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Review article

Small animal models for the study of bone sarcoma pathogenesis:characteristics, therapeutic interests and limitations

Camille Jacques[a], Nathalie Renema[a], Frederic Lezot[a], Benjamin Ory[a], Carl R. Walkley[b,*], Agamemnon E. Grigoriadis[c,*], Dominique Heymann[d,e,*]

[a] INSERM, U1238, University of Nantes, France
[b] St. Vincent's Institute of Medical Research, Department of Medicine, St. Vincent's Hospital, University of Melbourne, Australia
[c] Centre for Craniofacial and Regenerative Biology, King's College London Guy's Hospital, London, UK
[d] University of Sheffield, Medical School, Dept of Oncology and Metabolism. INSERM, European Associated laboratory «Sarcoma Research Unit», Beech Hill Road, S10 2RX Sheffield, UK
[e] Institut de Cancérologie de l'Ouest, INSERM, U1232, University of Nantes, «Tumour Heterogeneity and Precision Medicine», Bld Jacques Monod, 44805 Saint-Herblain cedex, France

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ABSTRACT

Osteosarcoma, Ewing sarcoma and chondrosarcoma are the three main entities of bone sarcoma which collectively encompass more than 50 heterogeneous entities of rare malignancies. In contrast to osteosarcoma and Ewing sarcoma which mainly affect adolescents and young adults and exhibit a high propensity to metastasise to the lungs, chondrosarcoma is more frequently observed after 40 years of age and is characterised by a high frequency of local recurrence. The combination of chemotherapy, surgical resection and radiotherapy has contributed to an improved outcome for these patients. However, a large number of patients still suffer significant therapy related toxicities or die of refractory and metastatic disease. To better delineate the pathogenesis of bone sarcomas and to identify and test new therapeutic options, major efforts have been invested over the past decades in the development of relevant pre-clinical animal models. Nowadays, in vivo models aspire to mimic all the steps and the clinical features of the human disease as accurately as possible and should ideally be manipulable. Considering these features and given their small size, their conduciveness to experiments, their affordability as well as their human-like bone-microenvironment and immunity, murine pre-clinical models are interesting in the context of these pathologies. This chapter will provide an overview of the murine models of bone sarcomas, paying specific attention for the models induced by inoculation of tumour cells. The genetically-engineered mouse models of bone sarcoma will also be summarized.

1. Introduction

The injection of a cell suspension of murine (allograft) or human (xenograft) cancer cells, in orthotopic sites (in close contact to the bone or into the bone medullary cavity) is the most common methods used to induce bone sarcomas in mouse [1,2]. It has also been possible more recently to utilise the limited material available from patient biopsies (e.g. needle biopsies), and implant such tumour material into immunodeficient [e.g. Patient-Derived Xenografts (PDX)] [3] or immunocompetent animals [4,5]. The advantage of these PDX bearing mouse models is the possibility of expanding the tumour tissues by retaining the original tumour architecture.

The cell-injection close to the bone is called "paraosseous induction", in contrast to the "intraosseous model" that consists in cell inoculation into the femur or fibula diaphysis. Immunocompetent (e.g. syngeneic model in C57/BL6 mice or Sprague-Dawley rats) or immunocompromised (xenografts in Nude or SCID mice) models can be used according to the main objective of the studies (Fig. 1). Other heterotopic cell injections are also described in the literature (e.g. subcutaneous, under the renal capsule) however, they do not engage the vicious cycle established between cancer cells and the bone microenvironment and do not mimic all steps of tumour development.

The choice of the model will depend on the goal of the study (e.g. analysis of local tumour growth, imaging of lung metastases). In addition, financial aspects (e.g. relative inexpensive models based on injection of established cell lines versus genetically-engineered models) and availabilities of research tools (e.g. antibodies) are also key parameters that could influence the choice. Independently of their costs,

* Corresponding authors
E-mail addresses: cwalkley@svi.edu.au (C.R. Walkley), agi.grigoriadis@kcl.ac.uk (A.E. Grigoriadis), dominique.heymann@univ-nantes.fr (D. Heymann).

