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Abstract For various reasons involving biological comparativeness, expansive technological possibilities, accelerated experimental speed, and competitive costs, zebrafish has become a comprehensive model for cancer research. Hence, zebrafish embryos and full-grown fish have been instrumental for studies of leukemia, melanoma, pancreatic cancer, bone tumors, and other malignancies. Although because of its similarities to human osteogenesis zebrafish appears to be an appealing model to investigate osteosarcoma, only a few osteosarcoma specific studies have been accomplished yet. Here, we review interesting related and unrelated reports of which the findings might be extrapolated to osteosarcoma. More importantly, rational but yet unexplored applications of zebrafish are debated to expand the window of opportunities for future establishment of osteosarcoma models. Accordingly technological advances of zebrafish based cancer research, such as robotic high-throughput multicolor injection systems and advanced imaging methods are discussed. Furthermore, various use of zebrafish embryos for screening drug regimens by combinations of chemotherapy, novel drug deliverers, and immune system modulators are suggested. Concerning the etiology, the high degree of genetic similarity between zebrafish and human cancers indicates that affected regions are evolutionarily conserved. Therefore, zebrafish as a swift model system that allows for the investigation of multiple candidate gene-defects is presented.

Keywords Bone tumor • Metastasis • Angiogenesis • Drug screening • Immune system • Fish

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General Introduction

The high quantity of worldwide research with dozens of weekly reports registered to PubMed emphasizes the international interest for osteosarcoma investigation. Partly this is explained by the clinical questions which yet need to be answered to improve patient care. On the other hand, it is the complex genesis and pathophysiology of osteosarcoma that attracts researchers from diverse fields of expertise to study this highly malignant bone neoplasm. However, the magnitude of the ongoing research is disproportional to the limited number of satisfying results achieved within the past decades [1, 2]. Here, the complexity of the tumor together with its rareness is the main limiting factor [3, 4]. This chapter provides a brief review of the zebrafish as a model for cancer and more specifically for osteosarcoma and provides some ideas for future zebrafish based studies of osteosarcoma.

Introduction to Zebrafish as a Cancer Model

During recent years zebrafish (*Danio rerio*) models have been increasingly generated to study malignancies, qualifying these fish as illustrative animal systems for the study of human cancer. Some experiment-specific characteristics of the zebrafish make it superior to other model systems; the main advantages include the following. Zebrafish embryos—which in the first few days after fertilization are not larger than just a couple of millimeters—undergo a full external development. Therefore, together with their transparency before pigmentation appears, the embryos provide miniature and optically advantageous model systems. Moreover, the high fecundity and short generation time of these fish make them ideal organisms for in vivo studies [5]. From a genetic point of view, advantageous of zebrafish is that the genome has been fully sequenced, showing many conserved genes as compared to the human genome, and the animals are relatively easily accessible for genetic manipulation [6]. More specifically for osteosarcoma studies it is relevant that zebrafish are vertebrate animals with developmental processes comparable to human osteogenesis. For these reasons, many cancer zebrafish models have been developed [7–11]. Although these models include hematologic (both myeloid and lymphatic lineages) and solid tumors (rhabdomyosarcoma, Ewing's sarcoma, hepatocellular carcinoma and other malignancies), they represent malignancies which are mainly referred to as tumors with relatively simple karyotypic changes [12–14]. This explains the lack of such models and especially transgenic systems for osteosarcoma because of its highly complex genomic alterations and stresses the need for xenograft models.

Miniature Laboratory

Background

The hallmarks of cancer [15] include (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating invasion and metastasis, (7) the capability to reprogram or modify cellular metabolism, and (8) evading destruction by the immune system. Broadly these hallmarks can be divided into two groups. The first group including the first four and number 7 contains internal characteristics of malignantly transformed cells while the second group including hallmarks 5, 6, and 8 defines common traits of the interaction between malignant cells and their host. Investigating these hallmarks in osteosarcoma biology shows that many characteristics of the tumor are resulting from a tremendous level of genomic instability. How a normal cell can gain and maintain such genomic instability is not the subject of this chapter; however, data point towards a single master mutation which allows the cell to continuously proliferate accumulating mutations while escaping cell cycle checks and apoptosis [16–20].

Especially for the second group of hallmarks a model is required which would allow for objectification of all the processes which the cancer cells induce inside the body of their host. For this zebrafish embryo models are proven to be ideal as processes like tumor growth, local aggressiveness, angiogenesis, metastases, etc. can be followed in a fast and real-time manner (Fig. 1) [7–9, 21–24]. Regarding osteosarcoma, previously models were established for both groups of hallmarks. First, since osteosarcoma cells show an array of genomic alterations by the time they are isolated from the human tumors that makes them very difficult to study, we established a mouse mesenchymal stem cells (MSCs) based model [25, 26]. This model allowed for addressing processes from the first group of hallmarks such as sustaining proliferative signaling, evading growth suppressors, and resisting cell death [26]. Next, the model was implemented into a zebrafish embryo model system to study aspects from the second group of hallmarks like angiogenesis, migration, and the immune system response of the zebrafish [27]. As for the osteosarcoma cells, any adequate normal cells for comparison on genetic level are lacking, selection for the driver-affected genes would be impossible. Therefore, MSCs were used of which we possessed normal parental cells before transformation and transformed ones, which produced osteosarcoma-like tumors after injection into mice. In short this experiment was conducted as described in Box 1 and Fig. 2.

