

# Rhabdomyosarcoma xenotransplants in zebrafish embryos

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## Abstract

Rhabdomyosarcomas (RMS) are the most common pediatric soft tissue sarcomas. High-risk and metastatic disease continues to be associated with very poor prognosis. RMS model systems that faithfully recapitulate the human disease and provide rapid, cost-efficient estimates of anti-tumor efficacy of candidate drugs are needed to facilitate drug development and personalized medicine approaches. Here, we present a new zebrafish-based xenotransplant model allowing for rapid and easily accessible drug screening using low numbers of viable tumor cells and relatively small amounts of water-soluble chemicals. Under optimized temperature conditions, embryonal RMS-xenografts were established in zebrafish embryos at 3 hours post fertilization (hpf). In proof-of-principle experiments, chemotherapy drugs with established clinical anti-RMS efficacy (vincristine, dactinomycin) and the MEK inhibitor trametinib were shown to significantly reduce the cross-sectional area of the tumors by 120 hpf. RMS xenograft models in zebrafish embryos henceforth could serve as a valuable addition to cell culture and mammalian models of RMS and represent a rapid and cost-effective solution for pre-clinical candidate drug testing.

## Introduction

Representing approximately 6% of all pediatric malignancies, soft tissue sarcomas constitute the third most frequent extracranial solid malignant tumor entity in children. Rhabdomyosarcomas (RMS) are the most common pediatric soft tissue sarcomas and a heterogeneous group of cancers. The main subgroups of RMS in children and adolescents are alveolar and embryonal, which differ in their histology and genetic underpinnings, but also their clinical behavior. Approximately 80% of alveolar RMS (ARMS) are associated with the chromosomal translocations  $t(2;13)(q35;q14)$  or  $t(1;13)(p36;q14)$ . These translocations lead to fusions of the *FOXO1* gene on chromosome 13 to the *PAX3* gene on chromosome 2 or the *PAX7* gene on chromosome 1. The resulting fusion genes *PAX3-FOXO1* and *PAX7-FOXO1* are considered drivers of malignancy in ARMS. Embryonal RMS (ERMS) show a higher degree of genetic diversity, including frequent RAS pathway gene mutations and genetic imbalances such as loss of heterozygosity (LOH) at chromosome locus 11p15. This genomic region contains several genes that play important roles in growth control and are therefore associated with different types of embryonal tumors. There are major differences in clinical characteristics, response to therapy and outcomes between embryonal and alveolar RMS. The 5-year overall survival rate of pediatric patients with RMS is approximately 70%. ARMS are generally associated with substantially worse outcomes than ERMS. There is an urgent need to establish the anti-RMS efficacy of candidate drugs and improve long-term survival, especially for patients with relapsed or metastatic disease, for whom survival rates continue to be dismal. In this study, we sought to establish a rapid RMS research platform in zebrafish embryos by implanting 3 hours-old zebrafish embryos with RMS cells. RMS xenografts were shown to shrink in response to exposure to anti-RMS drugs in proof-of-principle experiments.

## Methods

**Cell culture.** RD (*PAX3:FOXO1* -negative; *NRAS Q61H*) and Rh30 (*PAX3:FOXO1* -positive) cells were

maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 20% fetal bovine Serum (FBS; Thermo Fisher Scientific) (Rh30) cells and 10% FBS (RD), respectively. 1% Penicillin/Streptomycin (Thermo Fisher Scientific) was added to the medium for both cell lines. Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere containing incubator. The identity of the cell lines was confirmed by short tandem repeat (STR) fingerprinting by Eurofins

**Zebrafish husbandry.** Zebrafish were maintained and bred under standard conditions at the Developmental Biology, Institute for Biology I, University of Freiburg, as approved by Regierungspräsidium Freiburg. Experiments were conducted only during the first 120 hours post-fertilization. The following fish lines were used for the experiments: *nacre* (*mitfa* mutant, deficient in body pigment cells) and *sandy* (also known *sdv*; *tyrosinase* mutant, deficient in melanin dark pigment). All experiments were carried out in accordance with German laws for animal care.

