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An osteosarcoma zebrafish model implicates *Mmp-19* and *Ets-1* as well as reduced host immune response in angiogenesis and migration

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Abstract

About 40% of osteosarcoma patients die of metastases. Novel strategies to improve treatment of metastatic patients require a better understanding of the processes involved, like angiogenesis, migration, and the immune response. However, the rarity of osteosarcoma and its heterogeneity make this neoplasm difficult to study. Recently we reported malignant transformation of mouse mesenchymal stem cells (MSCs) which formed osteosarcoma upon transplantation into mice. Here we studied these cells in zebrafish embryos and found that transformed MSCs induced angiogenesis and migrated through the bodies of the embryos, but this was never observed with non-transformed normal MSCs (progenitors of the transformed MSCs). Whole genome expression analysis of both the cells and the host showed that angiogenesis and migration-related genes *matrix metalloproteinase 19* (*Mmp-19*) and *erythroblastosis virus E26 oncogene homologue 1* (*Ets-1*) were overexpressed in transformed MSCs compared to normal MSCs. Investigating the host response, embryos injected with transformed MSCs showed decreased expression of immune response-related genes, especially *major histocompatibility complex class 1* (*mhc1ze*), as compared to embryos injected with normal MSCs. These findings contribute to the identification of genetic events involved in angiogenesis, migration, and host response providing targets as well as an appropriate model for high-throughput drug screens.

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Keywords: osteosarcoma; mesenchymal stromal cells; migration; angiogenesis; immune response; transgenic zebrafish; gene expression; model system; drug screening

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Introduction

High-grade (conventional) osteosarcoma is an aggressive neoplasm, most probably originating from mesenchymal stem cells (MSCs) mutated somewhere in the process of differentiation towards osteoblasts [1–3]. It is the most common skeletal neoplasm of non-haematopoietic origin diagnosed in adolescents (mean age of 17 years) and is the second leading cause of cancer deaths within this age group [4]. Despite aggressive therapies by surgery and chemotherapy, 30–40% of the patients experience progressive metastatic disease within 5 years after diagnosis and die [5,6]. In order to reduce the fatality rate associated with progressive or metastatic osteosarcoma, a better understanding of the hallmarks of progressive osteosarcoma—especially mechanisms involving angiogenesis, invasion, and metastasis [7]—is required. Although to some extent terminal differentiation is disrupted, osteosarcoma cells

exhibit differentiation, often along the osteoblastic lineage. Nevertheless, many other and mixed subtypes are found, resulting into a broad spectrum of histological variation [8] without large clinical differences between the subtypes [9]. Next to histological heterogeneity, full-blown osteosarcoma at presentation is genomically highly unstable and shows an array of alterations [10,11]. These facts together with the rarity of osteosarcoma make it hard to study the human disease by looking for common genetic alterations and emphasize the need for representative model systems. Previously we showed that murine MSCs undergo malignant transformation during *in vitro* culturing. Interestingly, upon xenotransplantation in mice, these transformed murine MSCs produced osteosarcoma [12], thereby providing an excellent model to study osteosarcoma genesis from normal, non-tumourigenic cells to a full-blown malignancy. Genetic characterization of these cells identified aneuploidization and loss of *CDKN2A* genes as the main underlying genomic events for this transformation

[1]. In addition, the loss of *CDKN2A/p16* in human osteosarcoma was shown to be caused by the same mechanism, ie genomic deletion, validating the model [13].

Implementing the MSC model in zebrafish embryos would not only provide an alternative and faster method to detect transformation of MSCs, but would also allow studies of the *in vivo* behaviour of these transformed cells in a high-throughput manner. Furthermore, the host response against these transformed MSCs compared with the response against normal MSCs could be investigated. External development, small size, transparency, and short generation time make the zebrafish (*Danio rerio*) embryo an ideal vertebrate organism for *in vivo* studies [14]. Moreover, the zebrafish genome has been fully sequenced, showing many conserved genes, and the animals are relatively easily accessible for genetic manipulation [15]. For these reasons, zebrafish models have been established to study human biology, diseases, and drug screening in a high-throughput manner [16]. Furthermore, many cancer-related zebrafish models have been developed [17–19], including models to study migration and angiogenesis [20,21], although osteosarcoma models are lacking.

In this study, we investigate clinically relevant characteristics of osteosarcoma-like migration and angiogenesis in zebrafish embryos. We show that zebrafish embryos provide an efficient and high-throughput model to screen for malignant transformation of normal MSCs. The transformed MSCs behave more aggressively *in vivo* compared with their normal parental cells and are comparable to cells from actual osteosarcoma-like tumours in terms of invasion, migration, and angiogenesis. In addition, whole genome gene expression analyses identified differentially expressed genes in transformed MSCs as opposed to normal MSCs that were involved in the processes of migration and angiogenesis. Regarding the host response of the fish, differentially expressed genes were shown to be mainly involved in the immune system. These genes provide potential targets to inhibit angiogenesis and migration or to stimulate the host response against cancer cells, facilitating new approaches to treat osteosarcoma patients.

Materials and methods

Cell culture

Two MSC cultures (coded as B6-BMMSC and BC-BMMSC) freshly harvested from the bone marrow of C57BL/6 and BALB/C mice, respectively, previously characterized and shown to transform into osteosarcoma-producing cells after a period of crisis [1] were included. These cell cultures and two previously characterized cell lines that produced metastatic osteosarcoma lesions [12], TmMSC and S1d/s, were cultured in α -minimal essential medium (Catalogue

No BE12-169F; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 2% Glutamax (Invitrogen), and 2% penicillin/streptomycin (GIBCO, Invitrogen) in T75 uncoated flasks (Greiner Bio-one, Frickenhausen, Germany). Cell cultures were kept at 37 °C with 5% CO₂. Upon 80% confluence, 5×10^5 cells were passaged to a new flask and the rest collected for RNA isolation. The total cell count of each passage was recorded to calculate the number of population doublings (PD).

