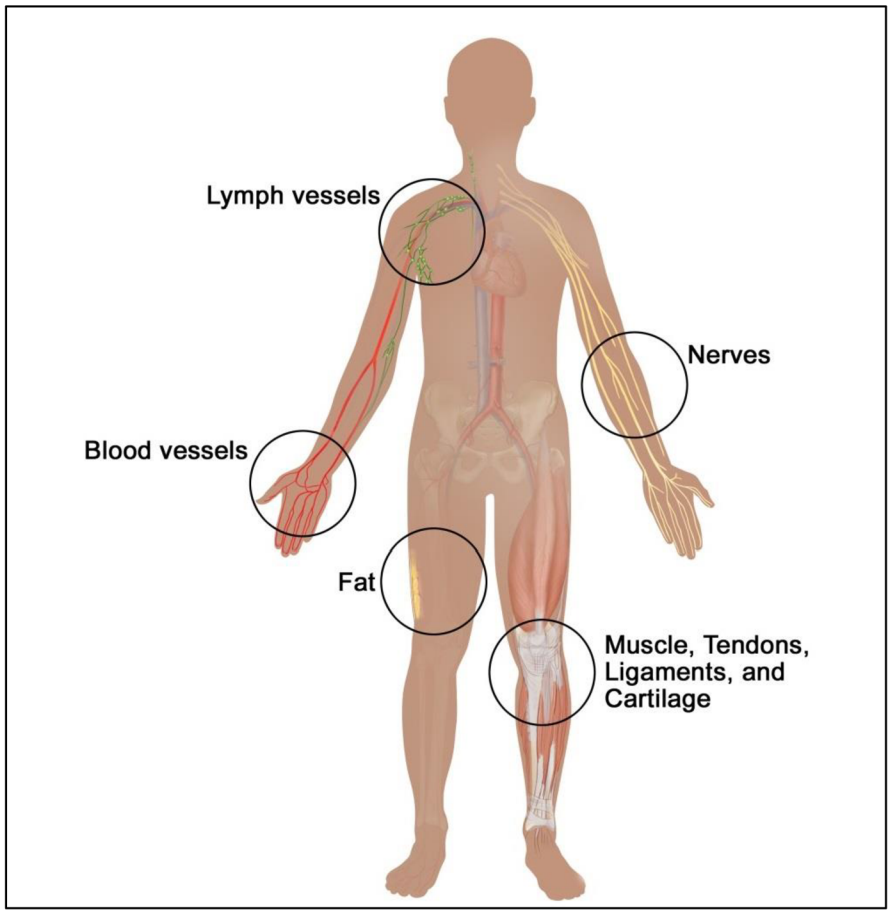

Fig. 2 The most common parts of the body affected by soft tissue sarcomas. The soft tissue sarcomas prefentially develops in arms and legs (*Koutecky et al. 2002*). (Image from: http://www.cancer.gov/types/soft-tissue-sarcoma)

1.2. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common type of the soft tissue sarcomas and the third most frequent extracranial solid tumor in children and adolescents. In adults RMS is joined with poor prognosis and with worse response to therapy *(Teicher 2012).* RMSs are associated with a disruption of myogenesis regulation and muscle differentiation. The origin of cells from which they arise is controversial. It could be muscle cells but it could develop even from non-muscle cells such as skull base (parameninges), salivary glands, biliary tree and genitourinary tract *(Merlino and Helman 1999; Xia et al. 2002; Hettmer and Wagers 2010; Keller and Guttridge 2013).*

1.2.1. Rhabdomyosarcoma subtypes

RMS are classified into four subtypes, each of them with different histological, genetic and clinical features: embryonal RMS, alveolar RMS, pleiomorphic RMS and spindle cell/sclerosing RMS. Embryonal and alveolar RMS are the major and the most frequent histological variants, and they are typical for children and young adults.

Chromosomal changes associated with certain subtypes of RMS were detected. On one hand translocations and amplifications of specific chromosomal region are typical for alveolar RMS, on the other hand, allelic losses and changes in chromosomal copy numbers are typical for embryonal RMS *(Kashi et al. 2015).*

Embryonal RMS

Embryonal RMS is the most common subtype of RMS (it counts about two thirds of all RMS) and occurs in children under 10 years of age. Chromosome gains (especially of chromosome 2, 8, 12, and 13) and allelic losses on chromosome 11, specifically in region 11p15.5, are typical for embryonal RMS. Within the region 11p15.5 are localized some tumor suppressor genes, namely *H19* (encoding a protein associated with cell differentiation) and *CDKN1C (p57/KIP2)* (encoding an inhibitor of several G1 cyclin/cyclin dependent kinase complexes), as well as *SLC22AIL (BWR1A)* gene (encoding a homolog to tetracycline resistance efflux proteins) and *IGF-II* gene (usually overexpressed in RMS). The region 11p15.5 is also associated with mutations of genes encoding RAS protein family, PIK3CA, CTNNB1 and FGFR4 *(Merlino and Helman 1999; Meyer and Spunt 2004; Xia et al. 2002; Chen et al. 2015; Dasgupta et al. 2016).*

Alveolar RMS

Alveolar RMS represents a RMS subtype with a worse prognosis and more aggressive growth than embryonal RMS. For this tumor are typical unique chromosomal translocations between chromosomes 2 and 13 [t(2;13) (q35;q14)] or between chromosomes 1 and 13 [t(1;13) (q36;q14)]. These translocations lead to the formation of *PAX3-FKHR* or *PAX7-FKHR* fusion oncogenes. The genes involved represent two transcription factor families: paired box transcription factor family (PAX family) and the forkhead transcription factor family (FKHR) *(Xia et al. 2002; Orkin et al. 2015).* The *PAX-FKHR* fusion proteins, negatively influence a function of myogenic genes especially it affect a suppression of terminal differentiation and protect tumor cells from apoptosis through the expression of antiapoptotic proteins, such as Bcl-xL *(Keller and Guttridge 2013).* In general the *PAX3-FKHR* fusion gene is more frequent and lead

to the more aggressive phenotype than *PAX7-FKHR* fusion oncogene *(Huh and Skapek 2010)*. Patients with alveolar RMS without the fusion protein PAX3-FKHR or PAX7-FKHR have a more favourable prognosis *(Arnold et al. 2016).*

Spindle cell / sclerosing RMS

Spindle cell RMS was originally classified as a variant of embryonal RMS, but it has been recently reclassified as a separate pathologic entity of RMS *(Rekhi and Singhvi 2014).* There have been identified biological similarities between spindle cell RMS and sclerosing RMS subtype and thus a new joint group in RMS classification was created. This RMS group displays two main characteristics: mutations in *MyoD1* gene with / without mutations in gene encoding PIK3CA or rearrangements in *NCOA2* gene *(Alaggio et al. 2016; Rekhi et al. 2016).* The most common tumor localization is paratesticular in children, which is associated with a better prognosis, and the deep soft tissue of the head and neck in adults, associated with worse prognosis *(Kashi et al. 2015).*

Pleomorphic RMS

Pleomorphic RMS represents a very rare RMS typical for adult population. There are no chromosomal aberrations typical for this subtype of RMS and an optimal treatment remains undefined. This tumor is diagnosed in males predominantly and it typically affects the head, neck, genitourinary tract and deep soft tissue of the extremities. It is associated with poor prognosis, high relapse rate, bad response to standard chemotherapy and high formation of metastasizes *(Kashi et al. 2015; Watanabe et al. 2016).*

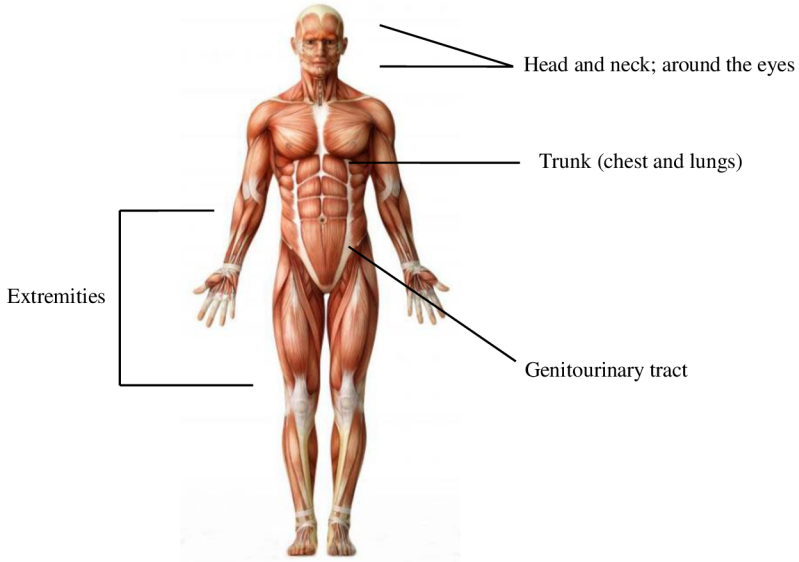

Fig. 3 The most commonly sites of rhabdomyosarcoma. The most common sites of rhabdomyosarcoma are presented (*Orkin et al. 2015*). (Image from:http://www.wisegeek.com/what-are-the-effects-of-exercise-on-the-muscular-system.htm#human-muscle-anatomy)