**Optimization of environmental temperatures.** Zebrafish embryos were maintained at three different temperature levels from 3 to 120 hours post fertilization (*hpf*). The control group (n=48) was maintained at 28.5°C, which is considered the standard temperature for maintenance of zebrafish embryos. Experimental group 1 (n=48) was kept at 33°C from 3 to 48 hpf, followed by a rise in environmental temperatures to 35°C until 120 hpf. The temperature for experimental group 2 (n=48) was maintained at 35°C from 3 to 120 hpf. Embryos were microscopically observed each day and classified as "abnormal" if they showed any visible signs of damage such as pericardial edema, scoliosis, or absence of heartbeats at 72 hpf.

To examine the effects of different environmental temperatures on RMS growth *in vitro*, RD and Rh30 cells were plated in 96-well plates at a density of 500 cells per well. Starting at the time of plating, cells were maintained at 33°C for two days, followed by a temperature rise to 35°C until day 5, analogous to the zebrafish experiment described above. For comparison, control cells were maintained at 37°C from the time of plating until day 5. Cell counts were determined on the day of plating as well as 2, 4 and 5 days after plating using a hemacytometer (4 replicates per day and condition). Mean cell numbers on day 5 were tested for statistical significance by Student's t-tests.

**Transplantation procedure.** RMS cells at 80% confluence were washed in PBS followed by incubation in staining-solution comprised of 10 µL lipophilic DiI (Thermo Fisher Scientific, V22885) in 2ml serum free DMEM for 20 minutes at 37°C. The freshly stained RMS cells then were washed twice in PBS to remove staining remnants, spun down and resuspended in 1 ml of PBS supplemented with 2% FBS and 2% PVP360 to prevent the cells from clotting in the injection capillaries. The cells were stored on ice until transplantation. Immediately before transplantation, cells were spun down and resuspended to a final concentration of  $\sim 1 \times 10^6$  / 100µL

1µL of the cell suspension was aspirated into a 100µm-diameter pulled borosilicate glass needle without filament (Science Products GmbH), using an oil-based microinjector (L.S. Starrett). The 3 hours-old zebrafish embryos were lined into injection molds made from agarose gel. Approximately 100 cells per embryo were transplanted into the blastoderm of 1000-cell to high stage zebrafish embryos. Transplanted embryos then were transferred into petri dishes containing E3 medium and kept at 33°C. One hour after transplantation embryos were screened for successful transplantation by fluorescence microscopy to detect the DiI-labeled xenografts.

Although transplantation and screening were performed at room temperature (24-28°C), development of embryos implanted with RMS cells and unmanipulated embryos at 28.5°C progressed without notable differences. All embryos were euthanized before exceeding 120 hpf.

**Xenograft imaging and analysis.** Embryos were anesthetized with 0.168 mg tricaine/ml E3 medium at 24, 72 and 120 hpf and placed in lateral position. Serial images were taken with Zeiss ZEN-Software using an Axio Examiner D1 (Zeiss) at 5X magnification. The cross-sectional area of the xenografts *in vivo* was measured sequentially at 24, 72 and 120 hpf to analyze changes in the size of the xenotransplants over time. For each timepoint, cross-sectional areas of the xenografts were measured using FIJI software. Xenografts were outlined using the default algorithm of the FIJI software; the z-plane with the largest cross-sectional

surface area was considered for analyses. To make changes in tumor size directly comparable, the standardized cross-sectional area (SCSA) was calculated for each timepoint by dividing the cross-sectional area measured at any given timepoint (24, 72, 120 hpf) by the value measured at 24 hpf.

**Histology.** Larvae were euthanized in tricaine at 72-120 hpf followed by fixation in 4% PFA overnight. Fixed embryos then were embedded in paraffin, sectioned into 5 $\mu$ M thick slices, and stained with Hematoxylin and Eosin, following standard staining procedures .