Zebrafish embryos

All experiments were performed at the larval stage of the zebrafish, ie up to 5 days post-fertilization (dpf), approved by the Leiden University Animal Experiments Committee (DEC), according to Dutch animal experiments guidelines. Two transgenic zebrafish, one from the Tg (Fli: EGFP) strain [22] and one from the Casper strain [23], were crossed to generate embryos with green fluorescent vasculature (Fli) and transparent bodies beyond the normal pigmentation stage (Casper). Next morning, the eggs were collected and kept in egg-water at 28.5 °C, a suitable temperature for zebrafish embryos.

Cell labelling and injection

Before injection, cells were labelled fluorescently as follows. After trypsinization, cells were spun down and re-suspended with 990 μ l of 1000 $\times$ diluted CM-Dil solution (Invitrogen, Breda, The Netherlands). Subsequently, tubes were wrapped in aluminium foil and incubated at 37 °C for 15 min, with gentle mixing every 5 min followed by a 5-min incubation at 4 °C. After washing with PBS, cells were re-suspended in 10 μ l of 5% Polyvinylpyrrolidone-40 (PVP; Sigma-Aldrich, Munich, Germany) to prevent cell clotting and sticking to the needle during injection. Since S1d/s cells already have a stable DsRed fluorescence label [12], they were directly re-suspended in PVP without labelling. Before injection, 2 dpf zebrafish embryos were mechanically dechorionated by using two forceps, placed on an agarose plate, and anaesthetized with 2 $\times$ tricaine mesylate solution (Sigma-Aldrich). Subsequently, cells were loaded in a needle and injected under a microscope by a FemtoJet microinjector (Eppendorf, Vienna, Austria), injecting approximately 500 cells into the yolk sacs of zebrafish embryos without entering any organ or blood vessels. After injection, embryos were kept at 34 °C, an optimal temperature between 28.5 °C and 37 °C (optimal temperatures for zebrafish embryos and mouse MSCs, respectively).

Imaging

After injection, all zebrafish embryos were screened daily using a fluorescent stereo-microscope (Leica, Wetzlar, Germany) to visualize the GFP-labelled blood vessels of the fish and the CM-Dil-labelled cells in fluorescent mode. The presence as well as the location of

newly formed green GFP-labelled blood vessels and red CM-Dil-labelled cells was recorded for angiogenesis and migration, respectively [20]. Meanwhile, at least five representative zebrafish embryos per injection group were selected and kept in 96-well plates. These plates were photographed daily for 3 days using the camera coupled with the microscope to follow each individual fish.

RNA isolation

At 5 dpf, all embryos of the same injection group were pooled in single tubes and frozen in liquid nitrogen. The frozen zebrafish and cell pellets of *in vitro* cultured MSCs were subjected to total RNA isolation using the miRNeasy Mini Kit (Catalogue No 217004; Qiagen, Maryland, USA). The concentration and quality of isolated RNAs were measured by a Nanodrop ND-1000 (Isogen, De Meern, The Netherlands) and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. All the RIN (RNA integrity number) values were above 7, implying that the integrity of the RNA samples was of sufficient quality for subsequent amplification and microarray hybridization [24].

Whole genome expression analysis

To investigate the genetic basis of the phenotypic differences between normal and transformed/tumourigenic MSCs—cultured *in vitro* and inside zebrafish embryos—gene expression profiles were studied in three experimental groups. The first group comprised *in vitro* cultured MSCs to analyse differences intrinsic to the cells. The second group consisted of total RNA from embryos injected with MSCs (or without in the case of the controls) hybridized to mouse arrays to study MSC gene expression profiles inside the embryos. The third group also contained total RNA from embryos injected with MSCs (or with PVP only in the case of the controls) but now hybridized to zebrafish arrays to investigate the embryos' responses. The extent of cross-hybridization between mouse and zebrafish was assayed by hybridizing mouse RNA to a zebrafish array and vice versa. From each sample, 500 ng of total RNA was amplified with the Quick Amp Labeling Kit (Agilent Technologies) after adding spike-ins (Two-Colour RNAs Spike-In Kit, Agilent Technologies). After column purification, the yield and quality were assessed with a Nanodrop ND-1000 (Isogen) and a 2100 Bioanalyzer (Agilent Technologies). Each zebrafish 4 × 44 k array (design ID: 021626) was hybridized with 825 ng of Cy5-labelled pooled material which was mixed by samples of all injected zebrafish (common reference channel) and 825 ng of Cy3-labelled material (test channel). Each mouse 4 × 44 k array (design ID: 026687) was hybridized with 825 ng of Cy5-labelled pooled material which was mixed by samples of different mouse MSCs (common reference channel) and 825 ng of Cy3-labelled material for cultured cells or 5 × 825 ng Cy3-labelled material for zebrafish injected