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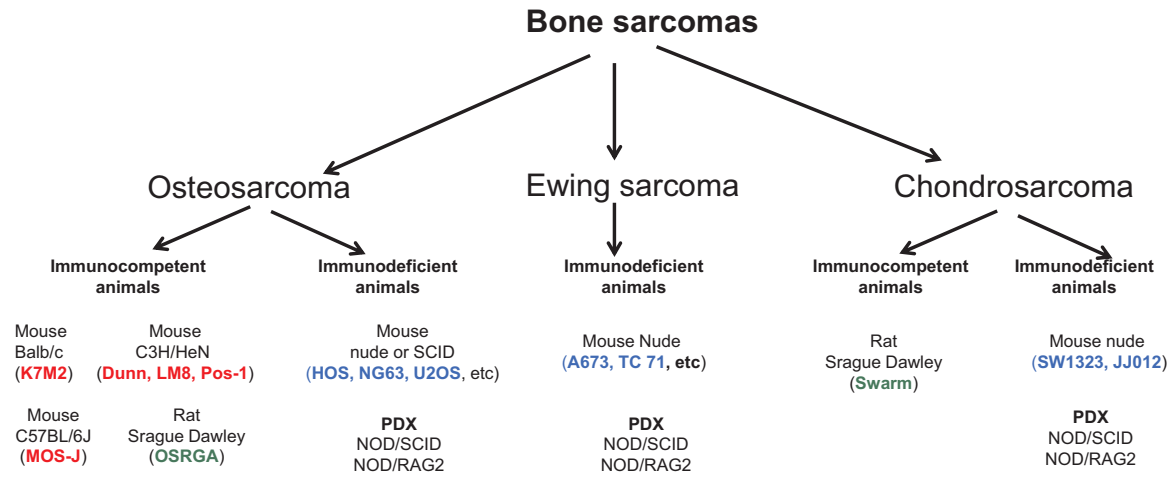

Fig. 1. Smal animal models available in the literature for the study of primary bone tumours. Cell lines: human (in blue), mouse (in red), rat (in green) orgin. PDX: Patient derived xenograft. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

each of these models have several advantages and limitations: i) inoculation of established cell lines may not represent the genetic heterogeneity of the human tumours; ii) genetically engineered models characterised by a spontaneous tumour development can mimic the natural history of the disease with an adapted tumour microenvironment (murine cancer cells in a murine microenvironment); iii) PDX models can maintain the cellular heterogeneity of the initial tumour fragments in a non human microenvironment. The current state of the art concerning the murine strains, the cell lines used, the number of cells injected per animal and some other specific technique-related features will be described in the paragraphs below.