**Toxicity screening.** 24 hours-old embryos were manually dechorionated and transferred to a 96-well plate containing E3 medium (one embryo per well). Vincristine, dactinomycin and trametinib were added to the wells in increasing concentrations as indicated in Figure 3A-C. Control embryos were maintained in standard E3 medium without exposure to any chemicals. Embryos in the carrier group were exposed to carrier solution only using the same carrier volume that was necessary to add the highest drug concentration tested. Embryos were microscopically analyzed at 120 hpf for visible damage. If they showed signs of toxicity such as pericardial edema, scoliosis, or absence of heartbeats at 72 hpf, or if they were already dead, the embryos were classified as “did not survive”. Each experimental group contained 16 embryos per experiment; the experiment was repeated 3 times. Maximum tolerated concentrations of each drug were determined based on differences in the survival rates of embryos exposed to increasing drug concentrations.

**Testing the effects of candidate drugs on RMS-xenografts in zebrafish embryos.** For drug testing experiments, approximately 100 DiI-labeled RD cells were transplanted into zebrafish embryos at the 1k-cell stage (3hpf). One hour after the transplantation procedure, embryos were inspected for successful transplantation via fluorescence microscopy. The embryos were then transferred to a 24-well plate containing E3 and maintained at 33°C until they reached 48 hpf, followed by a temperature rise to 35°C until 120 hpf. Chemicals were added to the E3-medium at 24 hpf. The following concentrations were tested: vincristine at 2 and 20  $\mu$ M; dactinomycin at 0.1 and 1  $\mu$ M; trametinib at 5 and 50 nM. Furthermore, embryos were exposed to 2 $\mu$ M vincristine and 0.1 $\mu$ M dactinomycin in combination.

To measure the effects of vincristine and dactinomycin on RD xenografts *nacre* embryos were used, whereas the effects of Trametinib on RD-xenografts were tested in transplanted *sdly* embryos, due to availability of the respective fish lines.

Observations from animals exposed to the same chemicals in 9 independent experiments were pooled and compared to the control group, which consisted of 30 *nacre* embryos and 15 *sdly* embryos, respectively, from 9 independent experiments. The following number of xenograft-bearing embryos were considered for analyses, after “dead or disformed” ones were removed from the experiments: 17 embryos exposed to 2  $\mu$ M vincristine, 30 exposed to 20  $\mu$ M vincristine, 16 to 0,1  $\mu$ M dactinomycin, 27 to 1  $\mu$ M dactinomycin, 16 to 2  $\mu$ M vincristine and 0,1  $\mu$ M dactinomycin, 11 to 5 nM trametinib, 21 to 50 nM trametinib and 17 to 10  $\mu$ l DMSO.

**Statistical Analysis.** Experiments were analyzed using Prism GraphPad 9.1.2. All Experiments with two experimental groups were analyzed using the Student’s t-test, whereas experiments with more than two groups were analyzed using ANOVA followed by Turkey’s multiple comparisons test. Differences were considered significant when *p*-Value was below 0.05. *P*-Values were illustrated in figures using asterisks:  $p < 0.05$  (\*),  $p < 0.005$  (\*\*),  $p < 0.0005$  (\*\*\*),  $p < 0.00005$  (\*\*\*\*).

## Results

**Optimization of environmental temperatures.** In laboratory environments, zebrafish embryos commonly are raised at 28.5°C , whereas human cells are maintained at 37°C . To enable engraftment of RMS xenotransplants in zebrafish embryos, it was necessary to establish a temperature level that allows for normal development of both zebrafish embryos and human tumor cells. To determine optimal environmental conditions, zebrafish embryos were exposed to different temperatures. The percentage of surviving embryos at 120 hpf was significantly lower at 35°C compared to the control group maintained at 28.5°C (Fig.1A,  $p = 0.0016$ ). This is consistent with previously published observations . Considering the higher mortality rate at 35°C, we tested a gentler two-step approach using temperatures of 33°C from 3 hpf to 48 hpf, followed

by a temperature rise to 35°C until 120 hpf, which did not have a negative impact on the survival rates of the zebrafish embryos compared to those in the control group (Fig.1A,  $p = 0.6244$ ). Next, these temperature settings were applied to RMS cells in culture, to examine possible differences in cell proliferation at 1, 48, 96 and 120 hours after plating. Lower temperatures did not affect RD cell growth, and the number of RD cells kept at 37°C did not differ significantly 120 hours after plating (Fig.1B, 4750 cells/well at 37°C vs. 5000 cells/well at 33/35°C,  $p = 0.6753$ ). In contrast, proliferation of Rh30 cells slowed down significantly at lower temperatures compared to the control cells maintained at 37°C (Fig.1C, 8750 cells/well at 37°C vs. 3438 cells/well at 33/35°C,  $p < 0.0001$ ).