with mouse cells (test channel) because of the low amounts of mouse cells in the injected zebrafish. Hybridization and washing were performed according to the manufacturer's instructions (Two-Colour Microarray-Based Gene Expression Protocol Manual version 5.5, Agilent Technologies). Slides were scanned with an Agilent G2505C scanner at 2 µm resolution and 20 bit scan-depth, and data were extracted with Feature Extraction version 10.7.3.1. MIAME-compliant data have been deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>, accession number GSE32428). Data analysis was performed using Agilent GeneSpring 11.0 software (Agilent Technologies) for fold change analysis, pathway analysis (Ingenuity Pathway Analysis integrated), and cellular processes analysis. For the pathway analysis, the software makes use of the connectivity between a gene and other genes in the list of the genes of interest. By default, a gene should be functionally connected to at least two other genes from the list to be included in the predicted pathways.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Bio-Rad iQ SYBER Green Supermix and an iCycler thermo-cycler were used to run a protocol including 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. Primer sequences are given in the Supporting information as supplementary data. The expression values were normalized using the $2^{-\Delta\Delta CT}$ method, correcting for the expression of the housekeeping genes as well as the positive controls. SPSS 17.0 software was used for statistical correlation analysis between the gene expression values found by qRT-PCR and whole genomic expression arrays.

Results

In vitro transformation after a period of crisis

The two MSC lines, B6-BMMSC obtained from the bone marrow of a C57BL/6 mouse and BC-BMMSC obtained from the bone marrow of a BALB/C mouse, were cultured for 8 months and characterized for their MSC origin as previously described [1]. Between 20 and 40 PD cells experienced a period of crisis, which was later than the crisis found previously in other MSCs [1], indicating a process of random selection. During crisis, many cells died while other cells survived and gradually dominated the entire culture. These cells exhibited higher proliferation rates than before crisis, shown by the steeper slopes of the growth curves (Figure 1).

Survival of the fittest

In total, eight groups of zebrafish, each containing 50 embryos, were included for the *in vivo* studies. As summarized in Table 1, embryos received injec-

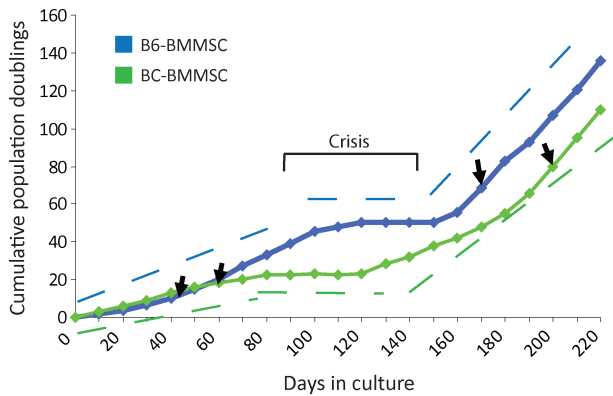


Figure 1. *In vitro* transformation. Bone marrow-derived mesenchymal stem cells (MSCs) from two mouse strains were cultured *in vitro* for about 8 months. During this long-term period of culturing, cells were counted at every passage to calculate the number of population doublings. After 60–100 days, cells started to go through a period of crisis, during which many died while others proliferated faster, resulting in a net PD number of around zero (indicated as 'crisis'). Dashed lines indicate the approximate growth rates of the cells in population doublings per day before, during, and after crisis. After crisis, cell cultures were stabilized and grew faster. The black arrows indicate the time points when MSCs were sampled for injections into zebrafish embryos.

tions of low-passage (normal) MSCs, high-passage (transformed) MSCs, and previously confirmed osteosarcoma-producing (tumourigenic) MSCs [12]. Two control groups were included: one group was untreated and the other injected only with PVP. Directly after injection, embryos were screened and only those with the correct injection site (caudal part of the yolk, Figure 2A) and without damage were selected for further study (Table 1). Fluorescent imaging showed that normal MSCs spread through the lower part of the yolk and lay down as a thin layer without invading any blood vessels (Figure 2B). The different effects of normal versus transformed and tumourigenic MSCs on zebrafish embryos were already detected 2 days after injection, ie 96 hpf. The aggressiveness of transformed and tumourigenic MSCs was demonstrated by the dead embryos that were injected with these cells. As shown in Figure 2C, the dead fish were attached to the plastic bottom of the plates with a large mass of CM-Dil positive remnant, indicating substantially

faster growth of the MSCs compared with the embryos. Consequently, the cells grew out of the embryos' bodies and attached to the plastic dish. This phenomenon was not observed in any embryo injected with normal MSCs.

Tumourigenic transformed MSCs migrate through the embryos

The phenomenon that MSCs migrated out of the original injection location towards other parts inside the zebrafish embryo was defined as migration (Figure 2A). Migration could not be detected in any of the embryos injected with normal MSCs of either C57BL/6 or BALB/C origin, whereas it was confirmed in the great majority of embryos injected with transformed MSCs and osteosarcoma-derived cells (S1d/s) (Table 1). In most cases, cells were found in the tail part of the embryos, but migration towards the head was found as well (Figure 2D).

Tumourigenic transformed MSCs induce angiogenesis

At the time of injection, in 48 hpf zebrafish embryos, subintestinal veins (SIVs) start to develop from the duct of Cuvier. During the next 24 h, SIVs form a vascular plexus located dorsally in the yolk sac that will provide a blood supply to the digestive system together with the suprainstestinal artery [25]. The formation of sprouting vessels from SIVs towards and around the injected MSC mass was defined as angiogenesis [26] (Figure 2A). Angiogenesis was not found in any embryos injected with normal MSCs, while it was clearly present in a number of embryos injected with transformed and tumourigenic MSCs (Table 1 and Figure 2D).

