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Generation of erythroid cells from fibroblasts and cancer cells *in vitro* and *in vivo*☆,☆☆

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Abstract

Bone marrow is generally considered the main source of erythroid cells. Here we report that a single hypoxia-mimic chemical, CoCl₂, can the size of fibroblasts and cancer cells and lead to formation of polyploidy giant cells (PGCs) or polyploidy giant cancer cells (PGCCs), activation of stem cell marker expression, increased growth of normal and cancer spheroid, and lead to differentiation of the fibroblasts and epithelial cells toward erythroid lineage expressing hemoglobins both *in vitro* and *in vivo*. Immunohistochemical examination demonstrated that these cells are predominantly made of embryonic hemoglobins, with various levels of fetal and adult hemoglobins. Ectopic expression of c-Myc induced the generation of nucleated erythroid cells expressing variable levels of embryonic and fetal hemoglobins. Generation of these erythroid cells can be also observed via histological examination of other cancer cell lines and human tumor samples. These data suggest that normal and solid cancer cells can directly generate erythroid cells to obtain oxygen in response to hypoxia and may explain the ineffectiveness of conventional anti-angiogenic therapies for cancer, which are directed at endothelium-dependent vessels, and offer new targets for intervention.

Keywords

Solid tumor cells; Erythroid cells; Hypoxia; Cobalt chloride; c-Myc

1. Introduction

Oxygen plays a critical role in energy production, and organisms including tumor cells have developed a programmed response to hypoxia that increases glucose utilization and stimulates erythropoiesis and angiogenesis to compensate for the decrease in available

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☆☆Author contributions: S.Z. and J.L. designed the study. S.Z., I.M. performed the key experiments. S.Z. performed cell culture and made pathological observations. I.M. performed the immunohistochemistry experiments. S.Z. and J.L. wrote the manuscript.

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Appendix A. Supplementary material: Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2013.01.037>.

oxygen [1]. Erythropoiesis, the process by which erythroid cells are produced, is stimulated by decreased O₂ in circulation. However, in humans with certain diseases, erythropoiesis also occurs outside the bone marrow, within the spleen or liver [2]. Erythroid cells consist mainly of hemoglobin, a complex metalloprotein containing heme groups whose iron atoms temporarily bind to oxygen molecules in the lungs and release them throughout the body via vessels [3]. Hemoglobin is the iron-containing oxygen-transport metalloprotein in erythroid cells. Hemoglobin molecules consist of four globin chains, each of which has a heme moiety attached. Different hemoglobins have different capacities for oxygen affinity and release. In humans, mature erythroid cells are oval and flexible biconcave disks. They lack nucleus and most organelles, allowing maximum space to accommodate hemoglobin [4,5]. There are high-level expressions of Hb Gower-1 ($\zeta 2\epsilon 2$), Hb F ($\alpha 2\gamma 2$), and Hb A ($\alpha 2\beta 2$) during the embryonic, fetal, and adult developmental stages, respectively [6,7]. Human α and β globins are expressed at moderate levels in developing embryos and fetuses, whereas various amounts of embryonic ζ and ϵ globins and their encoding mRNAs can be detected in fetal erythroid cells and in adult-stage reticulocytes, respectively [8]. Intact erythroid cells containing a complex mixture of embryonic, semi-embryonic, and fetal hemoglobins have been shown to bind O₂ strongly [9].

While the bone marrow as a source of hematopoietic cells, it has been shown that human embryonic stem cells and induced pluripotent stem cells with a combination of one or more transcription factors can also generate these cells *in vitro* [10–13]. Szabo et al. demonstrated the ability to generate multilineage blood progenitors from human dermal fibroblasts without establishing pluripotency [11]. Furthermore, erythroid differentiation can be induced in K-562 cells by different kinds of chemical reagents, including hemin [14], butyric acid [15,16], 5-azacytidine [17], and chromomycin and mithramycin [18]. Bianchi et al. reported that human leukemic K562 cells can be induced *in vitro* to erythroid differentiation by cisplatin; they found that differentiation of K562 cells is associated with an increase in the expression of embryo–fetal globin genes [19]. These findings open a new possibility that the fibroblasts can also serve as an alternative source for hematopoietic cells, although this idea has not been tested vigorously at different experimental settings.