1.2.2. Clinical aspects and therapeutic approaches

RMS can be localised in all parts of the body. The localization may vary with sarcoma subtype: head/neck (the orbit, pharynx, sinuses) and genitourinary system (vaginal wall, paratesticular tissues) are typical for embryonal RMS, whereas alveolar RMS occurs within skeletal muscles of trunk and limbs (Fig. 3) *(Xia et al. 2002; Kashi et al. 2015; Orkin et al. 2015).*

Prognosis of a specific patient depends on sarcoma subtype, tumor size, tumor localization, stage of the disease, affecting of lymph nodes, presence/absence of metastases, and also on age at the time of diagnosis *(Hiniker and Donaldson 2015).* Patient suspected for RMS is examined with appropriate imaging system (MRI, CT, FDG/PET) to detect primary tumor followed. After biopsy and histopathological examination, molecular techniques are used to precise a diagnosis *(Orkin et al. 2015).* In general, RMS are diagnosed using muscle-specific and myogenesis-related markers such as MyoD, myogenin, myoglobin, myosin heavy chain, skeletal α-actin and desmin *(Keller and Guttridge 2013; Merlino and Helman 1999).*

Tab. 1 Main clinical features of ERMS and ARMS *(Orkin et al. 2015).*

	ERMS	ARMS
Age	3	0 - 19
Location	Central, not muscular	Peripheral, muscular
Invasiveness	Low/moderate	High
Metastases	Lung	Everywhere

Nowadays, risk-stratification system developed/used by the Children's Oncology Group Soft-Tissue Sarcoma Committee (COG) is used for set-up of effective RMS treatment. This stratification system encompasses the pretreatment (TNM) staging system (based on the location and size of tumor and presence/absence metastases) and clinical grouping system (based on the removal of tumor by surgery and extension of cancer). According this system patients with diagnosed RMS are stratified into three groups:

- Low-risk RMS patients
- Intermediate-risk RMS patients
- High-risk RMS patients

In the next step an appropriate treatment combining chemotherapy, radiation therapy and surgery is chosen for specific patient *(Huh and Skapek 2010; Dasgupta et al. 2016).*

Before introduction of chemotherapy into treatment of RMS, survival rate of patients was very poor. Nowadays used different protocols are used for patients in different groups of risk, but the most useful combination for RMS treatment still includes vincristine, dactinomycine (actinomycine D) and cyclophosphamide (VAC) *(Huh and Skapek 2010; Malempati and Hawkins 2012; Orkin et al. 2015; Dasgupta et al. 2016).*

Low-risk patients show the best results in treatment of RMS. The treatment of these patients is currently focused on minimalization of long-term effects, toxicity and duration of therapy. Patients in this group are treated with VAC, but better results are achieved with a combination with vincristine-irinotecan (VI) therapy. Comparing to the previous studies marked reduction is possible in cyclophosphamide doses, which can

cause infertility, myolosuppresion and problems with infections *(Orkin et al. 2015; Dasgupta et al. 2016).*

Standard therapy for intermediate-risk patients consists of alternations in blocks of VAC and vincristine/irinotecan therapy for 1 year. In this group of patients, irradiation is also involved at week 4 of therapy *(Huh and Skapek 2010; Malempati and Hawkins 2012).*

The poorest results are achieved for high-risk RMS patients. A treatment combining vincristine, doxorubicin and cyclophosphamide alternating with ifosfamide and etoposide and with courses of vincristine and irinotecan is usually used, where some of these compounds are accompanied by radiation therapy *(Huh and Skapek 2010; Orkin et al. 2015).* The COG considers also addition of temozolomide and cixutumab to the treatment of RMS *(Malempati and Hawkins 2012).*

Radiation therapy (RT) is used together with chemotherapy and surgery almost in all patients suffer for RMS. The key role play in cases, where is unable to use surgery, as in cases, where tumor disrupt skull base, cause spinal cord compression and advance into intracranial space *(Meyer and Spunt 2004).* Application of radiotherapy brings also questions like time, when and how long use RT and in what cases bring benefits or not. In RMS patients with good prognosis is using of RT very rare, cause damage of surrounding non-tumor tissues and decrease late-effects of radiotherapy. In other worse-prognosis cases is used of RT necessarily with doses ranging from 36 to 50.4 Gy *(Dasgupta et al. 2016).*

Nowadays are applied new approaches into irradiation to spare normal surrounding tissue and avoid secondary neoplasms. Promising methods for RMS patients are brachytherapy, intraoperative radiation therapy, intensity-mediated radiation therapy, but the most promising method is proton therapy (Ladra *et al.* 2014; Orkin *et al.* 2015; Leiser *et al.* 2016).

To improve the outcome of RMS patients, especially those of intermediated-risk and high-risk, it is very important to better understand the biology of RMS. The RMS origin is often unclear, because there may be present evidences for muscle cell origin (i. e. from muscle satellite cells, embryonic myoblast, myocyte) *(Huh and Skapek 2010)* but also for non-muscle cells of origin such as from mesenchymal stem cell *(Charytonowicz et al. 2009).*

Another possibility how to achieve better survival rate for RMS patients is identification of new therapeutic targets. As a promising therapeutic aims were identified Inhibition of mTOR pathway by FANCD2 (mediator of DNA repair pathway) *(Singh et al. 2014*) by rapamycin *(Houghton et al. 2008; Kaylani et al. 2013)* inhibition of fusion protein PAX3-FOXO1 or its targets (such as PDGFRA by thapsigargin) *(Epstein et al. 1998; Taniguchi et al. 2008; Jothi et al. 2013)*; fibroblast growth factor receptor 4 *(Crose et al. 2012)*; MET *(Taulli et al. 2006; Hou et al. 2011)* and simultaneous inhibition of anaplastic lymphoma kinase and insulin-like growth factor-1 receptor *(van Gaal et al. 2013)* were identified as promising new therapeutic targets for RMS treatment.

High-risk RMS patients, especially those, who have worse response to the standard chemotherapy and who have metastases, are appropriate candidates for high-dose chemotherapy followed by autologous peripheral blood stem cell transplantation. These protocols of experimental therapy showed promising results in some cases *(Kim et al. 2012; Ishi et al. 2016),* but other studies consider this treatment not sufficiently effective *(Bisogno et al. 2009; Peinemann et al. 2011)* .

Another promising target for new therapeutic approaches is focused on cancer stem cells in RMS. Recently, cancer stem cells were identified in many human malignancies including sarcomas and they were characterized in detail and analysed for their possible use as therapeutic targets.

1.3. Cancer stem cells

Cancer stem cells (CSCs), also called tumor-initiating cells (TICs), are present in the solid tumors as a small subpopulation of tumorigenic cells that can be distinguished from the majority of other tumor cells within hierarchically organized tumor tissue *(Akbari-Birgani et al. 2016)*. The first hypothesis concerning hierarchic organization of solid tumors is dated back more than 40 years but the first evidence of CSCs was given for acute myeloid leukemia cells by Bonnet and Dick in 1997. They showed that a specific group of leukemic cells expressing CD34 cell surface antigen and lacking CD38 were capable initiate leukemic disease after injection into immunosuppressed NOD/SCID mice *(Frank et al. 2010; Allegra and Trapasso 2012)*.

The most important characteristics of CSC are a capability of self-renewal and an ability to give rise more differentiated cell populations containing one or more cell types; these features allow CSCs to produce heterogeneous lineages of specialized cells that comprise the tumor *(Komuro et al. 2007; Frank et al. 2010; Beck and Blanpain 2013)*. CSCs persist in the tumor tissue and may cause metastases and they are also responsible for recurrence of the disease *(Jiang et al. 2012; Ciurea et al. 2014)*. Presence of CSCs has been identified not only in hematologic malignancies but also in many types of solid tumors including colorectal, prostate, breast, ovarian, pancreatic, brain, head and neck cancers *(Podberezin et al. 2012)*.

1.3.1. Cancer stem cell theory

Despite intensive research in this field, the origin of CSCs remains unknown. There have been formulated two main theories concerning explanation of tumorigenesis: the clonal evolution theory and the cancer stem cell theory. These theories have been explaining common features of many human malignancies and the phenomenon of tumor heterogeneity. Both of these theories point out to the existence of cells within a tumor bulk that are capable initiate the tumors in distant locations.

The first model, i.e. the stochastic model or the clonal evolution model, assumes that cells within tumor bulk are biologically homogeneous with an identical tumorigenic potential (Fig. 4A). According this model, all tumor cells have the same potential to initiate new tumors. Tumor heterogeneity is a consequence of random influences (e.g. levels of transcription factors, activation of signalling pathways, microenvironment, immune response) and genetic mutations that alter the behaviour of individual cells within the tumor bulk *(Dick 2009; Podberezin et al. 2012; Beck and Blanpain 2013; Bradshaw et al. 2016)*.

