Novel immunological tolerance of human cancer cell xenotransplants in zebrafish

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Tolerance in zebrafish xenotransplantation

Abbreviations

cd3,cd3 ζ ; dpf, day post fertilization; e2f1/3, E2F family of transcription factor 1 and 3; foxp3, forkhead box P3; hpf, hour post fertilization; il-17, interleukin 17; ifn- γ , interferon gamma; IFNG, interferon gamma; klf1, Kruppel-like factor 1 (erythroid); KOr, Kusabira-Orange; mpf, month post fertilization; myod1, myogenic differentiation 1; MEK1/2, mitogen-activated protein kinase kinase 1/2; SNEA, subnetworkenrichment analysis; STAT5A, signal transducer and activator of transcription 5A; tbx20, T-box 20

Abstract

Immune deficiency or suppression in host animals is an essential precondition for the success of cancer cell xenotransplantation, because the host immune system has a tendency to reject implanted cells. However, in such animals, the typical tumour microenvironment seen in cancer subjects does not form because of the lack of normal immunity. Here, we developed a novel zebrafish (*Danio rerio*) model based on two-rounds of cancer cell xenotransplantation that achieved cancer-specific immunological tolerance without immunosuppression. We irradiated human cancer cells (PC-3, K562 and HepG2) to abolish their proliferative abilities and implanted them into zebrafish larvae. These cells survived for 2 weeks in the developing host. Three months after the first implantation, the zebrafish were implanted with the same, but non-irradiated, cell lines. These cancer cells proliferated and exhibited metastasis without immune suppression. To reveal the transcriptional mechanism of this immune tolerance, we conducted dual RNA-seq of the tumour with its surrounding tissues and identified several regulatory zebrafish genes that are involved in immunity; the expression of *plasminogen activator*, *urokinase* and *forkhead box P3* were altered in response to immunological tolerance. In conclusion, this xenograft method has potential as a platform for zebrafish-based anti-cancer drug discovery because it can closely mimic human clinical cancers without inducing immune suppression.

Introduction

Xenotransplantation of human cancer cells provides a valuable model for pathophysiological and therapeutic studies in cancer research. One challenge is the avoidance of immune rejection by the host animal. Induction of immune deficiency, for example using the NOD/SCID mouse[1], γ -irradiation, or immune-suppressing agents[2], has been used to facilitate xenotransplantation. Although these models have contributed greatly to the understanding of human cancers, the lack of systemic immunity, mainly T and B cell function, is a problem for further understanding of cancer mechanisms[3, 4]. Correspondingly, the drug response in cancer xenografted “nude” animals does not always correlate to the clinical situation owing to the changes in the immune system [5]. In addition, the immune suppression treatments can induce spontaneous tumours in the host, thus confounding xenotransplantation cancer studies [6].

Zebrafish(*Danio rerio*) have been successfully used for decades as a model organism for preclinical studies and drug discovery. In cancer research, cancer xenograft zebrafish represent a promising alternative model to study tumour proliferation[7, 8], angiogenesis [9, 10], metastasis[11, 12] and cancer stemness[13, 14]. To avoid immune rejection of the xenograft, cancer cell implantation has been

performed at the embryonic stage (24–72 hours post-fertilization, hpf) when the adaptive immune response is not established. Although the sites of haematopoiesis are different between zebrafish and mammals, the cellular and regulatory processes of haematopoiesis, including for immune cells, are highly conserved[15, 16]. In fact, most of the immune cells found in mammals have also been identified in zebrafish[17, 18]. Because of this similarity, the number of studies examining adult-zebrafish xenografts is small. Gamma-irradiation [19] and immune-suppressants [20] have been used to

ablate immune cells of zebrafish to establish adult-zebrafish xenografts. Recently, Tang *et al.* developed an immuno-compromised zebrafish model using *rag2* mutants, which allows long-term engraftment of human cancer cells [21]. In spite of these successful studies, such methodologies are limited owing to the lack of a normal immune system in the host.

In this study, we established a novel cancer xenograft zebrafish with immunological tolerance specific to the implanted cancer cells. The implanted cancer cells proliferated and exhibited metastasis without host immune suppression.