Hypoxia is a key regulator in stem cells, erythroid differentiation, angiogenesis, and tumor development [20] and is associated with the formation and maintenance of cancer stem cells [21,22]. Cobalt chloride (CoCl₂) has been widely used as a hypoxia mimic to treat aplastic anemia and renal anemia [23,24]. Here we report that ovarian fibroblasts and cancer cells can directly generate hemoglobin and erythroid cells *in vitro* and *in vivo* using hypoxia mimic CoCl₂. Our study provides a novel insight how normal and neoplastic tissue can obtain O₂ during normal tissue and tumor development.

2. Materials and methods

2.1. Cell culture and generation of immortalized cell lines

Fresh specimens of human fallopian tube fimbria and ovarian tissue were obtained from patients at The University of Texas MD Anderson Cancer Center under a protocol approved by the Institutional Review Board. Culture of primary fallopian tube epithelial cells (FTEs) and normal ovarian fibroblasts (NOFs) was performed as described previously [25]. All FTE and NOF cells were maintained in a 1:1 mixture of medium 199/MCDB 205 (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Intergen), 10 ng/mL epidermal growth factor (Sigma–Aldrich), and 100 U/mL penicillin/streptomycin (Sigma–Aldrich). Primary FTE187, NOF151, and NOF137 cells were infected sequentially with a retrovirus containing pBabe-hygro-hTERT and pBabe-puro-p53 siRNA against mRNA [26]. NOF137p53ihT was infected sequentially with retrovirus containing pLNCX-neo-c-Myc cDNA. FTE187hTERT was infected sequentially with a retrovirus containing pBabe-zeo-SV40 early region and

pBabe-puro-HRASV12 as described previously [25]. Infected cells were selected in Zeocin (500 µg/mL), hygromycin B (100 µg/mL), and puromycin HCl (1 µg/mL) for 5–10 d following each of the respective rival infections. MDA-MB-231, and BT-549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Phoenix, WI-38, and BJ cells were purchased from the American Type Culture Collection and maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

2.2. Cell treatment with CoCl₂

The cells were cultured in medium with FBS and antibiotics until the cells reached 90% confluence. We treated the cells with different concentrations of CoCl₂ for different times (Supplementary Table 1). After being rinsed with 1× phosphatebuffered saline (PBS), the cells were cultured in medium with FBS and antibiotics. After cells recovered from CoCl₂ treatment, they were cultured with stem cell medium contained 80% DMEM/nutrient mixture F-12, 20% knockout serum replacement (Gibco/Invitrogen), 1% non-essential amino acid, 1 mM L-glutamine (Gibco/Invitrogen), 0.1 mM 2-mercaptoethanol, and 4ng/ml of basic fibroblast growth factor (Gibco/Invitrogen).

2.3. Immunofluorescent staining of spheroids

The cell lines listed above formed multiple spheroids after treatment with CoCl₂ when cultured in stem cell medium. The spheroids were detached gently via pipetting and centrifuged at 400 g for 5 min to obtain spheroid pellets. The spheroids attached to coverslips after culture with complete medium for several hours. The spheroids were then fixed in ice-cold acetone for 10 min. After washing in Tris-buffered saline and Tween-20 three times for 5 min each, the spheroids were incubated with 1% bovine serum albumin in PBS and Tween-20 for 30 min to block unspecific binding of antibodies. Primary and secondary antibodies in PBS and Tween-20 with 1% bovine serum albumin were added to the coverslips (for detailed antibody information, see Supplementary Table 2) and then incubated in a humidified chamber for 1 h at room temperature. The spheroids were stained with DAPI for 1 min and observed under a fluorescence microscope (Eclipse TE 2000-U; Nikon).

2.4. Surface marker analysis of NOF137p53ihTc-Myc by flow cytometry

To confirm the role of the C-Myc gene in erythroid cell differentiation, the infected NOF137p53ihTc-Myc cells were treated with CoCl₂. After CoCl₂ treatment, the recovered NOF137p53ihTc-Myc cells produced suspension cells. To characterize the nature of these suspension cells, we spun down the floating NOE137P53ihTc-Myc cells and then resuspended them in PBS buffer with 1% albumin to a concentration of $1-2 \times 10^7$ cells/ml. 50 µl of cell suspension was aliquoted into five tissue culture tubes to directly conjugate with antibodies at concentration suggested by e-Bioscience. The solutions were then mixed gently, incubated for 20 min on ice, washed with 1 × PBS buffer, resuspended in 300–400 µl of buffer, and analyzed by flow cytometry. Five samples including (cells only, CD45 FITC only, CD71 PE only, CD34 APC only, and CD45FITC + CD71 PE + CD34APC) were prepared for flow cytometric analysis.