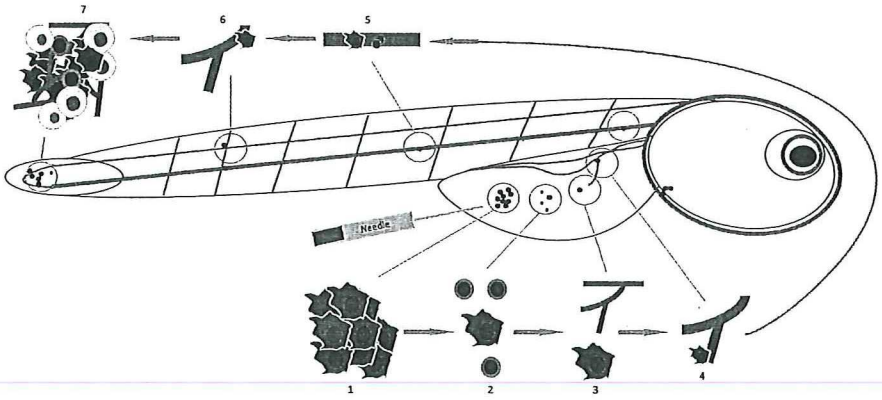

Fig. 1 In vivo tumor progression. A schematic representation of a zebrafish embryo with fluorescent green vasculature is shown. After injection of labeled tumorigenic cells—in this case colored *red*—into the embryos' yolks, important processes of tumor progression can be studied. As indicated by the *arrows*, (*1*) in vivo cell proliferation, (*2*) escaping immune cells (colored *blue*), (*3*) inducing angiogenesis, (*4*) intravasation, (*5*) migration through the circulation, (*6*) extravasation, and (*7*) proliferation at a secondary location can be observed respectively. Depending on how cells are labeled accurate proliferation rates and doubling times can be calculated to compare different cell lines or to examine anti-proliferative effects of selected compounds. Which components of the zebrafish immune system might be encountered by the injected cells is dependent on the embryonic stage. Within a couple of hours after fertilization the innate immune system is functional while the adaptive immunity is detectable within the first weeks. Studying migration of the cells involving angiogenesis, intravasation and extravasation, and homing at a new location is crucial from a clinical point of view as drugs selectively inhibiting any of these aspects might be useful to prevent osteosarcoma cells from metastasizing or to target micro metastases. From a technical point of view, it is important that cells are not directly injected into the blood vessels, as in that case regardless of their intrinsic characteristics cells would travel through the circulation system. Experiments show that "metastases" are most frequently found at the distal end of the tail or the head of the fish. Next to the plausible explanation that cells are entrapped at these locations due to the small vasculature network, it would be interesting to study alternative theories with a role for cytokines and a supportive niche. Please note that if instead of injecting cells, tumor tissue pieces are transplanted into the embryo's, migration of tumor cells and tumor formation elsewhere would be more representative of true metastasis

Cell Lines Versus Primary Tumor and Niche Support

For the model described in Box 1 cultured cells were used to inject into zebrafish embryos. Although within the first hours of injection the cells remain together and proliferate, this clump of cells does not fully represent a true tumor. Inside the yolk the cells lack interaction with stromal cells except for the early immune cells. One way to improve the model would be to inject the cells of interest—either MSCs or osteosarcoma cells—together with stromal cells, for example fibroblasts. Another possibility in case of MSCs would be to inject a mix of normal and transformed MSCs and to hypothesize that the normal MSCs would be stimulated by the transformed ones in a way to provide niche support, maybe by differentiating into other lineages. However, more elegantly, pieces of osteosarcoma directly dissected from patients' tumors could be xenografted into zebrafish embryos. In mouse studies it is shown that such "fresh" pieces of osteosarcoma as well as many osteosarcoma cultured cell lines are able to survive and grow subcutaneously [30, 31]. Unfortunately in those studies the course of tumor growth, angiogenesis, and metastasis could mainly be examined after the

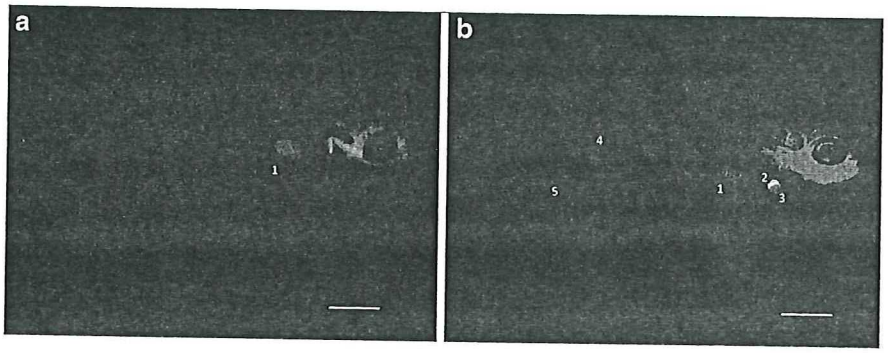

Fig. 2 Normal versus tumorigenic. Two pictures of zebrafish embryos obtained by a camera coupled to a fluorescent microscope are depicted. The transgenic embryos at 2 dpf with green vasculature were injected with cells labeled in *red* and these pictures were taken after 3 days (5 dpf). Magnification bars represent approximately 1 mm. (**a**) A zebrafish embryo injected with normal mouse MSCs is depicted. Despite the strong red signal of the cells (*1*), no signs of angiogenesis were found and the cells remained inside the yolk. (**b**) This embryo was injected with transformed mouse MSCs which were shown to be tumorigenic when transplanted into mice. Already within 24 h sprouting of the subintestinal vein (SIV) was observed indicating angiogenesis (*2*). Furthermore, cells migrated to these vessels (*3*) could be found inside the circulation (*4*) and accumulated at the distal part of the tail (*5*) or the head (not shown here)