The second model, i.e. cancer stem cell model or hierarchical model, represents one of the most topical and controversial issues in the field of cancer research (Fig. 4B). This model assumes that a tumor represents morphologically and phenotypically distinct populations of cells, in which CSC are found at the beginning of this cellular hierarchy and they are essential for tumor initiation, progression and metastasis *(Frank et al. 2010; Allegra and Trapasso 2012; Kreso and Dick 2014)*.

The evidence of cancer heterogeneity and CSC theory was confirmed by aldehyde dehydrogenase (ALDH)-positive cells isolated from human breast tumors that contained

the subpopulation of cells showing a differentiation potential and these cells have capability of self-renewal as well as the ability to generate tumors in NOD/SCID mice *(Ginestier et al. 2007)*. ALDH-positive cells, which also fulfill the criteria for CSCs, were also identified in melamona *(Luo et al. 2012)*, colon *(Huang et al. 2009)*, brain *(Corti et al. 2006)* and liver cells *(Ma et al. 2008)*. Presence of CSCs was also confirmed in other types of human malignancies including malignant germ cell tumors, leukemias and breast carcinomas *(Shackleton et al. 2009)*.

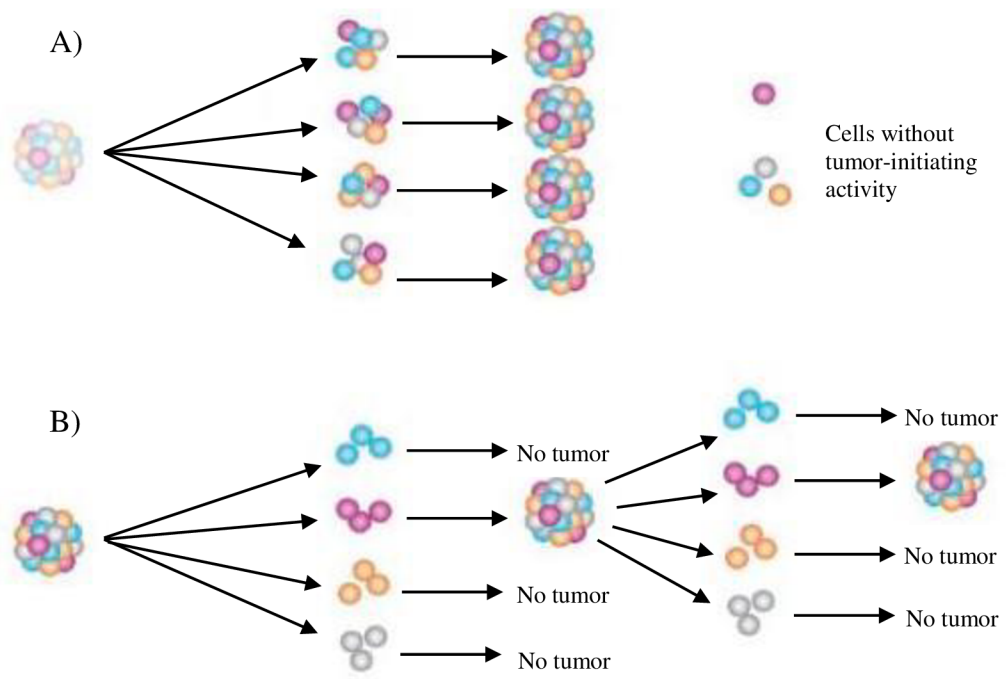

Fig. 4 Models of tumor heterogeneity: A) Stochastic model B) CSC model. (A)The stochastic model of tumor heterogeneity assumes that tumor cells are biologically equivalent and tumor heterogeneity is a consequence of random influences. (B) CSC model assumes the existence of biologically distinct classes of cells, where only subset of cells, CSCs, have the ability to initiate tumor growth (B) (Adapted and modified from *Dick 2009*).

Recently, some evidences support the possibility that clonal evolution and CSC models are not mutually exclusive. This dynamic model assumes a subpopulation of cancer stem cells that have potential to switch between non-tumorigenic and tumorigenic cell states in response to various stimuli or endogenous factors exist within the tumor bulk (Fig. 5) *(Cabrera et al. 2015)*. This concept of cancer stem cell plasticity was confirmed by the Weinberg group describing CSCs able to rise *de novo* from more differentiated cell *(Chaffer et al. 2011)*. Furthermore, these researchers found that the

dedifferentiation of non-CSC is enabled by the transcription factor ZEB1 (a key regulator of epithelial-mesenchymal transition), which allow the differentiated cells to respond to the microenvironment signals and to change the phenotype to the CSC state *(Chaffer et al. 2013)*. Two independent groups also confirmed the model of dynamic stemness in glioma and colon cancer *(Oshima et al. 2014; Suvà et al. 2014)*. These researchers identified two groups of transcription factors (POU3F2, SOX2, SALL2 and OLIG2 for glioblastoma cells; OCT3/4, SOX2 and KLF4 for colon cancer cells, respectively) which are capable to reprogram the fully differentiated cancer cells to the stem-like cells *(Oshima et al. 2014; Suvà et al. 2013)*.

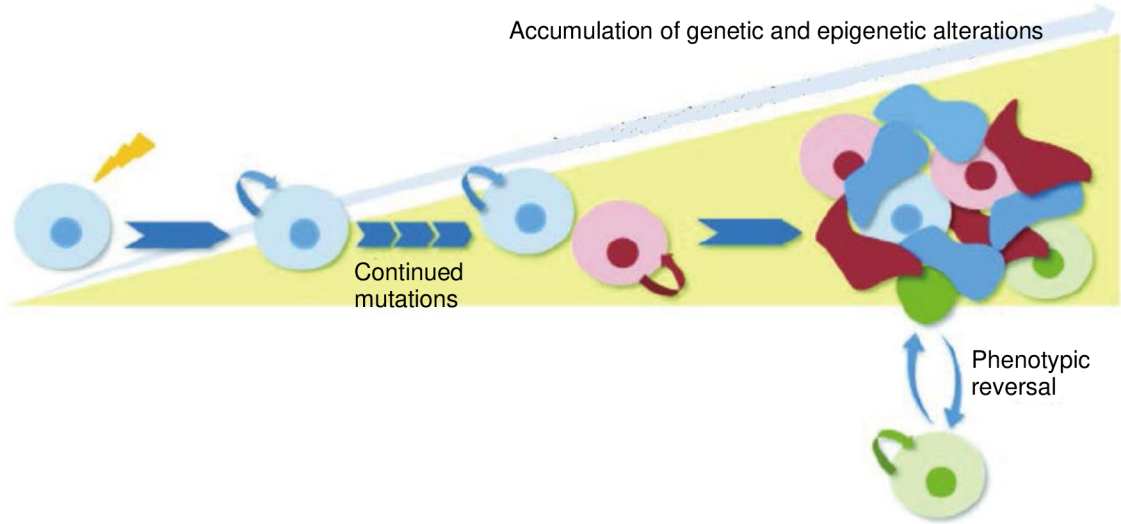

Fig. 5 Model of cancer stem cell plasticity. The model of cancer stem cell plasticity assumes that in tumor persist several subclones of CSC that over time accumulate epigenetic and genetic changes. Each subclones of CSC differs and could give rise to intermediate transit-amplifying progenitors (green) or more differentiated cancer cells. The progenitors would lack self-renewal capabilities, but still continue to accumulate genetic changes and could gain again the self-renewal potential (Adapted and modified from *Cabrera et al. 2015)*.

Nowadays, several in vitro and in vivo assays are available for studies and detection of the presence of CSCs in solid tumors *(Akbari-Birgani et al. 2016)*. The detection of putative or proven CSC markers and especially their combinations indicating CSC phenotype and the functional assays assess whether cancer cells have the ability to behave like CSCs or not *(Trucco and Loeb 2012)*. Moreover, there are several signalling pathways known to be associated with stem-like differentiation potential, e.g. Notch, Wnt/β-catenin or mTOR *(Jiang et al. 2012)*.

1.3.2. Specific markers of cancer stem cells in soft tissue sarcomas

Presence or absence of specific cell surface or intracellular molecules and their combination may indicate the CSC phenotype. Nevertheless, some of them were similar to markers associated with growth, metastasizing and recurrence of various solid tumors *(Jiang et al. 2012)*. Identification of specific markers or their combination followed by isolation of CSCs seems to be a first step in targeted cancer treatment *(Ciurea et al. 2014)*. Several protein markers including ABC transporters, CD133, nestin, aldehyde dehydrogenase *(Veselska et al. 2012; Dela Cruz 2013)*.