**Induction of RMS xenografts in zebrafish embryos.** To analyze the development of Rh30 and RD cells *in vivo*, DiI-labeled RMS cells were transplanted into zebrafish embryos at the 1000-cell stadium (Fig.2). Images of the transplants were taken, and cross-sectional areas were measured at 24, 72 and 120 hpf (Fig.3A-C). RD xenografts showed stable growth from 24 hpf until 120 hpf (Fig. 3D), whereas the cross-sectional areas of Rh30 xenografts decreased during the observation period (Fig. 3D). Histology sections from 72-120 hours-old, successfully transplanted embryos were prepared and stained with hematoxylin and eosin. The presence of tumor cell nests confirmed successful engraftment of xenotransplants (Fig. 3E).

**Toxicity screening.** Possible toxic effects of the candidate anti-RMS drugs on embryonal and larval development were explored until 120 hpf. Vincristine significantly reduced the proportion of surviving embryos starting at drug concentrations higher than 20  $\mu\text{M}$  (Fig. 4A, 94% surviving embryos in E3 vs. 69% surviving embryos in E3 medium with 25 $\mu\text{M}$  vincristine,  $p < 0.0001$ ). Dactinomycin treatment also led to significantly lower survival rates starting at concentrations exceeding 1  $\mu\text{M}$  (Fig. 4B, 88% surviving embryos in E3 vs. 73% surviving embryos in E3 medium with 2 $\mu\text{M}$  dactinomycin,  $p = 0.0398$ ). The mitogen-activated protein kinase kinase (MEK) inhibitor trametinib negatively affected the embryos starting at drug-concentrations higher than 50 nM (Fig. 4C, 88% surviving embryos in E3 vs. 67% surviving embryos in E3 medium with 100nM trametinib,  $p = 0.0007$ ).

**Testing the effects of candidate drugs on RMS xenografts in zebrafish embryos.** Successfully transplanted embryos were exposed to selected drugs to assess their effect on RD-xenotransplants *in vivo*. Vincristine concentrations of 2 and 20  $\mu\text{M}$  reduced the standardized cross-sectional area (SCSA) to 39% (Fig 5A;  $p < 0.0001$ ) and 15% (Fig. 5A;  $p < 0.0001$ ), respectively, compared to vehicle-treated RMS xenotransplants. A statistically significant, dose-dependent effect of vincristine at 2 and 20  $\mu\text{M}$  could also be observed (Fig. 5A;  $p < 0.0001$ ), further supporting the observation that vincristine has antitumoral effects in zebrafish RMS-xenotransplants.

Dactinomycin showed an analogous inhibitory effect on RMS xenografts in zebrafish with a reduction to 52% of the SCSA of non-treated xenotransplants (Fig. 5B;  $p < 0.0001$ ) at a concentration of 0.1  $\mu\text{M}$ , and to 24% of the SCSA of non-treated xenotransplants (Fig. 5B;  $p < 0.0001$ ) at concentrations of 1 $\mu\text{M}$  dactinomycin.

2 $\mu\text{M}$  vincristine and 0.1 $\mu\text{M}$  dactinomycin in combination showed stronger inhibitory effects compared to 0.1 $\mu\text{M}$  dactinomycin monotherapy therapy (Fig. 5C; reduction to 28% of the SCSA of untreated xenografts by 2 $\mu\text{M}$  vincristine and 0.1 $\mu\text{M}$  dactinomycin in combination vs. 52% by 0.1 $\mu\text{M}$  dactinomycin alone;  $p = 0.0055$ ). There were no significant differences in the efficacy of the combination treatment compared to exposure to 2 $\mu\text{M}$  vincristine alone (Fig. 5C;  $p = 0.4265$ ). Given that RD cells carry an *NRAS proto-oncogene, GTPase (NRAS)Q61H*- variant resulting in activation of mitogen-activated protein kinase (MAPK) signaling, the MEK-inhibitor trametinib was also tested for inhibitory effects on RMS xenotransplants in zebrafish embryos. At trametinib concentrations of 5 and 50 nM, we observed a reduction of the SCSA to 57% ( $p < 0.0001$ ) and 21% ( $p < 0.0001$ ) of the SCSA of non-treated RMS-xenotransplants, respectively (Fig. 5D).