Whole genome expression analysis of mouse and zebrafish genes

After a quality check and normalization of whole genome expression data, we concluded that murine genes could be detected among zebrafish RNA (after total RNA isolation of zebrafish injected with mouse MSCs) and that the levels of cross-hybridization were

Table 1. *In vivo* characteristics

Group	Embryos*	Injected with				Dead fish 1 dpi [†]	Migration 3 dpi [‡]	Angiogenesis 3 dpi [§]
		Name	Passage	Strain	Label			
1	19	B6-BMMSC	9	C57BL/6	CM-Dil	0	0	0
2	26	B6-BMMSC	28	C57BL/6	CM-Dil	5 (19%)	21 (100%)	7 (33%)
3	24	BC-BMMSC	14	BALB/C	CM-Dil	0	0	0
4	20	BC-BMMSC	34	BALB/C	CM-Dil	2 (10%)	13 (72%)	13 (72%)
5	17	TmMSC	X+3	C57BL/6	CM-Dil	2 (12%)	8 (53%)	7 (47%)
6	18	S1d/s	X+3	C57BL/6	DsRed	4 (22%)	12 (86%)	10 (71%)
7	26	PVP [¶]	N/A	N/A	N/A	0	N/A	N/A
8	45	Un-injected	N/A	N/A	N/A	0	N/A	N/A

*The number of embryos with injections at the correct site included for further study. [†]The number of dead embryos at 1 day post-injection (dpi). [‡]The number of embryos in which migration of cells was found at 3 dpi. [§]The number of embryos in which signs of angiogenesis were found at 3 dpi. ^{||}For established cell lines, the exact passage number is not of importance and is indicated by 'X'. [¶]PVP injections were used as a negative control to see the effect of injections on the embryos. N/A = not applicable.