2.5. Paraffin embedding of blocks of spheroids and floating cells

Media containing the spheroids and floating cells described above were centrifuged at 100g for 5 min. The supernatant was removed, 1 ml of 70% ethanol was added to the pellet to fix the spheroids, and 50 µl of eosin was added to the vial containing spheroids and floating cells. The samples of spheroids and floating cells were dehydrated in a graded ethanol series

(70%, 80%, 95%, and 100% for 15 min per grade). The vials were then infiltrated with acetone, absolute xylene, a mixture of 50% xylene and 50% paraffin, and purified paraffin at 65 °C for 15 min each. All of these steps were performed in a 1.5-mL vial. The spheroids were then embedded in paraffin and sectioned for hematoxylin and eosin (H&E) staining and immunohistochemical analysis.

2.6. H&E and immunohistochemical staining

For H&E staining, 4- μ m sections of formalin-fixed, paraffin-embedded spheroids were deparaffinized and rehydrated and then counterstained with hematoxylin for 1 min and eosin for 2 min. Immunohistochemical staining of the sections was performed by using the avidin–biotin–peroxidase method as described previously [27]. The sections were incubated with primary antibodies overnight at 4 °C in a humidified chamber (Supplementary Table 1 contains detailed antibody information). The nuclei in the sections were counterstained with hematoxylin.

2.7. Spheroid and BT-549 cells injection in nude and NOD.CS17-Prkdc SCID mice

NOF151p53ihT, NOF137p53ihT, and WI-38 spheroids were trypsinized and then centrifuged at 400g for 5 min. The supernatant was removed, and 0.1 mL of $1 \times$ PBS was added to resuspend the pellets. The spheroids were mixed with 0.1 mL of PBS buffer and 0.1 mL of Matrigel in syringes and kept on ice before injection. The spheroid–Matrigel mixtures were subcutaneously injected into the flanks of 6- to 8-week-old NOD.CS17-Prkdc SCID mice. Two months later, small nodules (0.2 cm in size) formed in two of four mice injected with NOF151p53ihT spheroids, one of four mice injected with NOF137p53ihT spheroids, and one of four mice injected with WI-38 spheroids. For BT-549 cell injection, BT-549 cells after CoCl_2 treatment were subcutaneously injected into the flank of nude mice (1×10^5 per mouse). The mice were killed by cervical dislocation following CO_2 inhalation, and the nodules were removed and fixed in 10% formalin for routine histologic examination and immunohistochemical staining. The care and use of the mice were approved by the MD Anderson Institutional Animal Care and Use Committee.

2.8. Human ovarian tumor samples

The use of human tumors and blocks for immunohistochemical and H&E staining was approved by the MD Anderson Institutional Review Board. For primary culture of human ovarian cancer, fresh human ovarian tumor samples were washed with PBS three times and then sterilized with penicillin and streptomycin. The samples were minced into pieces and cultured with complete Dulbecco's modified Eagle's medium. The medium was changed every 1–2 weeks.

2.9. Smears of supernatants

Supernatants from the primary culture of human ovarian cancer were pipetted out of the flasks and centrifuged at 400g for 5 min. Thirty to fifty microliters of $1 \times$ PBS was used to resuspend the pellets. A 10- μ l mixture of PBS and cells was dropped onto slides for smearing. The smear slides were fixed with 75% ethanol and then air-dried for H&E staining.

3. Results

3.1. Generation of spheroids, hemoglobin, and erythroid cells after CoCl_2 treatment

To determine how hypoxia affects the reprogramming of fibroblasts, we treated a panel of human fibroblast lines with CoCl_2 and examined the resulting changes in phenotype. We compared the spheroid formation abilities in normal ovarian fibroblasts (NOFs) NOF151,