2. Induction of primary bone tumour by cell injections in heterotopic sites

2.1. Induction of bone sarcoma by subcutaneous cell injections

Given the mesenchymal and bone/joint origin of bone sarcomas, their initiation through heterotopic subcutaneous cell injection does not take account of the proper interactions between the tumour cells and their normal bone/muscle/cartilaginous microenvironment. However, this model has the advantage of being technically easy to carry out, a large panel of cancer cell lines and diverse injection sites can be used and the resulting tumours are easily and directly accessible for experiments. Importantly, however, this approach can also address whether the transformed cells have the potential to form tumours in a cell-autonomous way in the absence of their normal environment. In the context of osteosarcoma, the human 143B cell line as well as several c-Fos-transgenic mouse osteosarcoma cells were reported to form tumour masses containing bone after subcutaneous injection [6,7]. Cancer cells have been also incorporated into acellular Matrigel™ based-matrix to provide an active bio-molecule scaffold from murine origin and facilitate cell engraftment. Utilising such an approach, Duan et al. established osteosarcoma tumours subcutaneously by resuspending KHOS osteosarcoma cells in a 1:1 Matrigel™ volume ratio and injected an amount of 2×10^6 cells per mouse [8]. The use of the Saos-2 human osteosarcoma cells combined with Matrigel™ was also reported. A recent study reports the injection of 3×10^6 cells resuspended in 100 µL of Matrigel™ mix (1:1) in this case [10]. Syngeneic models of osteosarcoma are also available. The Dunn cell line and its derivate LM8 subline are the most frequently used. Dunn cells were originally reported with a low metastatic profile in contrast to its LM8 subline which is highly metastatic. LM8 was initially obtained after 8 successive cycles of in vivo selection [10,11]. $1–10 \times 10^6$ Dunn or LM8 cells resuspended in 200–300 µL of PBS are inoculated subcutaneously into the flank of C3H mice (5- to 8- weeks-old) [12,13]. The inoculation of LM8 cells results in the development of a primary local tumour and the formation of metastases to the lungs within 4 weeks with an incidence of 100%. Finally, genetically-engineered osteosarcoma cells have also been reported to efficiently grow after subcutaneous injection [13–17] (Fig. 2). For instance, the low metastatic mouse RF43 osteosarcoma cells and their stable genetically-modified counterparts expressing sFRP2-were injected into nude mice[17] Similar studies have also been reported with Ewing Sarcoma cells, with A673 cells being one of the most commonly reported, for drug screening [18]. One to three million A673 cells are sufficient to generate a tumour mass after subcutaneous implantation into the flank or in the inguinal region of nude mice [19,20]. TC71 and SK-N-MC cell lines were also described to reproduce relevant non-osseous Ewing sarcoma models [21]. Similarly to osteosarcoma, Ewing sarcoma cells (5×10^6 of TC32 cells) suspended in 30% Matrigel™ have been inoculated subcutaneously [22]. Finally, the subcutaneous injection method is also employed to generate chondrosarcomas, as shown by Li et al. [23] and Wang et al. [24], who injected 5×10^6 of SW1353 cells and 10^6 c-Fos-transformed murine chondrosarcoma cells, respectively, into the hind limbs of nude mice. One million JJ012 human chondrosarcoma cells resuspended in 200 µL of serum-free medium [25] or diluted in 100 µL of medium,

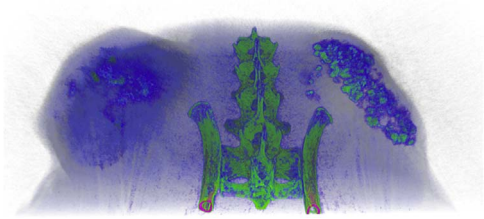

Fig. 2. Typical view of microCT image of luciferase expressing murine OS cell lines grown on the back flank of Balb/c nu/nu mice. Cells were implanted subcutaneously in matrigel. Mass on the left is control cells (control shRNA) and those on the right is expressing an shRNA directed against Pthr1. Pseudo coloring indicates intensity of the gray scale density of the tumour with green being most dense and blue least dense. (image generated by A. Goradia/M. Russell/C Walkley [13]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concomitantly with 300 µL of Matrigel™ can be also inoculated subcutaneously in the back of nude mice [26].

2.2. Induction of bone sarcomas after cell injection in a deep non-osseous microenvironment

Intraperitoneal, skeletal muscle and kidney are the main deep heterotopic sites of cancer cell inoculation. Recently, Saos-2 osteosarcoma cells (1×10^6 cells/mouse) were injected intraperitoneally in nude mice, resulting in the induction of osteosarcoma xenograft models [27]. SK-NEP-1 Ewing sarcoma cells were inoculated under the renal capsule of mice, this model displaying the advantage of reproducing the lung metastatic spreading after the inoculation of 1×10^6 cells [28]. Ewing sarcoma tumours were formed after injection of 2×10^6 TC71 Ewing Sarcoma cells into the gastrocnemius muscle [29]. The human JJ012 chondrosarcoma cells (5×10^6 cells resuspended in 100 µL of medium) were directly injected into the lateral tail vein of nude mice to generate a model of disseminated chondrosarcoma [30].