ABC transporters

The ATP-binding cassette (ABC) transporters superfamily consist of 48 genes classified into 7 subfamilies, ABCA through ABCG *(Dean et al. 2005)*. Typical structure of all ABC proteins have the same architecture: two transmembrane domains, containing 6 trans-membrane segments, and two highly conserved nucleotide binding domains (Fig. 6) *(Kavishe et al. 2009; Mo and Zhang 2011)*. These ABC transporters, function of which depend on ATP hydrolysis, serve in cells as efflux pumps for lipids, various drugs, natural products and peptides *(Choi 2005; Locher 2016)*. Ability of cells to efflux fluorescence dyes under in vitro conditions is considered as a promising method for identification of stem cells in different tissues including tumors *(Komuro et al. 2007; Golebiewska et al. 2011)*. Cell subpopulation achieved with this model is characterized by high efflux of a fluorescence dye binding to DNA, typically Hoechst 33342 or rhodamine123 *(Komuro et al. 2007)*. This subpopulation is termed side population due to its position in the dot plot achieved by flow cytometry *(Goodell et al. 1996)*. Side population was identified in many types of cancers and cells in this subpopulation mostly display all features of CSCs including self-renewal and tumorigenicity after transplantation into immunocompromised mice *(Golebiewska et al. 2011)*.

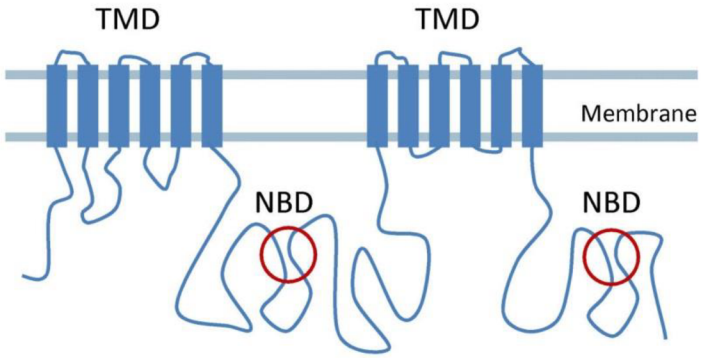

Fig. 6 Schematic structure of an ABC transporter. An ABC transporter consist of two transmembrane domains (TMD), which anchor the transporter into lipid membrane, and two nucleotide binding domains (NBD), which bind ATP molecules *(Kavishe et al. 2009)*.

ABC transporters are also considered as a causation of multi-drug resistance of cancer cells and subsequent failure of chemotherapy; especially ABCB1 (P-glycoprotein/P-gp/MDR1), ABCG2 (Breast Cancer Resistance Protein/BCRP) and ABCC1 (multidrug Resistance Protein 1/MRP1) transporters are usually discussed regarding multi-drug resistance *(Fletcher et al. 2016; Horsey et al. 2016)*. Side population assay has been successfully used for identification of a subpopulation of cells with increased resistance to chemotherapeutics and with CSC phenotype in cell lines derived from ERMS *(Komuro et al. 2007)*.

Prominin-1 (CD133)

Prominin-1 (CD133) is cholesterol-interacting pentaspan membrane glycoprotein originally identified in mouse neuroepithelial stem cells and later also in human tissue *(Fargeas et al. 2003)*. This protein is characterized by extracellular N-terminus, cytoplasmic C-terminus, 2 small cysteine rich cytoplasmic loops and 2 large extracellular loops each containing 4 potential sites for N-linked glycosylation (Fig. 7). AC133, its glycosylated epitope, is recently considered as a real universal marker of CSCs *(Kemper et al. 2010; Irollo and Pirozzi 2013)*. CD133 was found in three types of apical membrane protrusions: in microvilli, in primary cilium and in midbody, which are involved in remodelling of epithelial cells during (neuro)epithelial differentiation *(Corbeil et al. 2010)*. CD133 was frequently studied as a putative CSC marker, although its physiological role in the cell and a relationship between CSC phenotype and CD133

expression remains unclear *(Grosse-Gehling et al. 2013; Schmohl and Vallera 2016)*. Some studies discussed involvement of CD133 in canonical Wnt signalling *(Mak et al. 2012; Rappa et al. 2013)* and in PI3K/Akt pathway *(Takenobu et al. 2011; Kimura et al. 2012; Wei et al. 2013)*.

CD133 and nestin expression was identified in RMS and derived RMS cell lines, where in these cell lines were identified subpopulation of $CD133^{+}$/$nestin^{+}$ cells. Next was demonstrated the capability of NSTS-11 RMS cell line to form tumors in the immunodeficient mice and presence of putative cells with CSC phenotype *(Sana et al. 2011)*. These findings were supported by another research group, where CD133+ cells had tumorigenic potential and showed higher resistance to chemotherapeutic drugs *(Walter et al. 2011)*.

A subpopulation of putative CSCs with double positivity of $CD133^{+}$ and $ALDH^{high}$ was identified also in SW72 liposarcoma cell line. These cells displayed higher capability to form tumors in comparison with parental cell population or with cells positive for only one of these markers *(Stratford et al. 2011)*.

CD133 positive subpopulation in HT1080 human fibrosarcoma cell line showed an increased resistance to chemotherapeutics, high proliferation rate and ability to form tumors *(Feng et al. 2013)*. Expression of CD133 in synovial sarcomas was detected in five primary tumor samples and three synovial sarcoma cell lines but CSC properties of these CD133+ cells were not confirmed by any functional test *(Terry and Nielsen 2010)*.

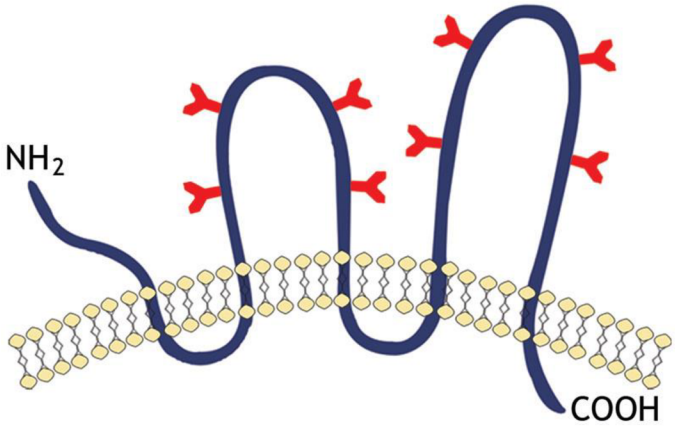

Fig. 7 Structural model of CD133 molecule. CD133 molecule consist of extracellular N-terminus (NH2), cytoplasmic C-terminus (COOH), 2 small cysteine rich cytoplasmic loops and 2 large extracellular loops each containing 4 potential sites for N-linked glycosylation (red labels) *(Irollo and Pirozzi 2013)*.

Nestin

Nestin is a cytoskeletal protein belonging to the class VI of intermediate filaments. It is known as a marker of neural stem/progenitor cells and its expression in nervous tissue during embryonic development and in subventricular zone in an adult organism *(Michalczyk and Ziman 2005)*. Expression of nestin was also detected in immature or progenitor cells in non-neuronal cells in normal tissues and during reparation processes *(Ishiwata et al. 2011)* as well as in primary tumors and metastases of various human malignancies, such as brain malignancies *(Strojnik et al. 2007)*, breast carcinoma *(Zhao et al. 2014)*, colorectal carcinoma *(Teranishi et al. 2007)*, pancreatic adenocarcinoma *(Ishiwata et al. 2011)* and prostate carcinoma *(Kleeberger et al. 2007)*. As putative marker of CSCs, nestin was identified in brain tumor stem cells for the first time and later in other types of human malignancies *(Neradil and Veselska, 2015).*

Although nestin expression was reported in rhabdomyosarcomas many years ago (Kobayashi et al. 1998) nowadays exists published work where in RMS and their derived cell lines was identified subpopulations of nestin positive and CD133 positive cells. In derived RMS cell lines was also identified subpopulation of $CD133^+$/$nestin^+$ cells and the presence of cells with CSC phenotype was demonstrated by tumorigenicity assay, where injected NSTS-11 cell line was capable of tumor growth after injection into immunocompromised mice *(Sana et al. 2011)*.

The same results were achieved with osteosarcomas and their derived cell lines, which showed positivity for subpopulations of nestin positive and CD133 positive cells, where in some of these osteosarcoma cell lines was proved $nestin^+$/$CD133^+$ double positivity, which suggest presence of cells with CSC phenotype *(Veselska et al. 2008)*.