NOF151 cells ectopically expressing human telomerase reverse transcriptase (NOF151hT), and NOF151 cells with knockdown of p53 expression (NOF151p53ihT) (Fig. 1A, a–c) following treatment with CoCl₂. After this treatment, the regularly sized cells were killed, while a few large cells (polyploidy giant cells, PGCs) survived and recovered (Fig. 1A, d–f). These recovered PGCs can form spheroids when cultured in stem cell medium. The NOF151hT and NOF151p53ihT had 3- to 4-fold more spheroids than did the NOF151 (Fig. 1A, g–i). However, there is no major difference in spheroid number between NOF151hT and NOF151p53ihT (Fig. 1B). We also observed spheroid formation by fibroblast lines WI-38 and BJ after CoCl₂ treatment (Supplementary Fig. S1A), suggesting that this spheroid formation is a general feature after CoCl₂ treatment. Results of immunofluorescent and immunohistochemical staining confirmed that the spheroids of NOF151hT and NOF151p53ihT were positive for SOX-2 (Fig. 1C) but not positive for Oct4, ABCG2 (data not shown). Because CoCl₂ is known to stimulate the generation of erythroid cells *in vivo* [24,28], we examined the spheroids to determine whether they directly produced erythroid cells *in vitro*. The NOF151hT and NOF151p53ihT spheroids were embedded in paraffin blocks and subjected to histologic analysis using H&E staining. As shown in Fig. 1D, erythroid cells were clearly generated by NOF151hT (Fig. 1D, a–c) and NOF151p53ihT (Fig. 1D, f–h) spheroids. Immunohistochemical analysis demonstrated that the spheroids and erythroid cells were positive for hemoglobin- $\beta/\gamma/\delta/\epsilon$ (Fig. 1D, d and i). Only erythroid cells were positive for hemoglobin- δ (Fig. 1D, e and j). Both the spheroids and erythroid cells were negative for hemoglobin- α , fetal hemoglobin, and hemoglobin- ζ (Supplementary Fig. S1B). Western blot analysis confirmed that treatment of the cells with CoCl₂ increased hemoglobin- $\beta/\gamma/\delta/\epsilon$, and hemoglobin- δ expression in NOF151hT and NOF151p53ihT (Fig. 1E).

3.2. Activation of erythroid cell differentiation by c-Myc

C-Myc protein plays a critical role in controlling self-renewal versus differentiation in hematopoietic stem cells [5,29]. We infected NOF137p53ihT (a fibroblast cell line from another patient) with retrovirus containing pLNCX-neo-c-Myc cDNA. After CoCl₂ treatment, the recovered NOF137p53ihTc-Myc cells produced suspension cells (Fig. 2A, a and b). To characterize the nature of these suspension cells, we embedded these cells into paraffin and stained them with H&E. Most of the cells had lost the spindle cell morphology of the parental cells but had a visible nucleus (Fig. 2A, c). Immunohistochemical analysis revealed that these cells were positive for hemoglobin- $\beta/\gamma/\delta/\epsilon$, fetal hemoglobin, and hemoglobin- δ (Fig. 2A, d–f) and negative for hemoglobin- α and - ζ (Supplementary Fig. S1C). Flow cytometric analysis demonstrated that 99% of suspension cells expressed CD71, a marker for erythroid precursors, whereas only a minimum population of these cells expressed CD34 and CD45 (Fig. 2B). Results of western blot confirmed that CoCl₂ treatment increased c-Myc expression in NOF137p53ihT and NOF137p53ihTc-Myc (Fig. 2C). We subcutaneously injected NOF151p53ihT, NOF137p53ihT, and WI-38 spheroids together with Matrigel into the flanks of 8-week-old NOD.CS17-Prkdc severe combined immunodeficiency mice. Two months later, we identified small nodules at the injection sites after the mice were killed. Staining of mouse xenografted tissue showed multiple erythroid cell clumps with fibroblasts admixed with Matrigel (Fig. 2D; Supplementary Fig. S1D, a and b). We confirmed the human origin of these tissues using immunohistochemical staining with human specific anti-mitochondrial antibody (Fig. 2E; Supplementary Fig. S1D, c). Erythroid cell differentiation was confirmed by human specific anti-hemoglobin- δ (Fig. 2F; Supplementary Fig. S1D, d).