2.3. Induction of bone sarcomas by orthotopic cell injections

The main advantage of the models described hereafter is that they reproduce the original site for the development of the primary bone sarcomas. Nevertheless, despite their location, these models do not allow reproduction of the process of the clonal selection associated both with the tumour growth and the metastatic spreading and may not fully recapitulate the tumour cell-immune interactions occurring in *de novo* tumours.

2.3.1. Primary-bone tumour induction by para-osseous cell injections

Syngeneic models of osteosarcoma have been generated by injecting 5-week-old male or female C57BL/6 J mice with 1×10^6 MOS-J cells in close proximity to the tibia, whereas the xenogenic models can be induced through the inoculation of 2×10^6 MNNG/HOS cells in Rj;NMRI nude mice with the same method [31,32] (Fig. 3). As no current syngeneic models of Ewing sarcoma are available, xenografts are conventionally used by injection of 1.5×10^6 TC71 or 1673 Ewing sarcoma cells directly into the nude mice [33]. Of note, this Ewing sarcoma model does not show any metastatic occurrence.

2.3.2. Induction of bone sarcoma by intraosseous injection of cancer cells

Among the currently available methods to generate models of bone sarcomas from cell injections, the intraosseous models are technically the most difficult to achieve and the operator needs to be specifically trained for properly inducing a series of tumour-bearing mice. In a recent study, osteosarcomas were induced using the intratibial injection method using either 1×10^5 human 143B or K7M2L2 osteosarcoma cells resuspended in 10 µL PBS/0.05% EDTA into SCID and BALB/c mice respectively, thus generating highly metastatic pre-clinical models [9]. Similarly, Tome et al. reported 5×10^5 143B-LM4 cells per nude mouse was able to generate tumours in immunodeficient [34]. Another study reports the use of the OS-1 and OS-2 canine osteosarcoma cells (1×10^5 cells resuspended in 10 µL PBS and intra-tibially injected into nude mice), to assess their tumour characteristics and metastatic features [35]. Finally, Shimozaki et al., injected a suspension of 5×10^5 143B cells diluted in Matrigel™, directly into the medullar cavity of the tibia of nude mice [36]. Similarly, isolated osteosarcoma cells isolated from c-Fos transgenic mice maintained on a C57Bl6/J background, were injected intratibially into four week-old female $Rag2^{-/-}$:$IL2R\gamma^{-/-}$ immunocompromised mice (2.5×10^5 per mouse, 5 µL injected with a 29-gauge Hamilton microsyringe). This model results in the formation of lung nodules 14 days after cell inoculation [37] (Fig. 4). In the context of Ewing Sarcoma, the intraosseous injections closely reproduces the human pathology, even if the tumours showed a slowed proliferation rate compared with the soft tissue injected models[1] and in osteosarcoma the microenvironment could influence the therapeutic response [38].