Aldehyde dehydrogenase

The human aldehyde dehydrogenase (ALDH) superfamily contains 19 know genes in 11 families and 4 subfamilies with distinct chromosomal locations. They are responsible for oxidation of endogenous and exogenous aldehydes, including drugs and environmental toxicants *(Black et al. 2009)*. After the discovery of ALDH activity in human and mouse bone marrow hematopoietic progenitor cells, the properties and locations of ALDH-positive cells have started to be investigated *(Jones et al. 1995).* Recent studies indicate that high activities of ALDH enzymes, especially of ALDH1A1 and ALDH1A3, are characteristic for stem cells as well as CSC *(Ma and Allan 2011;*

Marcato et al. 2011). Increased activities of ALDH1 enzymes described in leukemias *(Hoang et al. 2015)*, ovarian carcinoma *(Kryczek et al. 2012)*, melanoma *(Luo et al. 2012)*, prostate carcinoma *(Li et al. 2010)*, head and neck squamous cell carcinoma *(Clay et al. 2010)*, pancreatic adenocarcinoma *(Rasheed et al. 2010).* These studies showed that cells with high ALDH1 expression are highly tumorigenic and have enhanced stem cell characteristics both *in vitro* and *in vivo (Ma and Allan 2011; Marcato et al. 2011; Trucco and Loeb 2012).*

Activity of ALDH was originally measured by immunoblotting or by enzyme kinetics but these techniques require lysis of the cells. More useful approach is examination of ALDH activity in viable cells. At present "the gold standard" in measurement of ALDH activity is ALDEFLUOR^{TM} flow cytometric assay, originally used for isolation of hematopoietic stem cells but now used for isolation of CSCs of many cancers *(Marcato et al. 2011).*

In ERMS, it was showed that ALDH^{high} cells have a higher capacity of self-renewal, ability of tumor formation and they also were more resistant to chemotherapeutics. Detailed analysis also revealed high expression levels of transcription factor Sox2 in ALDH^{high} cells. These findings indicate that ALDH might be a putative marker of CSCs in ERMS *(Nakahata et al. 2015).*

As mentioned above, a subpopulation of putative CSCs positive both for CD133+ and ALDH^{high} was identified also in SW872 liposarcoma cell line and these cells were proven as tumorigenic *(Stratford et al. 2011).* Further, activity of ALDH1 was detected in MUG-Myx1 myxofibrosarcoma cell line: ALDH high cells displayed increased expression of c-Myc, Sox2 and E-cadherin, upregulation of ABC transporters ABCB1 and ABCG2, higher potential of tumorigenicity and higher proliferation rates. All these results suggest that ALDH^{high} cells in MUG-Myx1 myxofibrosarcoma cell line have a CSC potential and they could be a promise target for cancer treatment *(Lohberger et al. 2013).*

Sox2, Oct 4 and Nanog transcription factors

These transcription factors play a unique role in regulation of gene expression and they are associated with the maintenance of pluripotency, i.e. with the ability of cells to differentiate into any cell type of the organism. Despite of many putative markers of pluripotency, only four transcription factors (Sox2, Oct4, c-Myc and KLF4) were

proven as essential for pluripotency induction in adult mouse fibroblasts *(Takahashi and Yamanaka 2006)*. Published studies suggest that the altered expression of Sox2, Oct4 and Nanog transcription factors, their isoforms and pseudogenes play a crucial role in tumorigenesis and metastasizing. Expression levels of these three factors are usually higher in tumor cells or CSCs if compared with non-tumor tissue. Moreover, their expression in tumor tissue indicates poor prognosis for the patient *(Liu et al. 2013)*.

Nanog, a transcription regulator with DNA binding domain, is involved in embryonic stem cell proliferation, renewal and pluripotency and was detected also in several tumors *(Mato Prado et al. 2015)*.

Octamer-binding transcription factor 4 (Oct-4), also known as POU5F1 (POU domain class 5 transcription factor 1), is a transcription factor of the POU family, which contain the DNA binding POU homeodomain. Under i*n vivo,* occurs in the inner cells of the blastocyst and together with other regulators may activate target genes in specific cell types at defined developmental stage *(Pan et al. 2002)*. It was demonstrated that Oct-4 expression may regulate the maintenance the tumor survival by inhibiting of apoptosis through the specific Oct4/Tcl1/Akt1 pathway *(Mirza et al. 2004)*.

Sox2, also known as SRY - box 2, belongs to the SRY-related High Mobility Group transcription factor family. Sox2 regulates the self-renewal of the mouse and human embryonic stem cells and it maintains the self-renewal in adult tissues including the brain and trachea *(Driessens and Blanpain 2011; Abdelalim et al. 2014; Weina and Utikal 2014)*. Interesting association of Sox2 expression, EWS-Fli-1 fusion and microRNA-145 (miRNA145) was observed *(Riggi et al. 2010)*. They revealed repressive feedback loop between EWS-Fli-1 (associated with Ewing sarcoma family tumors) and miRNA145 and identify their common target gene, Sox2, in pediatric mesenchymal stem cells. Many studies showed amplification of Sox2 in human malignancies and it was presumed, that Sox2 could be responsible for initiated tumor formation in immunocompromised mice. Murine osteosarcoma cell lines were capable to form osteospheres and tumors in mice and they express the hematopoietic stem cell marker Sca-1. These results were verified by Sox2 knock-down cells, in which dramatically decrease expression of the Sca-1 antigen, tumor formation and fail to form osteospheres *(Basu-Roy et al. 2012)*. Similar findings were reported also by our research group, when only Sox2-positive sarcoma cell lines were capable initiate tumors in the immunocompromised mice *(Skoda et al. 2016)*.

1.3.3. Functional tests of cancer stem cell phenotype

Detection of CSC markers is used for identification of cells with the CSC phenotype, but for verification of the hallmarks of CSCs, namely self-renewal and lineage capacity is necessary to apply the functional assays. These assays are designed as *in vitro* assays (sphere formation assay, label retention cell assay and colony formation assay) and *in vivo* test of tumorigenicity, which is recognized as a "gold standard" for identification of CSCs *(Clarke et al. 2006)*.

Colony formation assay and clonogenicity assay

The colony formation assay is used for verification of the survival and proliferative capability of CSCs. This method is based on the determination of ability of CSCs to proliferate and create colonies in a semisolid medium or methylcellulose-based culture medium. Colonies with 30-70 cells per colony are formed in 14-21 days after inoculation. They are stained with crystal violet, counted and compare with colonies derived from non-CSC fraction *(Podberezin et al. 2012; Wang et al. 2014)*. For example, the increased capacity of CD133+ cells to form colonies *in vitro* was confirmed using this method in ERMS and ARMS.

The clonogenicity assay is used to evaluate the clonogenic capacity of cells. The single-cell suspension of cells is seeded in serum-containing medium on conventional cell culture plates, which could be pre-coated with Matrigel *(Cao et al. 2011)*. Automatic cell clonogenicity assay, combining the clonogenicity assay with flow cytometric cell sorting into 96-well microplate was established to simplify the assay protocol and to reduce steps with cell manipulations *(Fedr et al. 2013)*. This modification could be used in high-throughput screening of CSC marker or potential chemotherapeutic drugs.

Sphere formation assay

Sphere formation assay allowed the identification of CSCs using their capability of self-renewal and differentiation at the single-cell level under *in vitro* conditions *(Pastrana et al. 2011)*. First cultured spheres were obtained from isolated cells from subventricular zone of adult brain in non-adherent conditions in serum-free medium in the presence of epidermal growth factor *(Reynolds and Weiss 1992)*. In this assay, cells are analysed for

expression of selected CSC marker and subpopulations of positive and negative cells for specific marker/s are isolated using cell sorter. These isolated cells are then cultivated in non-adherent conditions, in serum-free medium with defined growth factors (epidermal growth factor, EGF, fibroblast growth factor FGF) and supplements. To verify the self-renewal potential, spheres should be serially passaged, generating secondary and tertiary spheres with a cellular composition similar to the primary spheres (Fig. 8) *(Alison et al. 2011; Dontu et al. 2005).*

Capability of cells to form spheres was proved in many types of tumor tissues and these spheres are usually described according to the histological origin of cells: neurospheres *(Reynolds and Weiss 1992)*, hepatospheres *(Cao et al. 2011)*, prostaspheres *(Rybak et al. 2011),* rhabdospheres *(Walter et al. 2011)* or osteospheres *(Tirino et al. 2008).*

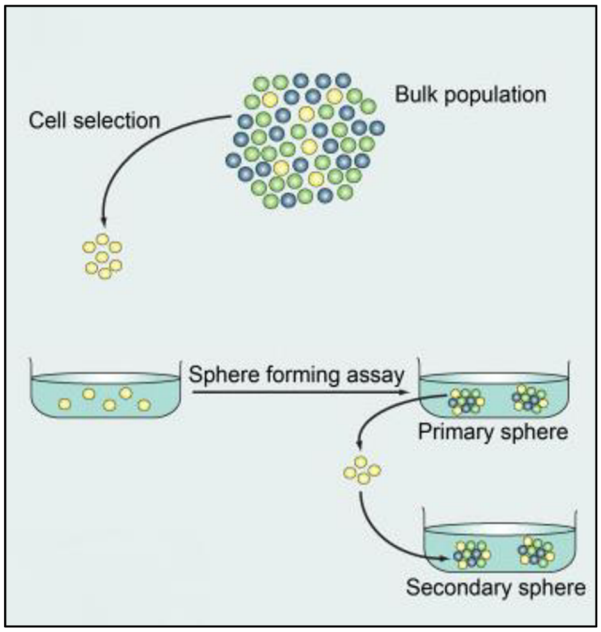

Fig. 8 Sphere assay. Subpopulation of selected cells (positive or negative for specific marker) is seeded in non-adherent conditions, in serum-free medium containing defined growth factors. After generating primary sphere are cells serially passaged, generating secondary and tertiary spheres to verify the self-renewal potential of cells *(Alison et al. 2011).*