Materials and Methods

Ethical approval

The animal experiments in this manuscript conformed to the ethical guidelines established by the Institutional Animal Care and Use Committee of Mie University, which follow the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Cell culture and fluorescent labelling

PC-3 cells (human prostate cancer), K562 cells (human chronic myelogenous leukaemia) and HepG2 cells (human hepatocarcinoma) were obtained from the RIKEN Cell Bank (Tokyo, Japan). The methods for cell culture and fluorescent protein labelling were performed as in a previous study [22]. We denoted *Kusabira-Orange* protein-expressing cells as PC3-KOr, K562-KOr and HepG2-KOr, respectively.

γ -Irradiation of cancer cells

PC3-KOr, K562-KOr and HepG2-KOr cells were cultured in 25cm² flasks containing 10 ml medium at a density of 4×10^5 cells/ml. Irradiation was delivered from a cobalt-60 gamma irradiation using a CAX-150-20 (ChubuMedical, Aichi, Japan). We prepared 10 Gy-irradiated PC3-KOr, 6 Gy-irradiated K562-KOr and 10 Gy-irradiated HepG2-KOr cells, all of which lacked proliferative capacity but did not die. We denoted the above irradiated cells as Ir-PC3-KOr, Ir-K562-KOr, and Ir-HepG2-KOr, respectively.

Zebrafish

Care and breeding of zebrafish were performed following protocols as described by Westerfield[23]. *nacre/rose/fli1:egfp* zebrafish were used because they lack pigmentation and are largely transparent, which facilitates *in vivo* monitoring of tumours[24].

Cancer cell xenotransplantation into zebrafish embryos

The xenotransplantation procedures used are described in our previous studies[24]. In brief, just before xenotransplantation, 48 hpf zebrafish were anaesthetized with 2-phenoxyethanol solution (100 ppm; Wako Pure Chemical Industries, Osaka, Japan), and arrayed on a holding sheet. Cancer cells (1×10^6 cells) were suspended in 30 μ L Matrigel (BD Biosciences, San Jose, CA, USA). The avascular region of the yolk sac was then injected with 10 nl of the cell suspension containing 100–200 cells using a glass needle and FemtoJet injection system (Eppendorf, Hamburg, Germany). For control fish, 10 nl of Matrigel was injected into the yolk sac.

Cancer cell xenotransplantation into adult zebrafish

Cancer cells were suspended at 3,000 cells/ μ L in Hanks' balanced salt solution. One microliter of cell mixture was injected into a site near the dorsal aorta of each recipient fish using a 10 μ L 701N syringe (Hamilton, Reno, NV, USA). Injected fish were placed in a recovery tank for 1 h and then returned to the tank system at 34°C.

Imaging

Zebrafish were anaesthetised with 2-phenoxyethanol solution (100 ppm; Wako Pure Chemical Industries). Images were captured using a MZ16F stereoscopic microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DP71 digital camera (Olympus, Tokyo, Japan). For fluorescence imaging, GFP2 (for *flil:egfp*) and DsRed (for KOr) filters were selected. Cancer cell proliferation was calculated by the ratio of KOr fluorescence intensity in the tumour area relative to that at day 0 using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as described previously [10, 22, 25].

RNA extraction, cDNA synthesis, and q-PCR

Total RNA was extracted from cells or tissues using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first-strand cDNA was synthesised from 200 ng of total RNA using the SuperScript III cDNA synthesis kit (Life Technologies) with random primers (Life Technologies). Quantitative polymerase chain reaction (q-PCR) was performed using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and a 7300 real-time PCR system (Applied Biosystems) as recommended by the manufacturer. The primers shown in Table S1 were used to amplify target genes.

RNA-seq

Total RNA was extracted from tumours and their surrounding tissues of adult stage PC3-KOr xenografts (7dpi) with or without Ir-PC3-KOr embryonic implantation using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Adult PC3-KOr xenografts without Ir-PC3-KOr embryonic implantation

were a control group. Each group (Ir-PC3-KOr and control) contained three fish. Before library construction, ribosomal RNA was depleted using a Ribo-Zero Magnetic Kit (Epicentre, Madison, MI, USA) according to the manufacturer's instructions. RNA libraries were then constructed using an Ion Total RNA-Seq Kit v2 (Life Technologies) according to manufacturer's instructions. The libraries were then sequenced to generate dual RNA-seq data using Ion Proton (Life Technologies).