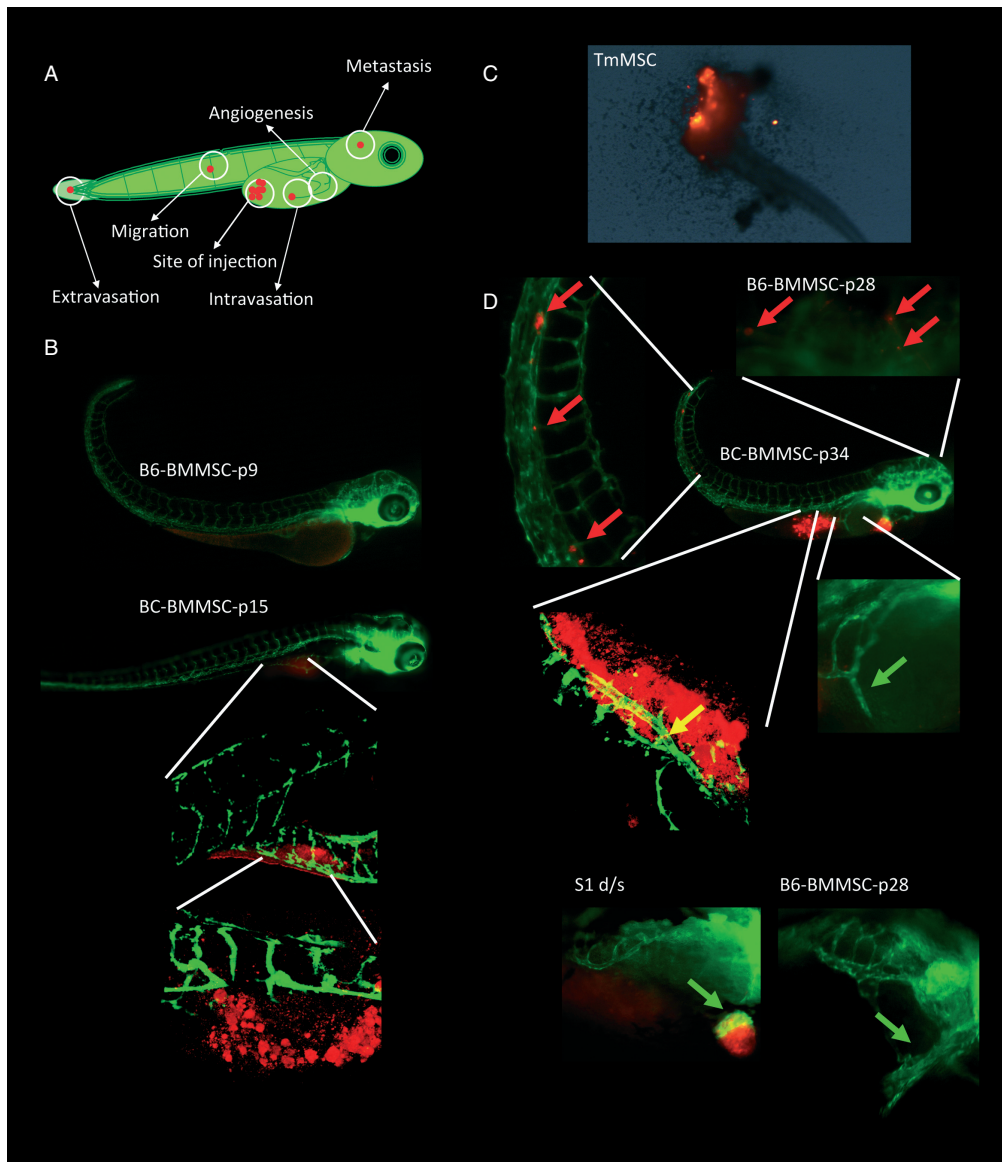


Figure 2. *In vivo* behaviour of normal versus transformed and tumourigenic MSCs. (A) Two MSC cultures before (normal) and two MSC cultures after (transformed) crisis (at time points indicated by the black arrows in Figure 1), as well as two previously confirmed osteosarcoma-like cell lines (tumourigenic), were injected into 48 h post-fertilization (hpf) zebrafish embryos as indicated in the schematic representation of a zebrafish embryo. After injection, embryos were screened daily for intra/extravasation, migration (metastasis) of the injected cells, and signs of angiogenesis. Per cell type, 50 embryos were injected. (B) A selection of representative pictures summarizing the findings after injection of normal pre-crisis MSCs where no signs of migration or angiogenesis were found. Pictures of higher magnification show that cells (red) did not enter the blood vessels (green) of the zebrafish. In total, 43 embryos were analysed. (C) The picture shows the grey-coloured remnant of the tail part of a zebrafish embryo attached to the plastic dish as cells grew rapidly out of the fish body into the culturing dish still expressing the red label. This was found in four embryos, two injected with transformed MSCs and two with tumourigenic cells. (D) Representative pictures of zebrafish embryos injected with transformed and tumourigenic cells. Magnified pictures repeatedly indicate migration of the cells to the embryos' tails and heads (red arrows), direct interactions with the blood vessels (yellow arrow), and signs of angiogenesis (green arrows). In total, 81 embryos were analysed.

not interfering with the generation of interpretable results. This can be appreciated from Figure 3, showing a principal component analysis which resulted in the designation of separate clusters. Subsequently, three groups of embryos –1, injected with normal; 2, injected with transformed; and 3, injected with tumourigenic MSCs, each containing two samples—were compared for differentially expressed genes with a log fold change (log FC) ≥ 2.0 . PVP-injected fish were used as a negative control group. First, MSCs injected into zebrafish, ie *in vivo*, were

compared for their gene expression profiles. Genes that were differentially expressed between normal and transformed MSCs were overlapping with those differentially expressed between normal and tumourigenic MSCs, thereby confirming the validity of this approach. Subsequently, the same was done for the *in vitro* cultured MSCs. To identify the most important genes that intrinsically differ between normal and transformed/tumourigenic MSCs causing their different behaviour inside the zebrafish embryos, the common differentially expressed genes between *in vivo*

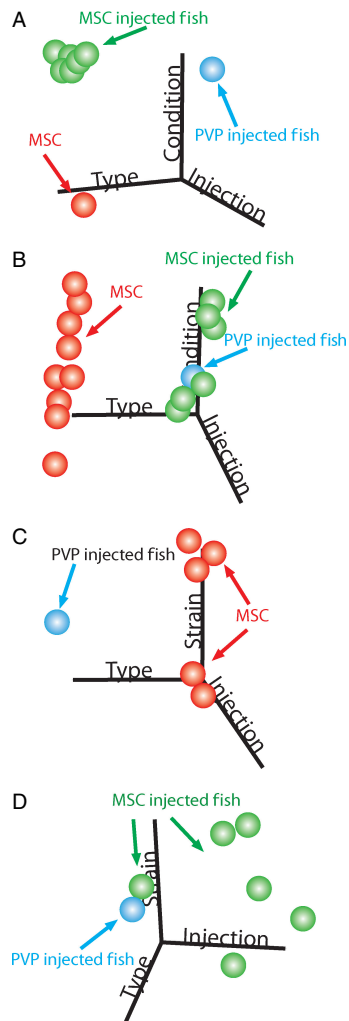


Figure 3. Whole genomic expression analysis. Total RNA was isolated from *in vitro* cultured MSCs as well as zebrafish injected with MSCs and hybridized to Agilent 44K whole genomic mouse and zebrafish expression arrays. Pictures show 3D principal component analysis (PCA) depicting the components of interest, including type (mouse- or fish-derived), condition (*in vitro* or *in vivo*), injection (MSC or PVP), and strain (C57BL/6 or BALB/C origin of the MSCs). (A) PCA represents hybridization to zebrafish arrays and shows that fish injected with MSCs have a different expression profile to that of fish injected with the matrix PVP only, indicating a biological difference. Hybridization of *in vitro* cultured MSCs into these arrays also results in separate clustering, indicating a manageable level of cross-hybridization. (B) Hybridization of all samples to mouse arrays, depicting separate clusters of mouse- and zebrafish-derived samples. (C) PCA of the *in vitro* cultured MSC samples from B together with a PVP-injected fish sample confirming a manageable cross-hybridization level in the reversed situation. (D) Comparison of fish samples injected with MSCs or PVP showing poor separation, as all samples have low expression levels.

and *in vitro* conditions were identified (Figure 4A). This resulted in a total of 28 genes (Supporting information, Supplementary Table 1). Five out of 28 differentially expressed genes showed involvement in shared cellular processes. While genes involved in transcription (*Myxd-4*) and T-cell response (*Fut-7*) were down-regulated in transformed and tumorigenic MSCs compared with normal MSCs, pro-angiogenic and pro-metastatic genes, *Ets-1* [27], *Mmp-19* [28,29], and *Kcnb-1*, were overexpressed (Figures 4B and 4C).

To validate these findings, the expression of the down-regulated gene *Myxd-4* and the up-regulated gene *Kcnb1* was analysed by qRT-PCR and showed a significant correlation with the array-based findings (Supporting information, Supplementary data).