Ability of CSCs to form rhabdospheres was proven in ERMS cell lines. Detail analyses of rhabdospheres showed the upregulation in gene expression of so-called stem cell genes such as *POU5F1, NANOG, SOX2, C-MYC, PAX3* and the higher ability of rhabdospheres to form tumors in comparison with adherent cells. Increased level of

CD133 was confirmed in rhabdospheres. Furthermore, the $CD133^+$ showed an increased tumorigenicity at lower cell densities and they were more resistant to cisplatin and chlorambucil, in contrast to $CD133^-$ cells *(Walter et al. 2011).*

The presence of CSCs was confirmed also in HT1080 fibrosarcoma cell line. In this study, the sphere-forming tumor cells were compared with their adherent counterparts. In case of sphere cells were proved stem-like potential with higher tumorigenicity, stem cell-related genes and drug resistance, compare to adherent cells without additional sorting to potential CSC marker *(Liu et al. 2012).*

Label retention assay

The label retention assay was designed to identify the asymmetric cell division of CSCs resulting in existence of two daughter cells, one of them with CSC phenotype and the second differentiating into tissue-specific cell phenotype. PKH26 and PKH6 dyes binding to the cell membrane are employed in this test. After symmetric cell division, both daughter cells contain the same amount of dye. In contrast, CSCs proliferate more slowly and thus they maintain higher quantity of this dye for longer period of time than non-CSCs (*Podberezin et al. 2012*). For example, this method was used for identification of CSCs in osteosarcoma cell populations (*Tirino et al. 2013).*

Tumorigenicity assay

Tumorigenicity assay *in vivo* is used for confirmation of self-renewal capacity of CSCs and their ability to form tumor during serial propagation in laboratory animal *(Liu et al. 2013).* In general, tested cell populations are injected into immunocompromised - typically NOD/SCID – mice. The injection into appropriate part of body related to the primary tumor is recommended, most frequently the subcutaneous to the back legs. The tumors are developed after several weeks. To show self-renewal potential of cells are cells from primary tumor are isolated and grafted into a second recipient animal to prove a presence and self-renewal potential of CSC within the primary tumor (Fig. 9) *(Clarke et al. 2006).* In this assay, the amount of cells injected into a mouse, the time to the first appearance of the tumor, the differences between mice with injected positive or negative cells should be taken into account during evaluation of experiments.

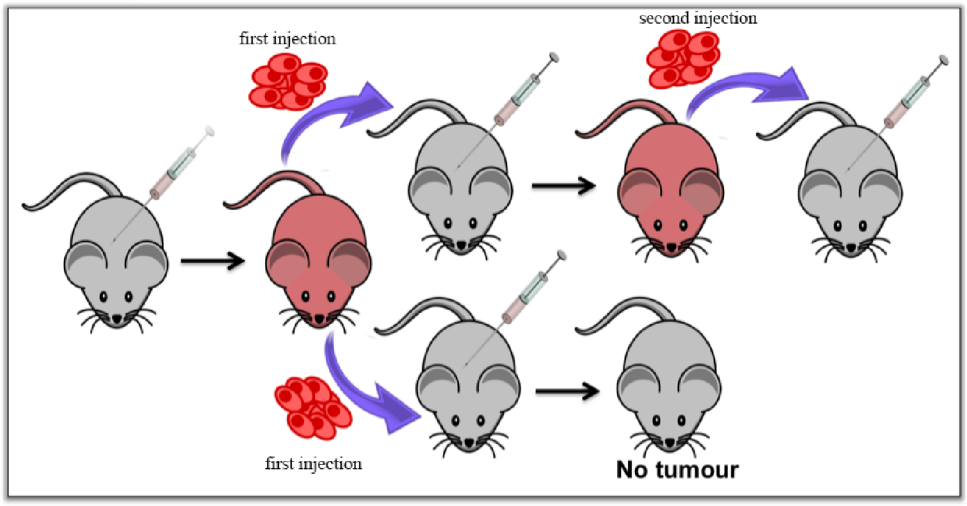

Fig. 9 Tumorigenicity assay: serial xenotranplantation of CSC. Population of tested cells is injected into immunocompromised mice. After several weeks primary tumors are generated. Cells from primary tumor are isolated injected into a second recipient animal (Image from: http://www.signalsblog.ca/inside-a-cancer-stem-cell-researchers-toolbox-xenotransplantation/).

In sarcoma research, the presence of cells with CSC phenotype using tumorigenicity assay was confirmed in ERMS cell line, where all injected mice with this cell line developed subcutaneous tumors *(Sana et al. 2011)*. Another study with ERMS cell lines (RD, Rh36 and Ruch2) showed higher tumorigenicity and chemoresistance in sorted CD133+ subpopulation of cells than CD133- subpopulation *(Walter et al. 2011)*.

Interesting results were achieved in the study of fibroblast growth factor receptor 3 (FGFR3) as a possible prognostic marker for RMS: $FGFR3^{+}$ cells were more tumorigenic than $FGFR^{-}$ cells *(Hirotsu et al. 2009)*. In SW872 liposarcoma cell line, higher capability of cells with double positivity for $CD133^{+}$ and $ALDH^{high}$ cells to form the tumors was showed using test *(Stratford et al. 2011)*.

2. Aims

Our cooperation with the Department of Pediatric Oncology (University Hospital Brno) gave us the opportunity to identify and to analyse CSCs in cell lines derived from RMS patients. The aims of this thesis were as follow:

1) Analysis of expression of putative CSC markers in rhabdomyosarcoma cell lines:
    a) Detailed evaluation of main putative CSC markers in sarcomas, i.e. ABCG2, CD133 and nestin
    b) Measurement of aldehyde dehydrogenase activity
    c) Analysis of the core stem cell markers, i.e. Nanog, Oct4 and Sox2
2) Verification of the CSC phenotype using colony forming assay, sphere assay and tumorigenicity assay
3) Comparative analysis of CSC markers mentioned above in xenograft cell lines during serial transplantation

3. Material and methods

3.1. Cell lines and cell cultures

Five rhabdomyosarcoma cell lines (Tab. 2) derived from tumor samples taken from patients of the Department of Pediatric Oncology (University Hospital Brno, Czech Republic) were used in this study. Furthermore, three xenograft cell lines LTB-1, LTB-5, LTB-24 resulting from a serial xenotransplantation of the NSTS-11 cell line were also included. The research ethic Committee of the School of Science (Masaryk University, Brno, Czech Republic) approved the study protocol, and a written statement of informed consent was obtained from each participant or his/her legal guardian prior to participation in this study.

All cell lines were derived in our laboratory using the same protocol to generate primary cultures as described below. The authentication of cell lines was performed by detection of muscle-specific transcription factor MyoD by indirect immunofluorescence (Fig. 10). The proportion of MyoD-positive cells varied in cell lines but expression of transcription factor was observed in all primary tumor lines included in this study.

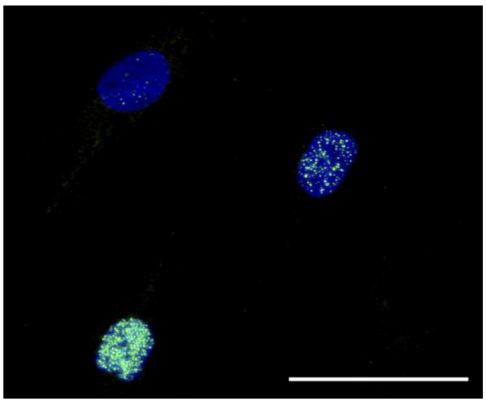

Fig. 10 Verification of histological origin of cell lines by detection of muscle-specific transcription factor MyoD. The representative example of is shown. MyoD was visualized by indirect immunofluorescence method using Alexa 488-conjugated secondary antibody. Positive signals for MyoD are green; nuclei labelled by DAPI are blue; scale bars, 50 µm.

3.1.1. Primary cultures

This method was used for processing of primary tumor samples and xenograft tumors. The tissue samples were briefly washed in 70% ethanol, followed by two washing in phosphate-buffered saline (PBS); the samples were then mechanically chopped into

pieces about 2 mm in diameter. After disaggregation, the pieces of tissue were washed three more times in PBS, this time with centrifugation. Next they were seeded into 25 cm^2 cell culture flasks containing 1 ml of complete medium, i.e. in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum, 2 mM glutamine and antibiotics: 100 IU/ml of penicillin and 100 µg/ml of streptomycin (all purchased from GE Healthcare Europe GmbH, Freiburg, Germany). The cells were cultivated under standard conditions at 37°C and in an atmosphere of 95% air: 5% CO_2. Once the tumor tissue pieces had attached, the volume of the DMEM was gradually increased to 5 ml over the next 48 hours. As soon as the outgrowing cells covered about 60% of the surface, they were trypsinized, diluted and transferred into a new flask.