Bioinformatic analysis of dual RNA-seq

Dual RNA-seq data were mapped and tag counts were performed using Genomics Workbench (CLC bio, Aarhus, Denmark). Then, we performed gene expression analysis using the R package and Tag Count Data Comparison (TCC). The package incorporates multi-step normalisation methods, whose strategy is to remove potential genuine differentially expressed genes before performing data normalisation [26]. After statistical tests, we performed further bioinformatic analyses (gene-set enrichment analysis (GSEA) and subnetwork enrichment analysis (SNEA) using Pathway Studio 9.0 (Elsevier, Amsterdam, Holland) according to the developer's instructions.

Statistical analysis

Data were analysed using statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). Differences between two groups were examined for statistical significance by independent Student's t-test. For multiple comparisons, assessments were analysed using one-way analysis of variance (ANOVA) followed by the Bonferroni–Dunn multiple comparison test. All data are presented as the mean $\pm$ SEM. Values of $P < 0.05$ were considered statistically significant.

Results

Implantation of irradiated human cancer cells induces immunological tolerance.

The key distinction of our human cancer cell xenotransplantation model is the double xenotransplantation strategy at embryonic and adult stages (Fig. 1A). We implanted Ir-PC3-KOr or naive (not irradiated) PC3-KOr cells into 48 hpf larvae (Fig. 1B). Seven days after implantation, the Ir-PC3-KOr cells had almost disappeared from the xenograft, while PC3-KOr cells were still present and remained until death of the hosts in the subsequent days (Fig. 1B). After 1 month, almost all PC3-KOr xenografted zebrafish were dead, while about 70% of Ir-PC3-KOr xenografted zebrafish survived and grew normally without any appearance of the implanted cells. When the embryonic Ir-PC3-KOr xenografted zebrafish and their littermates (control group without cancer cell implantation) reached 3 months post fertilization (mpf), we conducted xenotransplantation of naive PC3-KOr cells. Seven days after implantation at this adult stage, the Ir-PC3-KOr group exhibited a larger tumour size than the control group (Fig. 1C, left panels). Additionally, the Ir-PC3-KOr group developed metastasis in the tail region; however, none of the control zebrafish tails exhibited obvious fluorescence from cancer cells (Fig. 1C, right panels and Fig. 1D). In the following days, the tumours continued to proliferate while the tumours almost disappeared from control zebrafish (Fig. 1E).

To validate this novel method for xenotransplantation, we conducted the same experiments with human chronic myelogenous leukaemia K562 cells (Supplementary Fig. S1) and hepatocarcinoma HepG2 cells (Supplementary Fig. S2). K562-KOr and HepG2-KOr xenografts exhibited the same results as PC3-KOr xenotransplantation. These results demonstrate that this protocol should be applicable to the majority of human cancer cell lines.

Ir-K562-KOr xenografted zebrafish reject PC-3 cell xenotransplantation

To evaluate the selectivity of the immunological tolerance induced by cancer cell implantation into embryos, we performed cross-xenotransplantation with PC3-KOr cells into 3 mpf adult zebrafish that received embryonic Ir-K562-KOr xenografts. After 7 days, PC3-KOr cell implants produced smaller tumours than K562-KOr cells ($P < 0.05$; Fig. 2A and Supplementary Fig. S3) and these tumours were almost disappeared by day 14. *KOr* mRNA was only expressed in implanted cancer cells; therefore, its expression in the blood could be an indicator of circulating cancer cells [24]. We found that *KOr* mRNA levels in the blood of PC3-KOr xenograft fish (cross-xenograft with embryonic Ir-K562-KOr) were significantly decreased in comparison with K562-KOr xenograft fish on day 28 ($P < 0.05$; Fig. 2B).