animals were sacrificed. By implementing this system of xenotransplantation into zebrafish embryos, it would be possible to image a live tumor piece and to closely follow the subsequent processes in a real-time manner. Currently experiments are ongoing to establish optimal ratios of normal and transformed MSCs when injected together. This will point out if it is possible to create a primary tumor before the processes of angiogenesis and migration take place. Experiments aiming xenotransplantation of human tumors are hampered by technical difficulties due to the small size of the embryos and the aggressiveness of the tumors; however, overcoming these technical problems only seems to be a matter of time.

Immune Response

Another interesting finding from this model leading to new ideas was the fact that the immune response by the fish injected with normal MSCs was clearly different from the immune response of those injected with transformed MSCs as shown by gene expression patterns. The data suggested that the transformed MSCs could regulate the embryos immune system to their advantage. A shortcoming of the model was the lack of a label for the immune cells so this finding could be validated. Therefore, for future studies zebrafish based models are developed with certain labeled immune cells. Fundamental members of the zebrafish immune system, both innate and acquired, are shown to be similar to those in human [32, 33]. This provides the possibility to investigate crucial interactions between tumor cells and macrophages, antigen recognizing lymphocytes and immunoglobulins. Box 2 provides a more detailed overview of the zebrafish immune system in which some players might be important against tumor cells.

Box 1 Zebrafish Progression Model

Towards modeling clinical relevant aspects of osteosarcoma, such as its highly aggressive local growth and its progression in terms of invasion, angiogenesis, and metastasis, zebrafish embryos were used.

The night before the start of the experiment two mature zebrafish of both sexes were put together in an aquarium, but separated by a glass divider so that they could see but not reach each other. One transgenic zebrafish was expressing enhanced green fluorescent protein (EGFP) in all blood vessels [28] and the other was a transparent zebrafish called Casper [29]. Next morning when the divider was removed the female fish almost directly produced about 200–300 eggs which were fertilized by the male resulting into transparent embryos with green blood vessels. Subsequently the embryos started accelerated, simultaneous development outside the mother's body. Meanwhile, two types of MSCs, normal and transformed MSCs which produced osteosarcoma like tumors inside mice were cultured and labeled with a red dye. Next, both type of labeled MSCs were injected into the yolks of zebrafish 2 days post fertilization (dpf) so the red cells could be easily followed inside transparent zebrafish embryos with green vessels (Fig. 3). In contrast to the normal MSCs, the transformed MSCs showed within 3 days after injection excessive proliferation, migration towards the body of the fish, and induced angiogenesis. Whole-genome expression analysis of both the cells and the host showed that angiogenesis and migration-related genes were overexpressed in transformed MSCs as compared to normal MSCs.

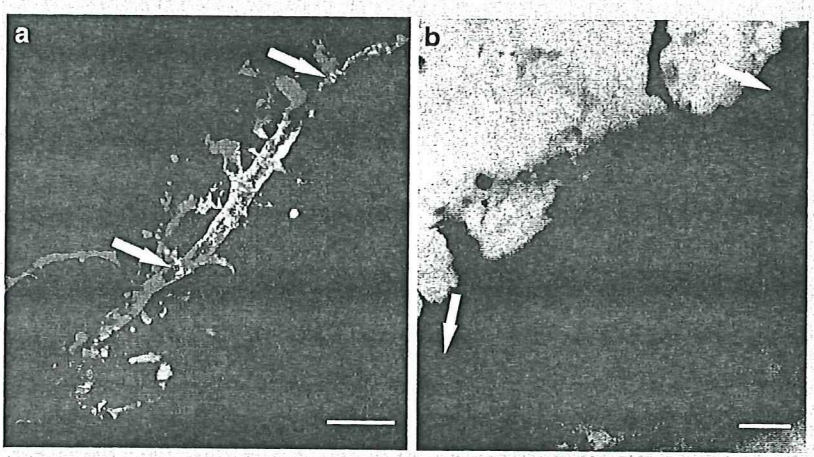

Fig. 3 Intravasation/extravasation of cells. Magnified pictures of the SIV complex—and newly formed vessels—of zebrafish embryos injected by transformed mouse MSCs are shown. Magnification bars represent approximately 100 μm. **(a)** High-resolution image by confocal microscopy depicts intravasation of the cells as indicated by the *white arrows*. **(b)** Three-dimensional reconstruction of **(a)** shows that cells are inside the blood vessels to exclude optical deception when cells and vessels would only overlap

(continued)

Box 1 (continued)

Investigating the host response, embryos injected with transformed MSCs showed decreased expression of immune response-related genes as compared to embryos injected with normal MSCs. The advantages of this model as compared to mouse models were its relatively low costs, its high statistical power by the large group sizes, its high speed—experiments were performed within 5 days—and its advantages in imaging by using transparent fish.