To investigate the embryos' responses to injection with normal or transformed/tumourigenic MSCs, zebrafish whole genomic gene expression analysis was performed. Gene expression levels of embryos injected with normal MSCs were compared with those injected with transformed and tumourigenic MSCs. PVP-injected embryos were used to determine the baseline of differential gene expression as a result of the injection shock to the animals. Zebrafish genes that were differentially expressed ($\log FC > 2.0$) between embryos injected with normal versus transformed MSCs were overlapped with those differentially expressed between normal and tumourigenic MSCs (Figure 5A). This resulted in 63 commonly differentially expressed zebrafish genes (Supporting information, Supplementary Table 2). These genes were subsequently translated to mouse gene orthologues as pathway analysis for zebrafish genes is not available. Pathway analysis showed that 11 differentially expressed genes were highly inter-connected by being involved in similar processes which were mainly related to the immune response of the embryos. Most evidently, zebrafish injected with transformed and tumourigenic MSCs showed lower expression of major histocompatibility complex-related genes (*H2-K1* and *H2-D1* mouse orthologues) involved in antigen presentation, immune cell processing, and immune response (Figures 5B and 5C). For validation, the expression of the zebrafish *mhc1ze* (zebrafish orthologue of the *H2-K1* and *H2-D1* mouse genes) was assayed by qRT-PCR and showed a significant correlation with the array-based findings (Supporting information, Supplementary data).

Discussion

Osteosarcoma patients need specific therapeutic strategies to target the metastases. For this, a better perception of the multistep process of metastasis is required. The histological and genetic variation of osteosarcoma and its rareness make it difficult to study and hamper scientific advance. This stresses the need for representative model systems and elucidates the use of MSCs and zebrafish embryos in this study. Instead of full-blown osteosarcoma cells, in this study transformed MSCs were used which contained notably less genomic alterations, as was confirmed by array comparative genomic hybridization [1]. Moreover, having the normal parental MSCs of the transformed cells allowed for comparisons to identify specific driver events that contributed to the malignant phenotype, ie angiogenic and metastatic capacity. In addition, previously confirmed tumourigenic MSCs that produce metastatic osteosarcoma upon injection in mice [1,12] were included in

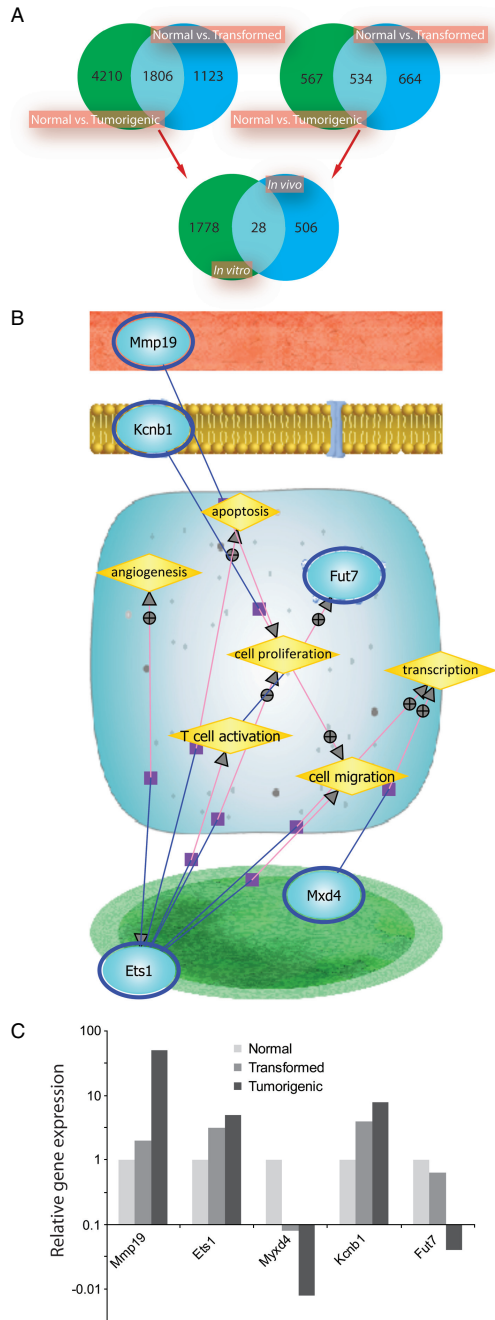


Figure 4. Migration and angiogenesis genes are up-regulated in transformed MSCs. Gene expression levels of all samples were normalized by using a pooled reference sample on a second channel. Subsequently, fish samples were baseline-transformed by using the median expression levels of the control PVP-injected embryos. (A) Work flow of the analysis. First, *in vitro* cultured normal MSCs were compared with transformed and tumourigenic MSCs, identifying 1806 differentially expressed genes with a log fold change ≥ 2.0 . Next, the same comparison for the injected MSCs identified 534 genes. Twenty-eight overlapping genes were further studied. (B) Cellular pathway analysis of the 28 differentially expressed genes identified five predicted to be functionally connected to at least two other genes inside the list of the 28 genes of interest. These genes are shown to be related and important for processes involved in migration and angiogenesis as seen in Figure 2. (C) Bar graph depicts differential expression of the above-mentioned genes showing higher expression of angiogenesis and migration-related genes in transformed and tumourigenic MSCs compared with normal MSCs. Please note the log scale of the Y-axis.