Transcriptome analysis of the immunologically tolerant zebrafish

The *forkhead box P3* (*FOXP3*) gene, a biomarker expressed in regulatory T cells (T_{reg}), plays a key role in immune modulation [27], a process central to immunological tolerance. It has been established that the cell population expressing *FOXP3* plays a significant role in inducing and maintaining immunological tolerance in human and model animals [28, 29]. Zebrafish *foxp3* has also been characterized and was found to have a similar role to that in mammals, although T_{reg} cells in zebrafish have yet to be purified [30]. Therefore, in this study, we examined the *foxp3* gene as a marker of functioning T_{reg} cells. Zebrafish *foxp3* mRNA levels in the blood of Ir-PC3-KOr xenograft fish were higher than that of control fish before and also after the second xenotransplantation ($P < 0.01$; Fig. 3A). We also found that zebrafish *foxp3* expression was higher in the tumour area than in the non-tumour tissue ($P < 0.01$;

Supplementary Fig. S4). In contrast to *foxp3* expression in the blood, after the second xenotransplantation, the expression of genes related to inflammation (*cd3ζ* [*cd3*], *interferon gamma* [*ifn-γ*] and *interleukin 17* [*il-17*]) was increased to a lesser degree in immunological tolerant fish compared with the control fish that did not receive the first xenotransplantation at their embryonic stage ($P < 0.01$; Fig. 3B).

To further reveal the mechanism of cancer progression in this model, we conducted RNA-seq analysis of the Ir-PC3-KOr xenografts. We collected tumours and their surrounding tissues at 7 days after the adult stage xenotransplantation and performed next generation sequencing. Adult PC3-KOr xenograft fish without embryonic Ir-PC3-KOr implantation, which still had tumours, were used as a control. Using GSEA [31], we found that 32 human genes and 627 zebrafish genes were significantly ($P < 0.05$) altered in Ir-PC3-KOr xenografted zebrafish compared with control fish that did not receive the first xenotransplantation at the embryonic stage. Supplementary Table S2 shows the list of genes with the greatest GSEA results. GSEA results of the human transcriptome showed alteration of some immune-related gene sets including those involved in the inflammatory response; however, these effects were not observed in the zebrafish transcriptome. To increase the sensitivity of the analysis, we performed SNEA [32] of the human and zebrafish RNA-seq data to identify key molecules regulating the expression of the genes that showed significant alteration (*false discovery rate* < 0.05). Table 1 shows pathways involved in this cancer xenograft in both human cancer cells and the zebrafish host. In humans, mitogen-activated protein kinase kinase 1/2 (*MEK1/2*), interferon gamma (*IFNG*) and signal transducer and activator of transcription 5A (*STAT5A*) were predicted to be involved in tumour proliferation. In zebrafish, Kruppel-like factor 1 (erythroid) (*klf1*), myogenic differentiation 1 (*nyod1*), T-box 20 (*tbx20*) and E2F family of transcription

factor 1 and 3 (*e2f1/3*) were predicted to be involved in host immunity. Expression of genes involved in these zebrafish pathways was confirmed by q-PCR (Fig.3C).

Discussion

Since the discovery in 1945 of immune-genetic interactions during vascular anastomoses in experiments involving a set of bovine twins [33], studies concerning immunological tolerance induced at the embryonic stage by artificial intervention have been performed in rat [34], chicken [35] and Syrian hamster [36]. These animal models had intact immune systems (were not nude animals) and the xenografted cancer grew and killed the host within a relatively short period, which made it difficult to study cancer progression or drug responses in adulthood.

The zebrafish immune system is similar to that of mammals in that they both have innate and adaptive immune systems [37]. Meanwhile, a study of lipopolysaccharide tolerance suggested the possibility that immune tolerance can be inducible in zebrafish [38]. In addition, human malignant melanoma cells implanted into zebrafish embryos were shown to persist until the adult stage (3 mpf), but could not form a tumour mass [39]. These studies indicated that xenotransplantation at the embryonic stage could impart immunological tolerance to implanted human cells.