Box 2 Zebrafish Immune System

Zebrafish model systems provide opportunities to identify members of the immune system which play a role in the defense mechanisms against cancer cells. Therefore, it is crucial to know how representative the fish immune system is for the human defense mechanisms.

Although the number of immune organs in zebrafish seems to be limited as compared to mammals and consist only of kidney, thymus, and spleen—so no bone marrow or lymph nodes—zebrafish possess both innate and acquired immunity [34]. The anterior kidney contains developing B lymphoid cells and is mainly involved in antigen processing, IgM formation, and immune memory [35–40]. The spleen plays an essential role in hematopoiesis, antigen trapping and degradation, and antibody production [41, 42]. Erythrocytes destruction by filtrating blood however is accomplished within melanomacrophagic centers in which macrophages are accumulated to capillaries. And finally the T-lymphocytes are produced in the thymus to control many of the previously mentioned effectors of the immune system [43]. The innate—or nonspecific—immune system of the zebrafish includes antibacterial peptides, lectins, and lysozyme expressed in cells of myeloid origin [44]. Furthermore, it is interesting to know that C-reactive protein (CRP) is a highly conserved acute phase protein and present in zebrafish [45]. Also both the classical and alternative complement pathways are comparable to those in human and play an important role in the link between the innate and adaptive immune responses [46–48]. As for the adaptive—or specific—immune response, the overall mechanisms are in zebrafish similar to those in human [49]. Main components of this system in zebrafish include MHC, recombination activating genes (*RAG* 1 and 2 which cause diversity in T cell receptors and antibodies), antigen recognizing lymphocytes and immunoglobulins [49, 50]. Finally important immune modulators such as toll-like receptors and cytokines, including interleukin-1b, TNF-alpha, and IL-6 are found in fish [51–55]. In conclusion, multiple high-quality recent studies have emphasized crucial similarities between the zebrafish and the human immune systems [56, 57]. This opens new windows of opportunity to investigate interactions between osteosarcoma cells—or cancer in general—and components of the immune system with consequences for novel drug screens.

High-Throughput Injection and Imaging

One of the major advantages of using zebrafish embryos as compared to other animal models is related to the small size of the larvae. Keeping 50–100 embryos in one normal sized petri dish or single fish in wells of 96-well plates, provides possibilities to study large groups increasing the power of the experiments. However, at the time the above-mentioned study was performed, which included manual injection of hundreds of embryos, it was clear that for future larger studies alternative methods for injection would be required. Furthermore, handling of the fish after injection and daily imaging to follow the cells were very time-consuming and labor-intensive. For these reasons experiments were performed to assay robotic injections and automated imaging systems. Recently several systems have been reported, by which a combination of robotic injections and several manners of automated imaging guarantee high-throughput screens with less labor intensity [58–60]. One remaining challenge when using robotic arms to inject zebrafish embryos is that the location of injection is less precise. As a consequence embryos might be injected at vital locations and not survive the experiment. Moreover, accidental injections directly inside the blood vessels would bias the results when migration of the cells is a primary outcome. However, robotic injection systems would speed up the experiments to such an extent that it is worthwhile to increase the size of the groups before injection and to select the correctly injected embryos afterwards (Fig. 4).

Drug Screens

Single Drugs

Traditionally zebrafish models were frequently used for screening of chemical compounds to assay carcinogenic effects by looking for tumor formation or mutant fish [61–65]. However, it is only recent that these fish exponentially gained the interest of cancer researchers for screening anticancer drugs. The main reason for this is the establishment of various zebrafish transgenic or xenotransplantation—as exemplified in Box 1—cancer models [61–63, 65–67]. With the availability of such models in which processes related to cancer progression, like angiogenesis and metastasis, can be followed in a live setting, it is only logical to screen drugs which could inhibit these processes. Moreover, when such drug screens are performed in a high-throughput automated fashion, significantly more drugs could be tested. This might decrease the pressure for in vitro selection steps for probable drugs and potentially increase the discovery of novel therapeutics based on in vivo results. Next to the previously mentioned advantages of zebrafish related to imaging, group size and simultaneous development of the embryos, there is another factor which makes the