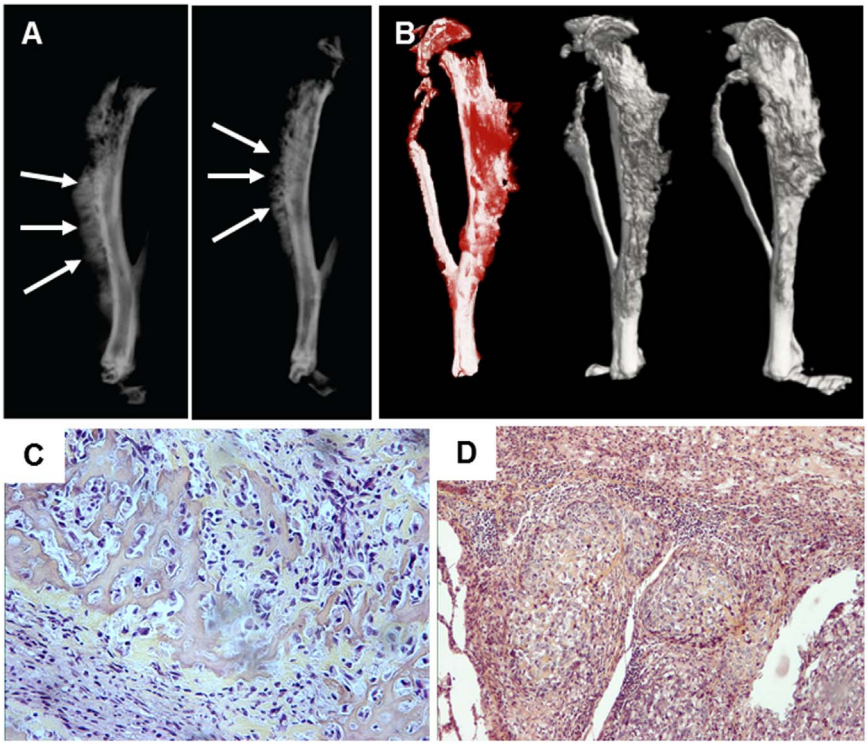

Fig. 3. Xenograft model of human osteosarcoma. Bone tumours are induced by para-tibial injection of human HOS-MNNG cells (A, B). Lung metastases can be detected two to three weeks after cell inoculation. Xray (**A**) and MicroCT (**B**) analysis of tibial osteosarcoma, three weeks after cell inoculation. leading to spontaneous lung metastases. Tumour are characterised by ectopic bone formation observed by Xray images generated by F. Lamoureux/F. Lézot/D. Heymann) and microCT (images generated by B. Gobin/S. Battaglia/D. Heymann [31]). (**C,D**) Osteosarcoma developed from intra-tibial injection of OSRGA cells in Sprague Dawley rat. Typical histological feature of an osteoblastic osteosarcoma (primary tumour) (**C**) leading to the development of lung metastases (D) (Images generated by D. Heymann [4]).