3.1.2. Cell culture conditions

The cell lines were maintained in DMEM supplemented with 20% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (all purchased from GE Healthcare Europe GmbH, Freiburg, Germany). The cultivation was performed under standard conditions at 37˚C in a humified atmosphere containing 5% CO_2 and the cell lines were subcultured once or twice per week.

Tab. 2 Characterization of primary RMS cell lines used in study.

Cell line	RMS type	Characterization of patients	
		Gender	Age (years)
NSTS-8	A	F	21
NSTS-9	A	M	17
NSTS-11	E	F	16
NSTS-22	A	F	5
NSTS-28	A	M	8

Abbreviation: RMS type: A – alveolar, E – embryonal; gender: M – male, F – female; age in time of diagnosis.

3.2. PCR methods

3.2.1. RT-PCR

RT-PCR was used to detected gene expression of chosen CSC markers in tumor cell lines at mRNA levels. RNA was isolated from cells using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Concentration and purity of isolated RNA were verified by nanophotometer (IMPLEN, Munich, Germany). Subsequently, RNA was converted to cDNA using M-MLV reverse transcriptase (Top-Bio, Prague, Czech Republic) and oligo-dT (Qiagen, Valencia, CA, USA) and for RT-PCR, RNA at concentration of 25 ng per µl was used. Each reaction involved 100 µM of dNTP mixture, 1U Taq polymerase (Top-Bio), 0.25 µM of primer and 10 µl of cDNA in total volume of 25 µl. PCR products were electrophoresed on a 1% agarose gel (Elisabeth Pharmacon, Croydon, UK) and visualized after Midori green (Elisabeth Pharmacon) staining. As endogenous reference control was used heat shock protein gene (*HSP90AB1*). The primer sequences used in the study are listed in Tab. 4

3.2.2. RT-qPCR

To analyse sorted ALDH-positive cells and to detect active ALDH isoforms in RMS cell lines, RT-qPCR method was chosen. Extraction and reverse transcription was completed as previously described. Quantitative PCR was performed in a volume of 10 µl using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data were analysed by 7500 Software v. 2.0.6 (Applied Biosystems) and relative quantification of gene expression were calculated using $2^{-\Delta\Delta CT}$ method *(Livak and Schmittgen 2001)*. As endogenous reference control was used heat shock protein gene (*HSP90AB1*) and NSTS-11 cell line served as the arbitrary calibrator. The primer sequences used in the study are listed in Tab. 4.

Tab. 3 Conditions of RT-PCR reactions.

	Temperature	Time	Cycles
Initial denaturation	94°C	1 min	1
Denaturation Annealing Extension	94°C 55-68°C 72°C	15 s 15 s 1 min na 1 kb	25-35
Final extension	72°C	7 min	1
Cooling	22°C		

Tab. 4 Primer sequences used in PCR reactions.

Gene	Primer sequence
ABCG2	F: TCACTACTTCCTTCCTTACCCCT
	R: ACAGAAACACAACACTTGGCTG
ALDH1A1	F: GTCAAAGGCTTCCTGCCCTA
	R: GGTTCTGATAGAGCACTTGGCT
ALDH1A3	F: TGGTGAACATTGTGCCAGGA
	R: TCACCCGCTTCAGATTGCTC
CD133	F: CCATTGACTTCTTGGTGCTGT
	R: TGGAGTTACGCAGGTTTCTCT
HSP90AB1	F: CGCATGAAGGAGACACAGAA
	R: TCCCATCAAATTCCTTGAGC
Nanog	F: TGAACCTCAGCTACAAACAG
	R: CTGGATGTTCTGGGTCTGGT
Nestin	F: AGTGATGCCCCTTCACCTTG
	R: GCTCGCTCTCTACTTTCCCC
Oct4	F: TGGAGAAGGAGAAGCTGGAGCAAAA
	R: GGCAGATGGTCGTTTGGCTGAATA
Sox2	F: AAGAGAACACCAATCCCATCC
	R: TCCAGATCTATACAAGGTCCATTC

Abbreviation: F- forward sequence, R – reverse sequence

3.3. Immunodetection methods

3.3.1. Immunofluorescence

Fluorescence microscopy combined with indirect immunofluorescence method was employed to visualise putative CSC markers and core stem cell markers.

The cells were cultivated on coverslips in Petri dishes for one day and then rinsed with PBS and fixed with 3% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 20 min. After washing again with PBS, non-specific binding was blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 10 min. The cells were then incubated with primary antibody at 37˚C for 60 min, washed three times in PBS and then incubated with the corresponding secondary antibody at 37˚C for 45 min. After a final wash with PBS, the cell nuclei were counterstained with 0.05% Hoechst 33342 (Life Technologies, Carlsbad, CA, USA) for 10 min, and the coverslips were mounted using Dako fluorescence mounting medium (Dako, Glostrup, Denmark).

The Olympus BX-51 microscope was used for evaluation of immunofluorescence; micrographs were captured using the Olympus DP72 CCD camera and analysed using the Cell P imaging system (Olypmpus, Tokyo, Japan). For each slide, 200 cells were

counted at least in total within discrete areas of each sample. Immunofluorescence was semiquantified by determination of a total immunoscore for each slide. The immunoscore was calculated by multiplying the percentage of positive cells by the respective immunoreactivity (1 = weak, 2 = medium, 3 = strong) in accordance with quantitative scoring method for immunohistochemistry *(Mikulenkova et al. 2015).*

For verification of the nuclear localization of protein CD133 was used the Olympus FluoView-500 confocal imaging system combined with the inverted Olympus IX-81 microscope. The images were recorded using the Olympus DP70 CCD camera and analysed with analySIS FIVE software (Soft Imaging System GmbH, Munster, Germany) and an Olympus FluoView Confocal Laser Scanning Microscope System 4.3.

3.3.2. Transmission electron microscopy

For detail analysis and verification of the CD133 localization transmission electron microscopy (TEM) combined with immune-gold labelling was used.

The cells were seeded and grown on coverslips for several days. Then the cells were rinsed with PBS and fixed with 3% paraformaldehyde and 0.1% glutaraldehyde (AppliChem GmbH, Darmstadt, Germany) in PBS at room temperature for 60 min. After that were cells rinsed with PBS and dehydrated and embedded in LR White medium (Polysciences Inc., London, UK). The labelling of the ultrathin sections was performed on grids using primary antibodies and a gold particle-conjugated secondary antibody. The primary antibody against α-tubulin was used as a control. After immunodetection, the specimens were contrasted with 2.5% uranyl acetate (PLIVA-Lachema, Brno, Czech Republic) for 10 min and with Reynolds solution (Sigma-Aldrich) at room temperature for 6 min.

The specimens were then observed under the Morgagni 268(D) transmission electron microscope (FEI Co., Hillsboro, OR, USA). The images were captured using an Olympus Veleta TEM CCD camera and analysed using iTEM Olympus Soft Imaging Solution.

3.3.3. Immunoblotting

To confirm presence or absence of the proteins in question in tumor cell lines, western blotting combined with immunodetection was used.

A 20 µl of cell lysate was loaded onto an 8% sodium dodecyl sulfate (SDS)−polyacrylamide gel and separated by electrophoresis. Electroblotting of proteins

was performed onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) on ice for 60 min followed by blocking in 5% non-fat milk at room temperature for 60 min. Subsequently, membranes were incubated with primary antibody at 4°C overnight, washing with Tris-buffered saline (TBS)-Tween-20 and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 60 min. Signal detection was performed using ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's instructions.

For detection and verification of nuclear localization of CD133 protein, a Nuclear Protein Extraction kit (Thermo Fisher Scientific, Rockford, IL, USA) was used. Samples were processed according to the manufacturer's instructions. Isolated cell lysates were analysed by Western blotting and immunodetection. For the controls of purity of the cytoplasmic and nuclear cell fractions, anti-α-tubulin and anti-lamin B2 were used, according their specificity for the cytoplasm and cell nucleus, respectively.

Tab. 5 Primary antibodies used in the immunodetection methods.