A key distinction of our xenotransplantation model is the double xenotransplantation strategy, which avoids cancer-induced death following the first implantation in the early developmental stages. At 48 hpf, a viable stage for the first cancer implantation, zebrafish embryos begin to develop an innate immune system; however, this stage precedes thymus development [40], resulting in the avoidance of immune rejection of implanted cells, as observed in this study. Xenotransplanted cancer cells usually proliferate in immune-compromised animals and finally kill the host; however, our irradiated cells disappeared and had no effect on the host survival

ratio. Furthermore, they induced specific immunological tolerance for the implanted cells. This methodology was applicable to not only human prostate cancer (PC-3) but also to CML (K562) and hepatocarcinoma (HepG2) cells, suggesting that this phenomenon is common to most kinds of human cancer cells. Additionally, the naive cancer cells without irradiation implanted into embryonic control fish persisted for more than 12 days and finally killed the host, similar to results in rodent studies [34]. These results indicate that the zebrafish immune system can recognize the antigenicity of each cancer cell line. These results were similar to those of another study that examined LPS response in zebrafish [38]. Moreover, zebrafish with immunological tolerance for a particular cell type still rejected other cell types that are cross-xenotransplanted, which suggests that they maintain immune tolerance, but not immune deficiency.

The induction of immune tolerance in xenografts may require several steps, especially deletion, anergy and the emergence of T_{reg} cells. T_{reg} cells induce and maintain immunological tolerance by inhibiting effector B and T cells [28], which promote autoimmune diseases [41] and tumour progression [42]. Thus, T_{reg} cells are an important therapeutic target, and inhibition of their activity or numbers is thought to enhance host-derived immunological attack against cancer cells [43, 44]. T_{reg} cells express *FOXP3* [27], a gene that is also present and functional in zebrafish [30]. However, zebrafish T_{reg} cells have not yet been identified and purified. Our results demonstrated the involvement of zebrafish *foxp3* in immunological tolerance during cancer progression, similar to findings in mammals.

Dual RNA-seq analysis was first used in zebrafish to analyse the pathogen-host interaction of infectious diseases [45]. While protein sequences are similar between human and zebrafish, RNA sequences tend to be different. Thus, deep sequencing is appropriate for transcriptome comparisons in mixed-species tissues, while DNA

microarrays are of limited utility. In the human transcriptome, pathways involving mitogen-activated protein kinase 1/2 (MEK1/2), interferon gamma (IFNG) and signal transducer and activator of transcription 5A (STAT5A) were activated during cancer progression. For the MEK 1/2 pathway, aberrant signalling through the RAS/RAF/MEK pathway leads to cell transformation [46], and several MEK inhibitors are now under clinical trials against many types of cancer [47]. This suggests that malignant transformation would continue in the implanted human cancer cells. Interferon- γ (IFNG) is a cytokine whose biological activity is conventionally associated with cytostatic / cytotoxic and anti-tumour mechanisms during the cell-mediated adaptive immune response [48]. IFNG regulates *solute carrier family 7, member 7* (*SLC7A7*), which was up-regulated in our study and induces immunosuppression in tumour-infiltrating lymphocytes [49]. SLC7A7 is a marker for glioblastoma prognosis and is involved in malignant transformation [50]. STAT5A is highly expressed in human prostate cancer cells and promotes metastasis [51], and is thus considered a promising therapeutic target against prostate cancer [52, 53]. In the zebrafish transcriptome, we found that urokinase-plasminogen activator (*plau*) down-regulation occurred in the *e2f1* and *e2f3* sub-networks, which has been reported to induce TGF- β activation, thus contributing to impaired T cell activation and proliferation with T cell tolerance [54, 55]. These results indicate modulation of the host immune system to accommodate xenotransplanted cancer cell growth in the immunological tolerance model.

In conclusion, we developed a novel cancer xenograft model in zebrafish that has immunological tolerance and that will overcome the limitations of traditional xenotransplantation observed in immuno-compromised animals. Our dual RNA-seq

analysis revealed the transcriptional networks underlying cancer-host interaction. This analysis has the potential to provide new therapeutic targets against cancer progression in the context of normal immunity. Thus, this model offers a useful platform for the evaluation of intact mechanisms of cancer progression and for zebrafish-based drug discovery. In addition, the immunological tolerance of this model will facilitate studies aimed at improving the success of organ transplantation.