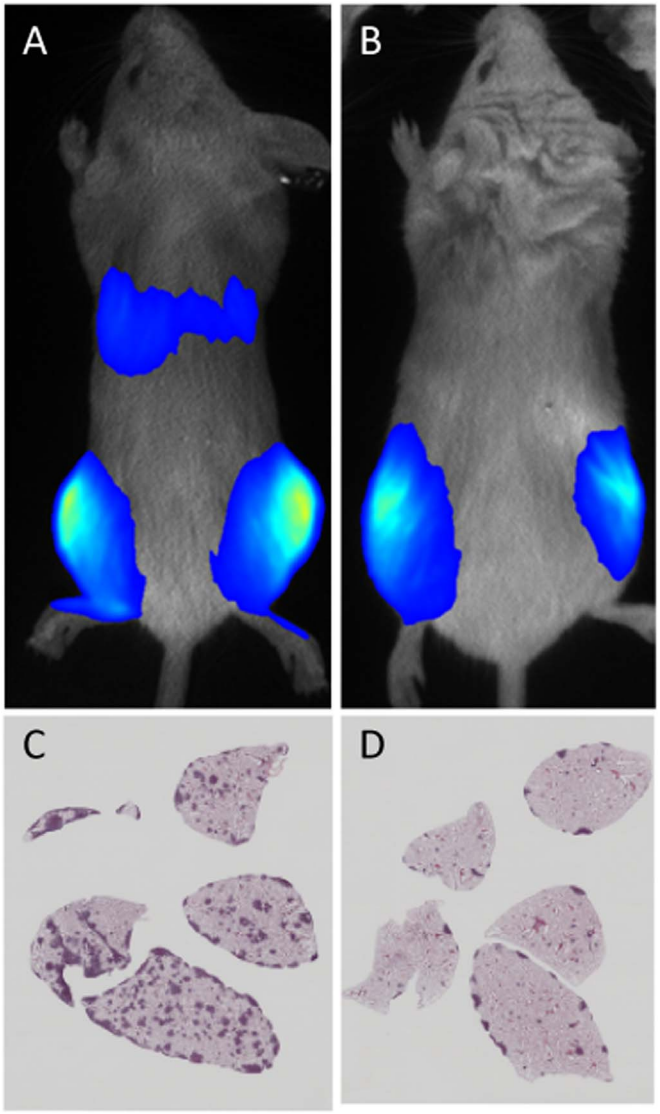

Fig. 4. Bioluminescence imaging and histology of immunocompromised mice following orthotopic injection of c-Fos transformed murine OS cells showing marked primary tumour formation and lung metastasis. Control OS cells (control shRNA) (A) showing lung metastases are inhibited by expressing an shRNA directed against Fgfr1 (B). H&E stained sections of control (C) and Fgfr1 knockdown (D) lungs show reduced lung nodules. Images generated by C. Zandueta/F. Lecanda (CIMA, Pamplona, Spain) [37].

2.4. Primary bone tumour induction by tumour transplantations

The transplantation of tumour fragments from a donor to a recipient is also another possible strategy to maintain the cellular heterogeneity and the genetic background of the tumour. The engraftment success is variable depending upon the model used and the tumour studied [2,4,5]. Such transplantation can be done subcutaneously or directly in close contact to the bone of the animals. A recent study reports the passage of 2 × 2 × 2 mm tumour-fragments from human OHS osteosarcoma cells through subcutaneous transplantation into the rear flank of nude mice as an efficient model to test the targeting ability of a murine monoclonal radio-labeled antibody to the CD146 [39]. Lamoureux *et al.*, described a transplantation method in which the murine POS-1 osteosarcoma cells were first inoculated in the hind footpad of C57BL6 mice until tumour formation was observed [40]. Similarly, 2 × 2 × 2 mm tumour fragments were then excised from these donor mice and transplanted along the tibia in other acceptor mice. A similar approach was used for chondrosarcoma [41,42] and for genetically engineered mouse models [43,44].

Several studies indeed report the use of osteosarcoma or Ewing sarcoma PDXs as useful models to perform personalized therapeutic tests. However, these models are still limited by the availability of patient samples, the low rate of engraftment and the cost of immunodeficient animals and the constraining process of mandatory quality control. The most recent work describing osteosarcoma and Ewing sarcoma PDX models have been reported by Stewart et al. [45,46]. These authors have conducted a comprehensive genetic characterization of both diseases using whole-genome sequencing from tumour fragments or original cell lines isolated from patient biopsies and implanted in NOD/SCID/IL-2Rγ-null mice. They identified recurrent somatic alterations in cancer genomes that may be missed using other methods. Murakami et al., recently performed a subcutaneous implantation of 5 mm fragments from freshly obtained human sarcoma samples, directly onto the flank of nude mice [47]. After three weeks when the tumour diameter reached more than 10 mm, 3 mm^3 tumour fragments were then reimplanted in orthotopic sites for reinducing a tumour mass. In the study of Goldstein et al., 3 mm fragments of the DAR PDX (generated from the malignant pleural effusion of a patient suffering from osteosarcoma) and the LR PDX (generated from a pulmonary metastasis of an Osteosarcoma patient) were implanted into either the flank or the pretibial side of mice [48]. The serially passaged tumours were then transplanted in the hindlimb of a single NSG mouse, from which the tumour was grown. This mouse was then sacrificed and 3 mm fragments were washed in Matrigel™ prior to be transferred into the hindlimb of NSG pups. In a study employing PDXs from Ewing sarcomas, NOD/SCID mice were used for the initial tumour engraftment (HSJD-ES-004 and HSJD-ES-006 models, originated from mediastinum-metastasis and from lung-metastasis respectively) and were then passaged into athymic nude mice prior to the assessment of the therapeutic response [3]. Ambati et al., developed PDX models of Ewing sarcoma by passaging initial tumour material two times into NSG mice prior to start the animal treatment [49].