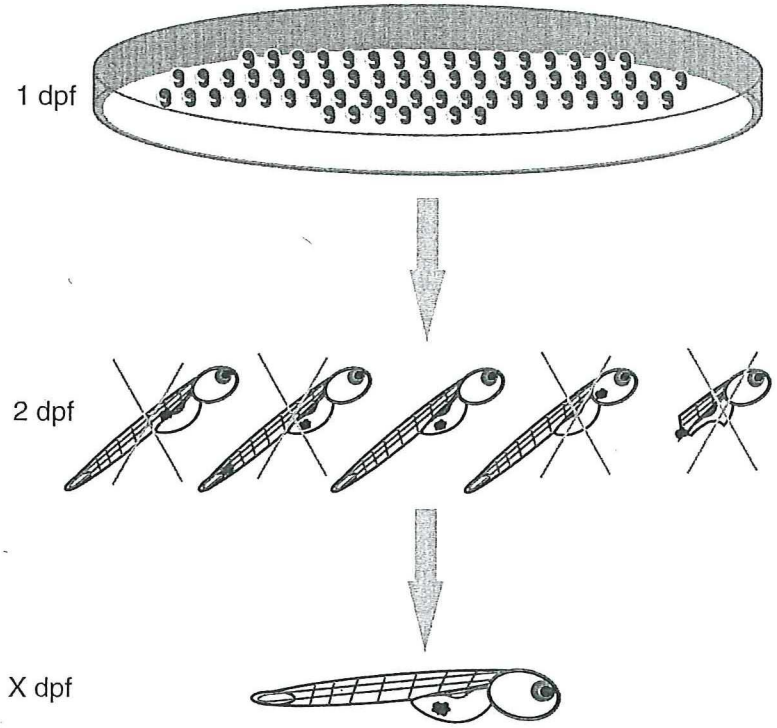

Fig. 4 High-throughput injection and imaging. A schematic work flow is provided for high-throughput injection and screening of zebrafish embryos. At 1 dpf embryos are still inside the chorion and can be mounted in rows in petri dishes coated with agarose. At these stage embryos dot not move, which simplifies robotic injections. Although the embryos will be injected at random locations, its high speed and reduction of labor intensity rationalize robotic injections. The next day embryos which were injected at the right location—in this case only inside the yolk—and without any sign of deformity can be selected. For the following days depending on the duration of the experiment, selected embryos can be imaged on a regular base. Automatic imaging systems could reduce the workload even further and provide a narrower selection for manual screening

fish superior to other animals for drug screening. Namely the drug of interest can be added to the swimming water of the fish which will secure equal uptake by all the members of the experimental group. A drawback here is that other routes of drug delivery, such as micro intravenous injections, are yet hampered by technical difficulties; however, solutions or alternative methods are being developed [59, 68]. Up to today only one report has been published with the specific aim to test anti-osteosarcoma drugs of which the developmental effects were assayed in zebrafish. In this study small molecule inhibitors of Ezrin [69] were shown to inhibit the invasive phenotype of osteosarcoma cells [70].

Combined Therapies

In addition to drug screens for single agents, high-throughput automated zebrafish model systems seem to be amenable for multiple drugs testing which allows for various combinations of different therapeutic regimens. Osteosarcoma patients' outcome considerably improved after the introduction of chemotherapy in the 1970s followed by adjustments in treatment protocols to reduce chemotherapy toxicity as much as possible [71]. However, in terms of survival no significant improvements were accomplished since [2]. Therefore, toxic chemotherapy next to surgery remains the key treatment, which fails in 30–40 % of the patients. Nevertheless, recently alternative or additional therapeutic options were reported to effectively kill osteosarcoma cells and cells of other sarcomas in vitro. Two of the promising protocols include addition of muramyl tripeptide to chemotherapy [72] and the use of anti-EGFR antibody cetuximab to increase the anti-osteosarcomic effect of NK cells [73]. An alternative method to stimulate NK cells cytolytic effect towards tumor cells was shown to be achieved by cytokine stimulation [74, 75]. Next to chemotherapy resistance yet another issue in the lack of specificity in current chemotherapy regimens. Recently the use of gold nanoparticles has been reported by which via the highly expressed folic acid in cancer cells enhanced therapeutic efficacy and reduced side effects were realized [76]. As osteosarcoma cells abundantly express folic acid—in fact methotrexate which is one of the most widely used chemotherapeutics against osteosarcoma is an antifolate—this might allow for better marking of the cancer cells [77]. These novel immune and chemical treatment options are just a few examples, which have proved to be effective for killing cancer cells in vitro. The next logical step is to investigate these alternative therapy regimens wherein combination of the current chemotherapeutics and novel agents can lead to more effective targeted therapy. Zebrafish embryo models provide excellent animal systems to implement such wide drug screens for assaying the effects on human tumor derived cells and even metastases.

Mimicking Human Osteosarcoma

In paradox to the frequent number of attempts, transgenic animal models, which fully represent human conventional osteosarcoma are lacking [78–80]. The main reason for this is the complex karyotype as a result of genomic instability which underlies the disease but is until now indefinable to single mutations which could be applied to animal models [3, 12]. Recently great strides have been made towards a better understanding of the complex pathogenesis of osteosarcoma. Processes likely underlying genomic instability were identified and investigated [17, 19, 26, 81] and possible susceptibility loci were identified [82]. As these type of clues are upcoming, new opportunities for—preferentially conditional—transgenic representative osteosarcoma models are rising. Since the number of possibilities for genomic engineering is

still broad, mouse models would be quite time consuming to start with. Alternatively, in a significantly swifter manner, zebrafish transgenic or knockout lines could be generated, as widely performed for other diseases and cancer [8, 83–95]. Recently detailed protocols have been published [59] for a sequence of experiments for generation of transgenic and knockout zebrafish lines, gene knockdown by using morpholinos, siRNA, or antibodies in a high-throughput manner and more. These protocols shorten the bridge between exciting in vitro data and transgenic zebrafish models.

Conclusion

After some relatively quiet years, great steps have been set towards a better understanding of the complex genomics and pathophysiology of osteosarcoma. Nevertheless, the lack of less toxic and more effective targeted therapy still remains. Therefore, alternative treatment protocols are urgently needed, especially for osteosarcoma patients with chemotherapy insensitive tumors. To accomplish this great challenge for such a multifarious and yet rare disease, the use of representative animal models is unavoidable. Certainly zebrafish embryo models would not always be sufficient as a link between in vitro data and clinical trials. However, current data strongly suggest that these models not only are outstanding alternatives to limit the number of mouse or other animal model studies but also provide new opportunities by allowing for much broader screens. Such screens should start by investigating the progression of osteosarcoma cells live inside the zebrafish bodies. In subsequent steps assaying key players which control tumor cells proliferation, migration through the circulation, metastasis, and the interplay with the immune system could provide novel specific targets for therapy. In addition for recently identified genes which might predispose osteosarcoma, zebrafish provide a speedy method for functional validation. When the targeted genes turn out to indeed be the true driver mutations, simultaneously new representative models are born. Finally all the established xenotransplanted and transgenic models could be utilized for comprehensive drug screens.