## Discussion

Rhabdomyosarcomas continue to be a formidable challenge in pediatric oncology. Especially patients with *FOXO1*- rearranged or metastatic RMS face extremely poor outcomes. In recent years, significant research efforts have provided insights into possible new therapeutic strategies. Also, molecular characterization of tumor tissue, aimed at identifying targets for individualized treatment of patients with refractory disease,

has proven feasible . There is an increasing need for RMS model systems that faithfully recapitulate the human disease and provide rapid, cost-efficient estimates of anti-tumor efficacy of candidate drugs.

Zebrafish represent an extremely cost-efficient model organism, that is used in many laboratories all over the world, easy to handle and well-suited for high-throughput experiments. In optimized conditions, female zebrafish can lay up to 200 eggs per clutch , allowing for large numbers of experimental animals and thereby reducing the statistical risk of incorrect measurements. RMS xenografts in zebrafish were previously reported in the literature. Chuan et al used ERMS and ARMS xenografts, established in 2 months-old zebrafish by injecting cells into the peri-ocular muscle and intraperitoneal spaces, to observe the effects of drugs, which were administered by oral gavage, at environmental temperatures of 37°C over a period of 28 days post injection. Xenograft induction in zebrafish embryos offers a number of advantages over xenotransplants in adult fish, including faster turnaround time, better scalability, easier drug application , and ease of repetitive imaging including time-lapse microscopy to assess xenograft dynamics. In this study, we set out to establish RMS xenografts in zebrafish embryos. Due to the small size of the zebrafish embryos, only few tumor cells were required to establish xenografts, and relatively small amounts of drugs were sufficient for testing.

The zebrafish platform established here allows for reliable induction of RMS xenografts, derived from the embryonal RMS cell line RD, in 3 hours-old zebrafish embryos. To reconcile viability and normal development of the embryos with engraftment of human cells, we selected optimized temperature conditions based on our own data (Fig. 1A) and previous reports . Temperatures higher than 33.5°C during early embryonic development are associated with abnormal embryonic development and higher mortality . Post gastrulation, higher temperatures at 35°C are tolerated more easily by the embryos. Indeed, we successfully kept embryos at temperatures of 33°C from 3 hpf to 48 hpf, followed by a temperature rise to 35°C until 120 hpf.

Proof-of-principle experiments revealed dose-dependent inhibitory effects of vincristine and dactinomycin - two chemotherapy drugs with long-established anti-RMS efficacy – on RMS xenografts in zebrafish embryos, validating the reliability of the model system in assessing antitumor effects of established anti-RMS drugs. The MEK inhibitor trametinib also led to a significant reduction in the size of xenografts, derived from *NRAS Q61H* -mutated RD cells . Of note, in the experiments reported here, PAX3:FOXO1 fusion-positive Rh30 cells did not engraft under the conditions described above. This corresponded to slower proliferation of Rh30 cells *in vitro* at lower environmental temperatures. Interestingly, lower temperatures did not exert any adverse effects on the *in vitro* growth of RD cells. Future experiments will determine if RMS cells, derived from other PAX3:FOXO1 fusion-positive cell lines or fresh patient-derived tumor tissue, are capable of engrafting in zebrafish embryos. Also, it will be interesting to observe possible differences in the location of primary tumors and patterns of metastases between xenografts derived from genetically distinct RMS tumors.

Zebrafish embryos as model organisms are generally associated with certain limitations. While there are substantial functional homologies between the zebrafish and the human genome , fundamental differences between these two vertebrate organisms must not be neglected. For example, the temperature requirements of zebrafish differ from those of mammals. The application of water-soluble chemicals to the embryo medium is remarkably easy, but the exact chemical dose that is absorbed into the embryo remains unclear, and larger or non-water-soluble molecules need to be injected into the embryo's yolk sac, necessitating more complex experimental procedures comparable to drug application procedures used in mice or older zebrafish. Furthermore, the development of the mature immune system in zebrafish embryos takes 4-6 weeks .