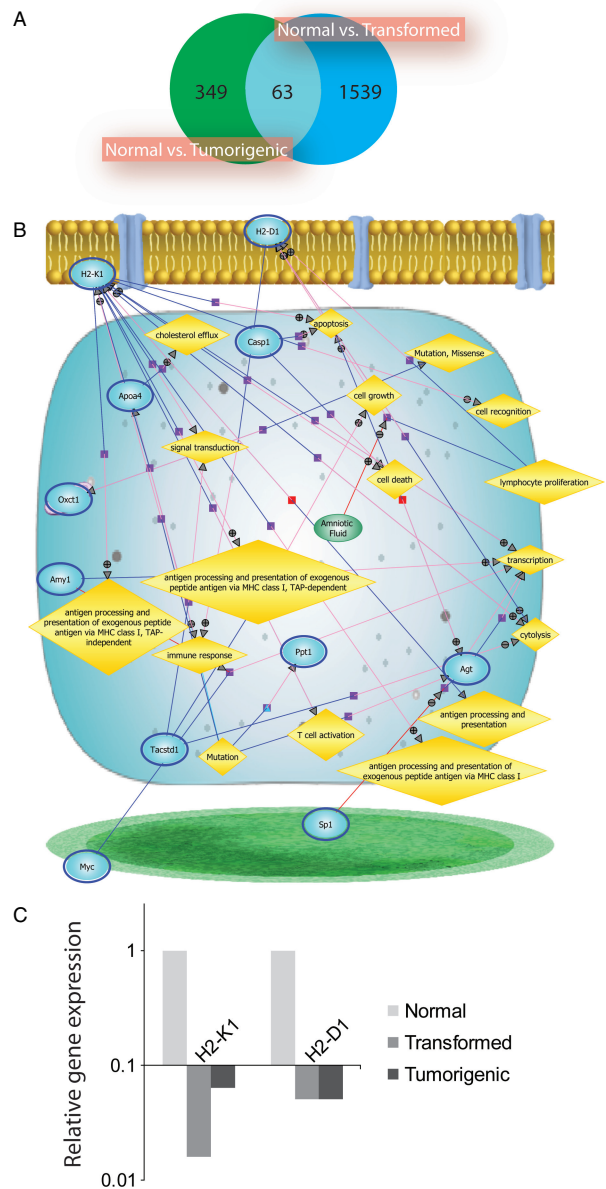


Figure 5. Immune response-related genes are down-regulated in embryos injected with transformed MSCs. (A) Overlap of the zebrafish differentially expressed genes after injection of normal or transformed/tumourigenic MSCs identified 63 differentially expressed genes. (B) Differentially expressed genes had to be translated to mouse genes to make pathway analysis possible. Forty-four mouse orthologues were found, of which 11 were involved in highly connected cellular processes involved in the immune system of the fish. (C) Bar graph shows that the two most important genes from the analysis, *H2-K1* and *H2-D1* (*mhc1ze* in zebrafish) related to the immune response, are hardly expressed in embryos injected with transformed and tumourigenic MSCs compared with those injected with normal MSCs.

this study to substantiate that transformed MSCs are representative of osteosarcoma-producing cells.

The comparison of genome-wide mRNA expression profiles between normal and transformed MSCs, both *in vitro* and *in vivo*, shows the feasibility of this approach. Remarkably, a number of differentially expressed genes between normal and transformed/tumourigenic MSCs were identified. The relatively small number of genes found is not surprising, given

the common origin of early and late passage MSCs, and it confirms that except for transformation-specific changes, these cells are comparable on the gene expression level. Furthermore, our results are in line with the new concept in tumorigenicity that primary tumour cells already have acquired metastatic and angiogenic properties [30]. Moreover, this approach was validated by the specific differential expression of genes involved in angiogenesis and metastasis, exactly the two phenomena that distinguished normal and transformed MSCs in the *in vivo* assays. The failure of many studies seeking driver mutations by comparing cancer cells with normal cells may be attributed to the nature of the 'normal' cells as well as the fact that cancer cells do not only carry the mutations making them cancer cells, but also additional 'hitchhiker' mutations due to their genetic instability, abnormally accelerated growth, and even culturing conditions. So when comparing certain features of interest, important alterations underlying those features can be identified without detecting background differences by keeping other factors as equal as possible. Here this approach is exemplified for features such as migration and angiogenesis by comparing cells that are almost the same except for differential characteristics before and after tumorigenic transformation. Moreover, using MSCs makes it feasible to identify mutations important in the early steps of osteosarcoma genesis, which would not be possible by studying fully developed osteosarcoma cells.

Next to the advantages mentioned before, using zebrafish embryos as *in vivo* models allowed for studies of the host response to MSC injections. Comparison of the response to injection of normal MSCs with that of transformed and tumorigenic MSCs strongly suggested that deregulation of the immune system—especially impairing the T-cell development—was already present at this early embryonic stage. This is interesting as in recent cancer studies immune destruction is considered to be an emerging general cancer hallmark [7]. More specifically, the innate immune system plays a pivotal role in explaining the outcome of osteosarcoma patients [31] and provides new tools for treatment [32]. Also for future studies, both to modulate MSC behaviour and to regulate the immune response, zebrafish embryos present efficient pre-screen models. Moreover, the zebrafish embryo model provides a high-throughput approach to study the effects of drugs against targetable genes identified in this study, such as *Mmp-19* and *Ets-1*. Subsequently, the most successful treatment can be validated in osteosarcoma mouse models by using the transformed MSCs as well as representative human osteosarcoma cell lines characterized before [33].

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Abbreviations

dpf	days post-fertilization
hpf	hours post-fertilization
MSCs	mesenchymal stem cells
PD	population doublings
PVP	polyvinylpyrrolidone-40

Author contribution statement

ABM, HPS, PCWH, and AC designed the study. ABM, WX, and RC conducted the study. ABM and WX collected data. ABM analysed data. ABM, HPS, PCWH, and AC interpreted data. ABM drafted the manuscript. ABM, PCWH, and AC revised the manuscript content. All authors approved the final version of the manuscript.