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References

1. Bielack SS, Kempf-Bielack B, Delling G et al (2002) Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20(3):776–790
2. Lewis IJ, Nooij MA, Whelan J et al (2007) Improvement in histologic response but not survival in osteosarcoma patients treated with intensified chemotherapy: a randomized phase III trial of the European Osteosarcoma Intergroup. J Natl Cancer Inst 99(2):112–128

3. Sandberg AA, Bridge JA (2003) Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer Genet Cytogenet 145(1):1–30
4. Cleton-Jansen AM, Buerger H, Hogendoorn PCW (2005) Central high-grade osteosarcoma of bone: diagnostic and genetic considerations. Curr Diagn Pathol 11:390–399
5. Stoletov K, Klemke R (2008) Catch of the day: zebrafish as a human cancer model. Oncogene 27(33):4509–4520
6. Deo RC, MacRae CA (2011) The zebrafish: scalable in vivo modeling for systems biology. Wiley Interdiscip Rev Syst Biol Med 3(3):335–346
7. Merlino G, Khanna C (2007) Fishing for the origins of cancer. Genes Dev 21(11):1275–1279
8. Langenau DM, Keefe MD, Storer NY et al (2007) Effects of RAS on the genesis of embryonal rhabdomyosarcoma. Genes Dev 21(11):1382–1395
9. Feitsma H, Kuiper RV, Korving J et al (2008) Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumors. Cancer Res 68(13):5059–5066
10. Etchin J, Kanki JP, Look AT (2011) Zebrafish as a model for the study of human cancer. Methods Cell Biol 105:309–337
11. He S, Krens SG, Zhan H et al (2011) A DeltaRaf1-ER-inducible oncogenic zebrafish liver cell model identifies hepatocellular carcinoma signatures. J Pathol 225(1):19–28
12. Bridge JA, Nelson M, McComb E et al (1997) Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genet Cytogenet 95(1):74–87
13. Rosenberg AE, Cleton-Jansen AM, Gd P et al (2013) Conventional osteosarcoma. In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds) WHO classification of tumours of soft tissue and bone. IARC, Lyon, pp 282–289
14. Kuijjer ML, Hogendoorn PC, Cleton-Jansen AM (2013) Genome-wide analyses on high-grade osteosarcoma: making sense of a genomically most unstable tumor. Int J Cancer 133(11):2512–2521
15. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5): 646–674
16. Mohseny AB, Hogendoorn PC (2011) Concise review: mesenchymal tumors: when stem cells go mad. Stem Cells 29(3):397–403
17. Stephens PJ, Greenman CD, Fu B et al (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144(1):27–40
18. Forment JV, Kaidi A, Jackson SP (2012) Chromothripsis and cancer: causes and consequences of chromosome shattering. Nat Rev Cancer 12(10):663–670
19. Davoli T, de Lange T (2012) Telomere-driven tetraploidization occurs in human cells undergoing crisis and promotes transformation of mouse cells. Cancer Cell 21(6):765–776
20. Gohring G, Lange K, Hofmann W et al (2012) Telomere shortening, clonal evolution and disease progression in myelodysplastic syndrome patients with 5q deletion treated with lenalidomide. Leukemia 26(2):356–358
21. Stoletov K, Montel V, Lester RD et al (2007) High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. Proc Natl Acad Sci U S A 104(44): 17406–17411
22. Lee LM, Seftor EA, Bonde G et al (2005) The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation. Dev Dyn 233(4):1560–1570
23. Herbomel P, Thisse B, Thisse C (1999) Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126(17):3735–3745
24. Lam SH, Chua HL, Gong Z et al (2004) Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. Dev Comp Immunol 28(1):9–28
25. Tolar J, Nauta AJ, Osborn MJ et al (2007) Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 25(2):371–379
26. Mohseny AB, Szuhai K, Romeo S et al (2009) Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol 219(3): 294–305