				Dilution		
Antigen	**Type/host**	**Clone (catalog no.)**	**Manufacturer**	**IF**	**WB**	**TEM**
ABCG2	Monoclonal/Mo	5D3	BD Pharmingen	1:50	-	-
ABCG2	Polyclonal/Rb	- (bs-0662R)	Bioss	-	1:1 000	-
ALDH1	Monoclonal/Mo	44/ALDH	BD Biosciences	-	1:1 000	-
CD133 (AC133)	- /Mo	AC133	Miltenyi	1:4	-	-
CD133	Monoclonal/Mo	17A6.1	Millipore	1:100	-	1:25
CD133 (PROM1)	- /Rb	- (70R-19543)	Fitzgerald	-	1:687	-
CD133	Polyclonal/Rb	- (ab19898)	Abcam	1:70	-	-
Lamin B2	Monoclonal/Rb	D8P3U	Cell Signalling	-	1:1 000	-
Nanog	Polyclonal/Rb	- (ab21624)	Abcam	1:20	1:200	-
Nestin	Monoclonal/Mo	10C2	Millipore	1:100	1:1 000	-
Oct4	Polyclonal/Rb	- (ab19857)	Abcam	1:100	1:1 000	-
Sox2	Monoclonal/Rb	EPR3131	Abcam	1:10	1:250	-
α-tubulin	Monoclonal/Mo	TU-01	Exbio	1:200	1:1 000	1:200
β-actin	Monoclonal/Mo	AC-15	Sigma	-	1:20 000	-

Abbreviations: IF – indirect immunofluorescence; WB – western blotting; TEM – transmission electron microscopy

Tab. 6 Secondary antibodies used in immunodetection methods.

Host	Specificity	Conjugate	Manufacturer	IF	WB	TEM
Goat	anti-Rb IgG	Alexa Fluor 488	Life Technologies	1:200	-	-
Goat	anti-Mo IgG	Alexa Fluor 488	Life Technologies	1:200	-	-
Goat	anti-Rb IgG	HRP	Cell Signaling	-	1:5 000	-
Horse	anti-Mo IgG	HRP	Cell Signaling	-	1:5 000	-
Goat	Anti-Mo IgG	20nm Gold	Abcam	-	-	1:40

Abbreviation: anti-Rb – anti-rabbit; anti-Mo – anti-mouse; IF – indirect immunofluorescence; WB – western blotting; TEM – transmission electron microscopy; HRP – horseradish peroxidase

3.4. Measurement of aldehyde dehydrogenase activity

To detect the activity of ALDH in viable cells, ALDEFLUOR® kit (Stem Cell Technologies, Grenoble, France) was used. This assay is based on the ALDEFLUOR® reagent system, which is able to identify the cells based on their ALDH activity. Non-toxic ALDH substrate (BODIPY-aminoacetaldehyde) freely diffuse into intact, viable cells and it is converted to the fluorescent product BODIPY aminoacetate by ALDH activity. Cells were prepared in accordance with the manufacturer's instructions. Cell at concentration of 1×10^4 of cells per ml were suspended in Aldefluor® assay buffer containing ALDH substrate and incubated at 37 °C for 45 min. Cells treated with a specific ALDH enzyme inhibitor were used as control.

Measurement of ALDH activity was performed with flow cytometer FACS Verse (BD Biosciences, San Diego, CA) and analysed with FACSuite software (BD Biosciences). For detail analyses of ALDH isoforms was used fluorescence-activated cell sorting. Cells after preparation were sorted using BD FACSAria Fusion (BD Biosciences) and analysed by BD FACSDiva™ software (BD Biosciences).

3.5. Functional assays for verification of CSC phenotype

3.5.1. Sphere formation assay

Sphere cultures were derived from cells grown in a defined serum-free medium consisting of neurobasal medium (PAA) and DMEM medium (GE Healthcare) supplemented with 10 ng/ml EGF (Sigma), 20 ng/ml FGF (Sigma) and B-27 supplement without vitamin A (Gibco, Therma Fisher Scientific, Waltham, MA, USA). Cells were seeded into ultra-low attachment 6-well plates (growth area 9.5 cm^2) (Corning, NY, USA) at concentration 2000 cells per well. Every three days, a fresh cultivation medium with growth factors was added in amount of 100 µl. Counting of spheroids in each well was performed after three weeks of cultivation.

3.5.2. Colony forming assay

To determine the colony formation efficiency, cells in DMEM were at a concentration of 1000 cells per culture dish (growth area 21 cm^2) (Sarstedt AG &Co, Numbrecht, Germany). Cells were cultivated under standard conditions as described above (see 3.1.2.). After 8 days, the colony forming ability of each cell line was assessed by counting the number of colonies under the Olympus CKX41 light microscope after Coomasie blue staining (Sigma). The colony was defined as a cluster of at least 50 cells.

3.5.3. *In vivo* tumorigenicity assay

To examine the tumorigenicity of cell lines, tumor cell lines were enzymatically dissociated into cell suspensions. Suspensions at doses of $1x10^6$ or $3x10^5$ cells were subcutaneously injected on the back into three 8- week-old female NOD/SCID gamma mice. Mice were examined for the presence of tumors every 3 days and after the appearance of a tumor, animal were sacrificed and the tumor was dissected (Fig. 11). After removing from mice, the tumor was splitted into two pieces: the first half of it was established as a primary culture and the second half was fixed in 10% buffered formalin and processed for histological examination. This study was approved by the Institutional Animal Care and Use Committee of Masaryk University and was registered by the Ministry of Agriculture of the Czech Republic as required by national legislation.

Fig. 11 Design of *in vivo* tumorigenicity assay rhabdomyosarcoma cell lines. Primary tumors were enzymatically dissociated into cell suspensions and injected subcutaneously into immunocompromised mice.

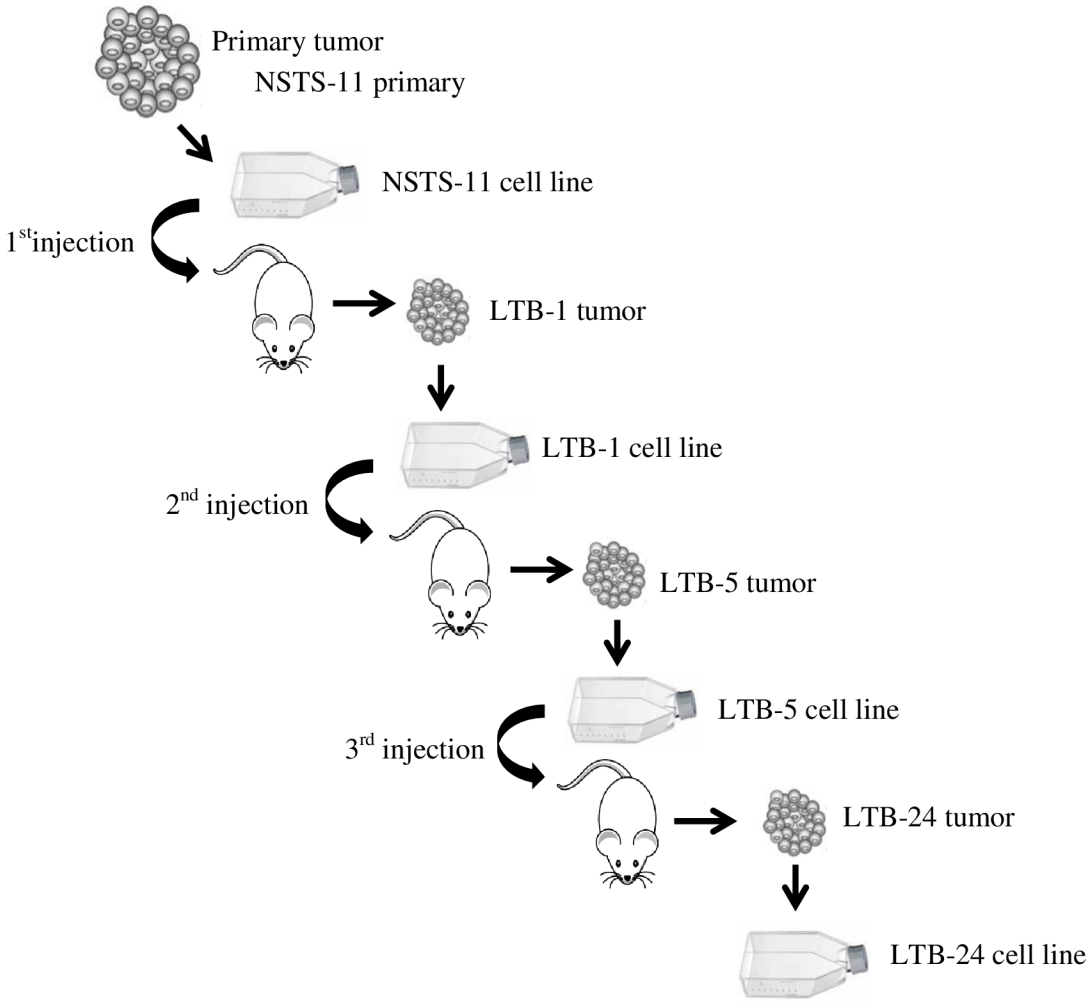

Fig. 12 Design of *in vivo* tumorigenicity assay NSTS-11 cell line and xenograft cell lines. Primary tumor was enzymatically dissociated into suspension and injected into immunocompromised mice. After the tumor appearance were mice sacrificed and the tumor was dissected. After removing was part of tumor enzymatically dissociated and repeatedly injected into mice. This process was repeated three times.