2.5. Genetically-engineered mouse models of bone sarcomas

Genetically-engineered mouse models have been characterised to be accurate models in oncology, especially in an attempt to study the tumour onset/development and to delineate the molecular drivers or the genetic initiator events responsible of these pathologies. The main advantage af such models is the formation of spontaneous tumours close to the human context, and can be imaged by conventional approaches (i.e. microCT) (Fig. 5). However, genetically-engineered odel can not summarize all events of human tumours and can not mimick the high molecular heteorgeneity (more specically of osteosarcoma). Ewing sarcoma is an exception in the list of three most frequent bone sarcoma (osteosarcoma, chondrosarcoma, Ewing sarcoma) for which all attempts for developping a genetically-engineered small animal mimicking the human disease has failed (64,65). Despites the technical difficulties, genetically-engineered models in small animals allowed a better understanding of molecular/genetic pathways surrounding bone sarcoma development (Figs. 6 and 7).

3. Conclusion

In this chapter we have outlined the currently available murine models for bone sarcomas induced both by injection of established tumour cell line and from transplantation of primary patient-derived

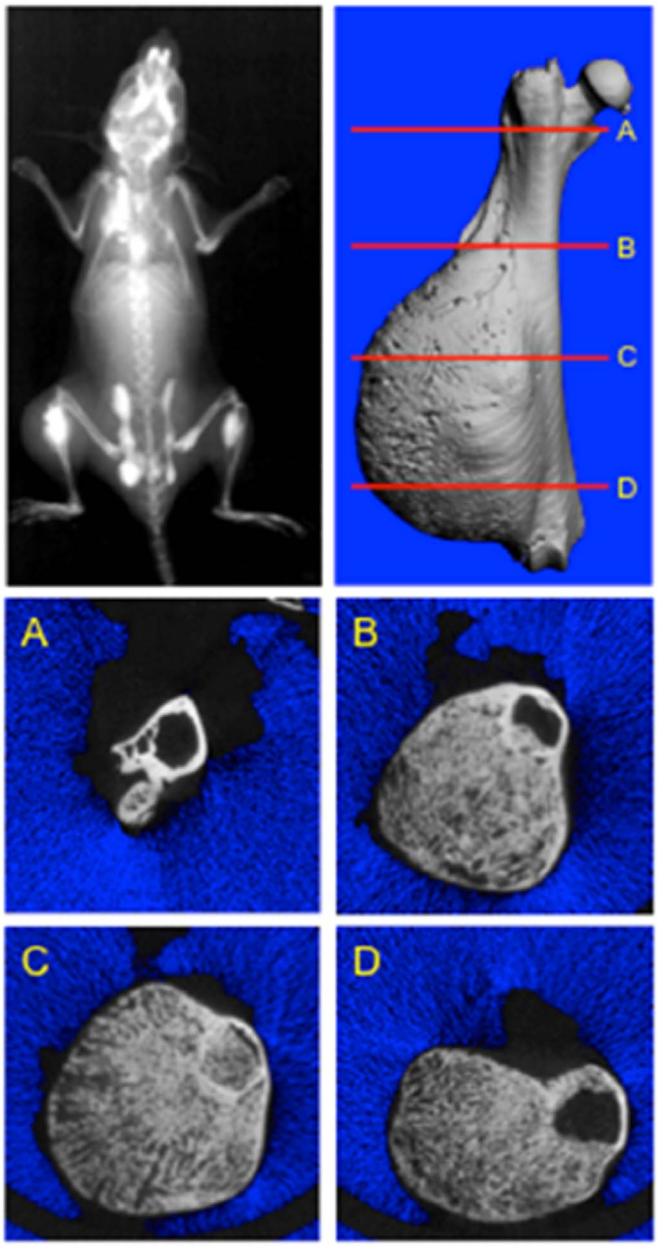

Fig. 5. Overview of the genetically-engineered models in small animals. +; gain; -: deletion; *: mutation; **: restricted expression of the intracellular domain of Notch1 in osteoblast; ***: inducible expression. §: description of metastases.