References

- Mohseny AB, Szuhai K, Romeo S, *et al.* Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. *J Pathol* 2009; **219**: 294–305.
- Mohseny AB, Hogendoorn PCW. Concise review: mesenchymal tumors: when stem cells go mad. *Stem Cells* 2011; **29**: 397–403.
- Tang N, Song WX, Luo J, *et al.* Osteosarcoma development and stem cell differentiation. *Clin Orthop Relat Res* 2008; **466**: 2114–2130.
- Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. *Cancer* 2009; **115**: 1531–1543.
- Bielack SS, Kempf-Bielack B, Delling G, *et al.* 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* 2002; **20**: 776–790.
- Lewis IJ, Nooij MA, Whelan J, *et al.* 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* 2007; **99**: 112–128.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
- Mohseny AB. Bone: conventional osteosarcoma. *Atlas Genet Cytogenet Oncol Haematol* 2008; [Accessed: 22 February 2012]. Available from: <http://AtlasGeneticsOncology.org/Tumors/ConvOsteoID5344.html>
- Hauben EI, Weeden S, Pringle J, *et al.* Does the histological subtype of high-grade central osteosarcoma influence the response to treatment with chemotherapy and does it affect overall survival? A study on 570 patients of two consecutive trials of the European Osteosarcoma Intergroup. *Eur J Cancer* 2002; **38**: 1218–1225.

10. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. *Cancer Genet Cytogenet* 2003; **145**: 1–30.
11. Cleton-Jansen AM, Buerger H, Hogendoorn PCW. Central high-grade osteosarcoma of bone: diagnostic and genetic considerations. *Curr Diagn Pathol* 2005; **11**: 390–399.
12. Tolar J, Nauta AJ, Osborn MJ, *et al.* Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 2007; **25**: 371–379.
13. Mohseny AB, Tieken C, Van der Velden PA, *et al.* Small deletions but not methylation underlie CDKN2A/p16 loss of expression in conventional osteosarcoma. *Genes Chromosomes Cancer* 2010; **49**: 1095–1103.
14. Stoletov K, Klemke R. Catch of the day: zebrafish as a human cancer model. *Oncogene* 2008; **27**: 4509–4520.
15. Deo RC, MacRae CA. The zebrafish: scalable *in vivo* modeling for systems biology. *Wiley Interdiscip Rev Syst Biol Med* 2011; **3**: 335–346.
16. Pardo-Martin C, Chang TY, Koo BK, *et al.* High-throughput *in vivo* vertebrate screening. *Nature Methods* 2010; **7**: 634–636.
17. Merlino G, Khanna C. Fishing for the origins of cancer. *Genes Dev* 2007; **21**: 1275–1279.
18. Langenau DM, Keefe MD, Storer NY, *et al.* Effects of RAS on the genesis of embryonal rhabdomyosarcoma. *Genes Dev* 2007; **21**: 1382–1395.
19. Feitsma H, Kuiper RV, Korving J, *et al.* Zebrafish with mutations in mismatch repair genes develop neurofibromas and other tumors. *Cancer Res* 2008; **68**: 5059–5066.
20. Stoletov K, Montel V, Lester RD, *et al.* High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. *Proc Natl Acad Sci U S A* 2007; **104**: 17406–17411.
21. Lee LM, Seftor EA, Bonde G, *et al.* 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* 2005; **233**: 1560–1570.
22. Marques IJ, Weiss FU, Vlecken DH, *et al.* Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC Cancer* 2009; **9**: 128.
23. White RM, Sessa A, Burke C, *et al.* Transparent adult zebrafish as a tool for *in vivo* transplantation analysis. *Cell Stem Cell* 2008; **2**: 183–189.
24. Schroeder A, Mueller O, Stocker S, *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* 2006; **7**: 3.
25. Nicoli S, Presta M. The zebrafish/tumor xenograft angiogenesis assay. *Nature Protoc* 2007; **2**: 2918–2923.
26. Nicoli S, Ribatti D, Cotelli F, *et al.* Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res* 2007; **67**: 2927–2931.
27. Hahne JC, Okuducu AF, Kaminski A, *et al.* Ets-1 expression promotes epithelial cell transformation by inducing migration, invasion and anchorage-independent growth. *Oncogene* 2005; **24**: 5384–5388.
28. Lettau I, Hattermann K, Held-Feindt J, *et al.* Matrix metalloproteinase-19 is highly expressed in astroglial tumors and promotes invasion of glioma cells. *J Neuropathol Exp Neurol* 2010; **69**: 215–223.
29. Muller M, Beck IM, Gadesmann J, *et al.* MMP19 is upregulated during melanoma progression and increases invasion of melanoma cells. *Mod Pathol* 2010; **23**: 511–521.
30. Van 't Veer LJ, Dai H, Van de Vijver MJ, *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530–536.
31. Buddingh EP, Kuijper ML, Duim RA, *et al.* Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage-activating agents. *Clin Cancer Res* 2011; **17**: 2110–2119.
32. Buddingh EP, Schilham MW, Ruslan SE, *et al.* Chemotherapy-resistant osteosarcoma is highly susceptible to IL-15-activated allogeneic and autologous NK cells. *Cancer Immunol Immunother* 2011; **60**: 575–586.
33. Mohseny AB, Machado I, Cai Y, *et al.* Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. *Lab Invest* 2011; **91**: 1195–1205.

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Table S1. Normal MSCs versus transformed/tumorigenic MSCs.

Table S2. Embryos injected with normal MSCs versus embryos injected with transformed/tumorigenic MSCs.

Supplementary data. qRT-PCR primers, data, and correlation with whole genomic expression array data.