A xenotransplant model similar to the system described here has recently been published and is based on injection of ERMS patient-derived xenograft (PDX) tumor cells into the yolk sac of zebrafish embryos at 48 hpf, with first addition of drugs in the medium at 72 hpf and continued exposure until 120 hpf. Embryos were kept at 28°C prior to injection of tumor cells and at 34 °C after injection of tumor cells . These observations are in line with those obtained from our experiments and confirm the potential of the zebrafish embryo xenograft platform. Important differences lie in the timing of transplantation and drug treatment with our model offering a longer treatment and observation period. Also, we argue that transplantation into the embryo itself compared to transplantation into the yolk sac offers the advantage of a cellular milieu.

In conclusion, the zebrafish embryo-based platform described here is very fast, cost-efficient, and easy to handle. It takes only 120 hours, low numbers of viable tumor cells and relatively small amounts of chemicals to obtain estimates on the anti-tumor efficacy of water-soluble chemicals. Laboratory requirements are easily accessible, and regulatory thresholds are low. By contrast, turnaround times range from weeks to months if xenografts are established in mice/ older zebrafish or if tumor cells are expanded in tissue culture. These demands increase when experiments are designed to cover replicate measurements and therefore require large numbers of cells. We argue that RMS xenograft models in zebrafish embryos lift rapid pre-clinical drug testing to a new level and henceforth could serve as a valuable addition to cell culture and mammalian models of this devastating cancer.

### Conflict of Interest statement

The authors declare that they have no conflict of interest.

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### References

#### Figure Legends

**Figure 1:** (A) The survival rate of zebrafish embryos at different environmental temperatures was examined during the first 120 hpf. Larvae maintained continuously at 35°C showed lower survival rates compared to the control group kept at 28.5°C. In contrast, embryos maintained at 33°C to 35°C exhibited similar survival compared to the control group. In parallel experiments, the development of (B)RD cells and (C) Rh30 cells was evaluated using the same temperature conditions. (B) RD cell numbers did not differ significantly at lower temperatures compared to control cells maintained at standard temperatures. (C) Rh30 cell numbers counted at 120 hours were significant lower when cells were maintained at lower temperatures.

**Figure 2:** Transplantation procedure: fertilized eggs were maintained at 28.5°C until 3 hpf. RMS cells were injected into the blastodisc, and environmental temperature was raised to 33°C. Embryos were screened at 4 hpf for successful transplantation. The embryos then were transferred to 24 well plates. Maintenance temperature was raised to 35°C at 48 hpf until the end of the experiments.

**Figure 3:** Development of RMS xenografts, derived from RD cells, is depicted at (A) 24 hpf, (B) 72 hpf and (C)120 hpf. (D) The development of RD and Rh30 cells xenotransplants was observed until 120hpf. The SCSA of RD tumors increased, whereas the SCSA of Rh30 tumors decreased during the observation period. (E) The head of a xenotransplanted zebrafish embryo was sectioned and stained with hematoxylin eosin. The arrow marks a nest of tumor cells.

**Figure 4:** Toxic effects of vincristine, dactinomycin and trametinib were screened in zebrafish embryos. Embryos tolerated exposure to (A) vincristine up to concentrations of 20 µmol/l, (B) dactinomycin up to concentrations up to 2 µmol/l and (C) trametinib up to concentrations of 50 nmol/l.

**Figure 5:** The anti-RMS effects of candidate chemicals on RD xenografts in zebrafish embryos. (A) Vincristine reduced the SCSA at concentrations of 2 and 20 µmol/l, respectively. (B) Dactinomycin exhibited analogous anti-RMS effects compared to vincristine. (C) Vincristine and dactinomycin in combination did not show stronger anti-RMS effects compared to vincristine alone but had stronger inhibiting effects compared to dactinomycin alone. (D) The MEK-inhibitor trametinib also exerted dose-depending anti-RMS effects *in vivo*.