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Figure legends

Figure 1. Immunological tolerance induced by irradiated PC3-KOr cell implantation at the embryonic stage. (A) Schematic representation of two-round cancer cell xenotransplantation. (B) Representative images of cancer cell-implanted zebrafish larvae at 1 dpi (day post implantation) and 7 dpi. The cancer cells (red) were detected in larvae injected with PC3-KOr cells, but not in those injected with Ir-PC3-KOr cells at 7 dpi. (C) Representative images of cancer progression after the second implantation in adulthood with naive PC3-KOr cells (7dpi) in Ir-PC3-KOr zebrafish (embryonic implantation with Ir-PC3-KOr) and control fish (embryonic implantation with Matrigel vehicle). Left panels: the cell injection site in the trunk. Right panels: tail regions showing cancer cell migration. (D) Quantification of cancer cells in the tail region at 7 dpi after the second xenotransplantation in adult zebrafish. KOr fluorescence intensity was measured to determine cancer cell area. (E) Quantification of the cancer proliferation after the second xenotransplantation in adult zebrafish. Cancer cell proliferation was calculated as the ratio of KOr fluorescence intensity in the tumour area relative to that at day 0. Differences between the two groups were examined for statistical significance by independent Student's t-test. n=10. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Immunological tolerance to the same but not different cancer cell lines.

(A) Representative images (the injection site is the same as that in Figure 1C) of xenotransplantation of the same (K562-KOr) or different (PC3-KOr) cell line into zebrafish (7 dpi and 14 dpi) that had been xenotransplanted with Ir-K562-KOr in their embryonic stage. Yellow framed areas indicate areas of magnification. (B) Kusabira-Orange (KOr) mRNA expression in the blood of zebrafish after the second cancer cell

xenotransplantation in adulthood (28 dpi). For multiple comparisons, the assessments were analysed by ANOVA followed by the Bonferroni–Dunn multiple comparison test. $n=3$. $*P < 0.05$.

Figure 3. Different gene expression profiles in the immunologically tolerant cancer xenografts. (A) Zebrafish *foxp3* gene expression in the blood of 3-mpf zebrafish before and after PC3-KOr cell implantation in adulthood (28dpi). $n=3$. $*P < 0.05$, $**P < 0.01$. (B) Gene expression fold differences of inflammatory factors *cd3*, *ifn- γ* , and *il-17* in the blood of 3-mpf zebrafish before and after PC3-KOr cell implantation (28dpi). $n=3$. $*P < 0.05$, $**P < 0.01$. (C) Expression of candidate genes was confirmed by q-PCR after PC3-KOr cell implantation (7dpi) in adult zebrafish. $n=3$. $*P < 0.05$, $**P < 0.01$. Differences between the two groups were examined for statistical significance by independent Student's t-test.

Table 1. Prediction of immunological tolerance mechanism using SNEA

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	Gene set seed	Name	Measured neighbours	P value
<u>Human</u>	<i>MEK1/2</i>	mitogen-activated protein kinase kinase ½	<i>BHLHE41, PLAGL1</i>	0.02
	<i>IFNG</i>	interferon gamma	<i>C4A, C4B, SLC7A7, CHRM2, ENPP2</i>	0.03
	<i>STAT5A</i>	signal transducer and activator of transcription 5A	<i>CEL, C4A</i>	0.03
<u>Zebrafish</u>	<i>KLF1</i>	Kruppel-like factor 1	<i>hbz, epor, hbe1, sptb, gata1, alas2</i>	7.14E-05
	<i>MYOD1</i>	myogenic differentiation 1	<i>tnni2, actg2, ccna2, myl2, smyd1, ccnb1, acta1, myl1, ccne1, myh4, tnnc2</i>	3.17E-04
	<i>TBX20</i>	T-box 20	<i>actc1</i>	3.84E-03
	<i>E2F1</i>	E2F family of transcription factor 1	<i>skp2, kif23, siva1, cdc45, cdt1, ppm1d, plk1, bmi1, dnmt1, melk, ncapd2, chek2, cdca8, ccna2, mcm10, ccnb1, fbxo5, plau, ccne1, stmnl, gmnn</i>	1.28E-07
	<i>E2F3</i>	E2F family of transcription factor 3	<i>gmnn, mcm10, cdt1, aurka, ccnb1, plau, ccnb2, ccne1, ccna2, cdc20, fbxo5</i>	4.08E-08

A

B

fig 3