27. Mohseny AB, Xiao W, Carvalho R et al (2012) An osteosarcoma zebrafish model implicates Mmp-19 and Ets-1 as well as reduced host immune response in angiogenesis and migration. J Pathol 227(2):245–253
28. Marques IJ, Weiss FU, Vlecken DH et al (2009) Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. BMC Cancer 9:128
29. White RM, Sessa A, Burke C et al (2008) Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2(2):183–189
30. Mayordomo E, Machado I, Giner F et al (2010) A tissue microarray study of osteosarcoma: histopathologic and immunohistochemical validation of xenotransplanted tumors as preclinical models. Appl Immunohistochem Mol Morphol 18(5):453–461
31. Mohseny AB, Machado I, Cai Y et al (2011) Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab Invest 91(8): 1195–1205
32. Rauta PR, Nayak B, Das S (2012) Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunol Lett 148(1):23–33
33. van der Vaart M, van Soest JJ, Spaink HP et al (2013) Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. Dis Model Mech 6(3):841–854
34. Press CMCL, Evensen O (1999) The morphology of the immune system in teleost fishes. Fish Shellfish Immunol 9:309–318
35. Bromage ES, Kaattari IM, Zwollo P et al (2004) Plasmablast and plasma cell production and distribution in trout immune tissues. J Immunol 173(12):7317–7323
36. Zwollo P, Cole S, Bromage E et al (2005) B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J Immunol 174(11):6608–6616
37. Zwollo P, Haines A, Rosato P et al (2008) Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. Dev Comp Immunol 32(12):1482–1496
38. Zwollo P, Mott K, Barr M (2010) Comparative analyses of B cell populations in trout kidney and mouse bone marrow: establishing "B cell signatures". Dev Comp Immunol 34(12): 1291–1299
39. Brattgjerd S, Evensen O (1996) A sequential light microscopic and ultrastructural study on the uptake and handling of Vibrio salmonicida in phagocytes of the head kidney in experimentally infected Atlantic salmon (Salmo salar L.). Vet Pathol 33(1):55–65
40. Kaattari SL, Irwin MJ (1985) Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. Dev Comp Immunol 9(3):433–444
41. Manning MJ, Nakanishi T (1996) The specific immune system: cellular defense. The fish immune system. Organism, pathogen and environment. Academic, San Diego, CA
42. Press CM, Dannevig BH, Landsverk T (1994) Immune and enzyme histochemical phenotypes of lymphoid and nonlymphoid cells within the spleen and head kidney of Atlantic salmon. Fish Shellfish Immunol 4(2):79–93
43. Bowden TJ, Cook P, Rombout JHMW (2005) Development and function of the thymus in teleosts. Fish Shellfish Immunol 19:413–427
44. Hall C, Flores MV, Storm T et al (2007) The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. BMC Dev Biol 7:42
45. Bayne CJ, Gerwick L (2001) The acute phase response and innate immunity of fish. Dev Comp Immunol 25(8–9):725–743
46. Carroll MC (2004) The complement system in regulation of adaptive immunity. Nat Immunol 5(10):981–986
47. Carroll MC (2004) The complement system in B cell regulation. Mol Immunol 41(2–3): 141–146
48. Holland JW, Pottinger TG, Secombes CJ (2002) Recombinant interleukin-1 beta activates the hypothalamic-pituitary-interrenal axis in rainbow trout, Oncorhynchus mykiss. J Endocrinol 175(1):261–267

49. Du Pasquier L (2001) The immune system of invertebrates and vertebrates. Comp Biochem Physiol B Biochem Mol Biol 129(1):1–15
50. Fischer U, Utke K, Somamoto T et al (2006) Cytotoxic activities of fish leucocytes. Fish Shellfish Immunol 20(2):209–226
51. Zou J, Grabowski PS, Cunningham C et al (1999) Molecular cloning of interleukin 1beta from rainbow trout Oncorhynchus mykiss reveals no evidence of an ice cut site. Cytokine 11(8):552–560
52. Bird S, Wang T, Zou J et al (2002) The first cytokine sequence within cartilaginous fish: IL-1 beta in the small spotted catshark (Scyliorhinus canicula). J Immunol 168(7):3329–3340
53. Roca FJ, Mulero I, Lopez-Munoz A et al (2008) Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol 181(7):5071–5081
54. Wang T, Secombes CJ (2009) Identification and expression analysis of two fish-specific IL-6 cytokine family members, the ciliary neurotrophic factor (CNTF)-like and M17 genes, in rainbow trout Oncorhynchus mykiss. Mol Immunol 46(11–12):2290–2298
55. Lee MS, Kim YJ (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu Rev Biochem 76:447–480
56. Renshaw SA, Trede NS (2012) A model 450 million years in the making: zebrafish and vertebrate immunity. Dis Model Mech 5(1):38–47
57. Mione M, Meijer AH, Snaar-Jagalska BE et al (2009) Disease modeling in zebrafish: cancer and immune responses – a report on a workshop held in Spoleto, Italy, July 20–22, 2009. Zebrafish 6(4):445–451
58. Chang TY, Pardo-Martin C, Allalou A et al (2012) Fully automated cellular-resolution vertebrate screening platform with parallel animal processing. Lab Chip 12(4):711–716
59. Spaink HP, Cui C, Wiweger MI et al (2013) Robotic injection of zebrafish embryos for high-throughput screening in disease models. Methods 62(3):246–254
60. Truong HH, de Sonneville J, Ghotra VP et al (2012) Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens. Biomaterials 33(1):181–188
61. Amatruda JF, Shepard JL, Stern HM et al (2002) Zebrafish as a cancer model system. Cancer Cell 1(3):229–231
62. Smolowitz R, Hanley J, Richmond H (2002) A three-year retrospective study of abdominal tumors in zebrafish maintained in an aquatic laboratory animal facility. Biol Bull 203(2):265–266
63. Stern HM, Zon LI (2003) Cancer genetics and drug discovery in the zebrafish. Nat Rev Cancer 3(7):533–539
64. Berghmans S, Jette C, Langenau D et al (2005) Making waves in cancer research: new models in the zebrafish. Biotechniques 39(2):227–237
65. Goessling W, North TE, Zon LI (2007) New waves of discovery: modeling cancer in zebrafish. J Clin Oncol 25(17):2473–2479
66. Berghmans S, Murphey RD, Wienholds E et al (2005) tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. Proc Natl Acad Sci U S A 102(2):407–412
67. Kari G, Rodeck U, Dicker AP (2007) Zebrafish: an emerging model system for human disease and drug discovery. Clin Pharmacol Ther 82(1):70–80
68. Peng K, Cui C, Tomatsu I et al (2010) Dextran based photodegradable hydrogels formed via a Michael addition. Soft Matter 7:4881–4887
69. Khanna C, Wan X, Bose S et al (2004) The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 10(2):182–186
70. Bulut G, Hong SH, Chen K et al (2012) Small molecule inhibitors of ezrin inhibit the invasive phenotype of osteosarcoma cells. Oncogene 31(3):269–281
71. Anninga JK, Gelderblom H, Fiocco M et al (2011) Chemotherapeutic adjuvant treatment for osteosarcoma: where do we stand? Eur J Cancer 47(16):2431–2445