tumour material, as well as providing an overview of the genetically-induced mouse models. In addition, the entire experimental procedure of the para- and intra-osseous injections are outlined here. The murine models are valuable tools in the field of oncology research, however each one has both advantages and limitations and the choice of a particular model should be careful considered depending upon the goals of the study. Furthermore, osteosarcomas naturally occur in large breed dogs (pet dogs/companion animals) and have been characterised to display clinical features comparable with human OS. In this context, it is conceivable that the spontaneous canine models will be further employed, in an attempt to better understand the normal biology of the bone as well as to find novel biomarkers and innovative therapeutic approaches [65–68].

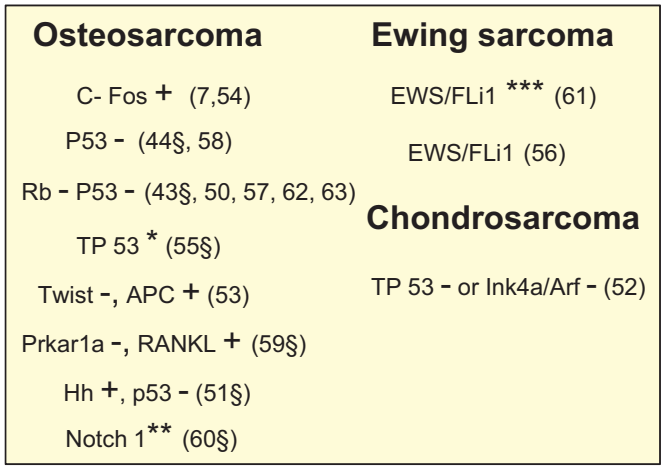

Fig. 6. Two different osteosarcomas arising in Osx-Cre $p53^{fl/fl}$ $pRb^{fl/fl}$ animals. Osteosarcoma arising in the tibiae (A) and in the vertebrae (B). Pseudo coloring indicates intensity of the gray scale density of the tumour with blue being most dense and black/crimson least dense. Images generated by A Ng/C Walkley [50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,68].

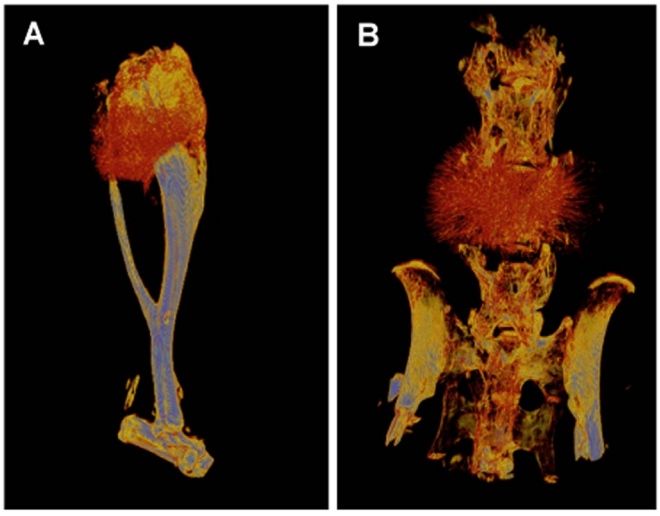

Fig. 7. Xray of an 8 week-old c-Fos transgenic mouse showing numerous calcified osteosarcomas throughout the skeleton. Tumours are at different stages of development. microCT analysis of a femoral osteosarcoma and serial cross-section images through the length of the femur show both extraosseous and intraosseous tumour growth. MicroCT image generated by L. Suva (Texas A&M University, USA) [7].

Conflicts of interest

The authors declare no conflicts of interest.

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