72. Chou AJ, Kleinerman ES, Krailo MD et al (2009) Addition of muramyl tripeptide to chemotherapy for patients with newly diagnosed metastatic osteosarcoma: a report from the Children's Oncology Group. Cancer 115(22):5339–5348
73. Buddingh EP, Schilham MW, Ruslan SE et al (2011) Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. Cancer Immunol Immunother 60(4):575–586
74. Lehner M, Gotz G, Proff J et al (2012) Redirecting T cells to Ewing's sarcoma family of tumors by a chimeric NKG2D receptor expressed by lentiviral transduction or mRNA transfection. PLoS One 7(2):e31210
75. Ahn YO, Weigel B, Verneris MR (2010) Killing the killer: natural killer cells to treat Ewing's sarcoma. Clin Cancer Res 16(15):3819–3821
76. Ganeshkumar M, Sathishkumar M, Ponrasu T et al (2013) Spontaneous ultra fast synthesis of gold nanoparticles using Punica granatum for cancer targeted drug delivery. Colloids Surf B Biointerfaces 106:208–216
77. Hagner N, Joerger M (2010) Cancer chemotherapy: targeting folic acid synthesis. Cancer Manag Res 2:293–301
78. Walkley CR, Qudsi R, Sankaran VG et al (2008) Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes Dev 22(12): 1662–1676
79. Parant JM, George SA, Holden JA et al (2010) Genetic modeling of Li-Fraumeni syndrome in zebrafish. Dis Model Mech 3(1–2):45–56
80. Mohseny AB (2008) Bone: conventional osteosarcoma. Atlas Genet Cytogenet Oncol Haematol. http://AtlasGeneticsOncology.org/Tumors/ConvOsteoID5344.html
81. Mohseny AB, Tieken C, van der Velden PA et al (2010) Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. Genes Chromosomes Cancer 49(12):1095–1103
82. Savage SA, Mirabello L, Wang Z et al (2013) Genome-wide association study identifies two susceptibility loci for osteosarcoma. Nat Genet 45(7):799–803
83. Bedell VM, Wang Y, Campbell JM et al (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491(7422):114–118
84. Bill BR, Petzold AM, Clark KJ et al (2009) A primer for morpholino use in zebrafish. Zebrafish 6(1):69–77
85. Cade L, Reyon D, Hwang WY et al (2012) Highly efficient generation of heritable zebrafish gene mutations using homo- and heterodimeric TALENs. Nucleic Acids Res 40(16): 8001–8010
86. Chen S, Oikonomou G, Chiu CN et al (2013) A large-scale in vivo analysis reveals that TALENs are significantly more mutagenic than ZFNs generated using context-dependent assembly. Nucleic Acids Res 41(4):2769–2778
87. Clark KJ, Voytas DF, Ekker SC (2011) A TALE of two nucleases: gene targeting for the masses? Zebrafish 8(3):147–149
88. Dahlem TJ, Hoshijima K, Jurynec MJ et al (2012) Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genet 8(8):e1002861
89. Huang P, Zhu Z, Lin S et al (2012) Reverse genetic approaches in zebrafish. J Genet Genomics 39(9):421–433
90. Moore FE, Reyon D, Sander JD et al (2012) Improved somatic mutagenesis in zebrafish using transcription activator-like effector nucleases (TALENs). PLoS One 7(5):e37877
91. Leacock SW, Basse AN, Chandler GL et al (2012) A zebrafish transgenic model of Ewing's sarcoma reveals conserved mediators of EWS-FLI1 tumorigenesis. Dis Model Mech 5(1):95–106
92. Onnebo SM, Condron MM, McPhee DO et al (2005) Hematopoietic perturbation in zebrafish expressing a tel-jak2a fusion. Exp Hematol 33(2):182–188

93. de Andrea CE, Prins FA, Wiweger MI et al (2011) Growth plate regulation and osteochondroma formation: insights from tracing proteoglycans in zebrafish models and human cartilage. J Pathol 224(2):160–168
94. Wiweger MI, Avramut CM, de Andrea CE et al (2011) Cartilage ultrastructure in proteoglycan-deficient zebrafish mutants brings to light new candidate genes for human skeletal disorders. J Pathol 223(4):531–542
95. Wiweger MI, Zhao Z, van Merkesteyn RJ et al (2012) HSPG-deficient zebrafish uncovers dental aspect of multiple osteochondromas. PLoS One 7(1):e29734