3.3. Generation of erythroid cells and embryo-fetal hemoglobin in solid cancer cell lines and human ovarian cancer tissue

To determine whether cancer cells can directly generate erythroid cells *in vitro*, we initially used the breast cancer cell line BT-549 as our working model. The BT-549 cells were irregular in shape with small apophyses (Fig. 3A, a). Treatment of these cells with CoCl_2 selectively killed the regularly sized cancer cells, only large cancer cells survived the treatment of CoCl_2 . We defined these large sized cancer cells three times greater than that of regular sized control cancer cells as polyploidy giant cancer cells (PGCCs). After removal of CoCl_2 from the medium, the remaining PGCCs began to proliferate several days (Fig. 3A, b). Following two to three treatments with CoCl_2 , multiple spheroids formed in the medium (Fig. 3A, c). We embedded these spheroids in paraffin and subjected them to histologic and immunohistochemical analysis. The spheroids were positive for the cancer stem cell markers CD44 and CD133 (Fig. 3A, d and e) and the normal stem cell markers SOX-2 (Fig. 3A, f and g). Histologic examination showed that the erythroid cells generating by BT-549 cells were biconcave disk shapes without nuclei, with an average size of 5–6 μm . These cells were flattened and depressed in their centers and had torus-shaped rims around the edges of the disks (Fig. 3B, a–c), making them morphologically indistinguishable from mature erythroid cells generated by bone marrow. To characterize the nature of hemoglobin in these erythroid cells, we performed immunohistochemical staining with anti-hemoglobin- α , - $\beta/\gamma/\delta/\epsilon$, - ζ , - δ and fetal hemoglobin antibodies. Almost all of the erythroid cells were strongly positive for hemoglobin- $\beta/\gamma/\delta/\epsilon$ (Fig. 3B, e) and fetal hemoglobin (Fig. 3B, f), and 70% were positive for hemoglobin- α (Fig. 3B, d). In comparison, 10–20% of the erythroid cells were positive for hemoglobin- ζ (Fig. 3B, g) or hemoglobin- δ (Fig. 3B, h). When BT-549 cells after CoCl_2 treatment were injected into nude mice (1×10^5 per mouse), numerous erythroid cells were found in the cytoplasm of BT-549 cancer cells in xenografted tumor in nude mice (Fig. 3C a–c). We confirmed the human origin of erythroid cells using immunohistochemical staining with human specific anti-hemoglobins (Fig. 3C, d). These results demonstrated that cancer cells can also produce embryonic and fetal hemoglobins from human but not from mouse.

To further validate above finding, we treated four additional cell lines including MDA-MB-231, H-ras-transformed fallopian tube epithelial cell line FTE187SV40hT-Hras, and Phoenix cells with CoCl_2 and examined the direct generation of erythroid cells by the spheroids induced by CoCl_2 . As shown in Fig. 4A, erythroid cells could be clearly observed among all of these cell lines. Furthermore, we primarily cultured human ovarian cancer. Smear of cancer cells in the medium confirmed the presence of erythroid cells by high-grade ovarian cancer cells after a 2-week culture *in vitro* (Fig. 4B, a). Histologic examination of paraffin-embedded slides also showed that erythroid cells appeared in the cytoplasm of high-grade ovarian cancer cells (Fig. 4B, b). Furthermore, multiple round and red bodies with variable sizes in the cytoplasm of and surrounding cancer cells could also be seen (Fig. 4B, c and d). Immunohistochemical staining of consecutive sections of ovarian cancer tissue demonstrated that these bodies were positive for hemoglobin- ζ but negative for fetal hemoglobin (Fig. 4B, h and g). A few round and red bodies were positive for hemoglobin- α or hemoglobin- $\beta/\gamma/\delta/\epsilon$ (Fig. 4B, e and f). We further examined the hemoglobin expression of human cancer cells. Immunohistochemical staining of hemoglobin showed that tumor cells expressed hemoglobin- α , - $\beta/\gamma/\delta/\epsilon$, - ζ , and fetal hemoglobin (Supplementary Fig. S2A, e–h). Also, mature erythroid cells within vessels were positive for hemoglobin- α and hemoglobin- $\beta/\gamma/\delta/\epsilon$ (Supplementary Fig. S2A, a and b). A few erythroid cells within tumor vessels were positive for fetal hemoglobin (Supplementary Fig. S2A, c), whereas all of the erythroid cells in them was negative for hemoglobin- ζ (Supplementary Fig. S2A, d). Taken together, all of these fibroblasts or tumor cell-derived erythroid cells exhibited greater activation of fetal and embryonic hemoglobin expression.

4. Discussion

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. Angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in granulation tissue [30,31]. Angiogenesis is generally believed to be required for supplying blood from the systematic circulation for tumor growth. Tumors, in particular, are believed to undergo an initial period of avascular growth followed by angiogenesis [32,33] or vasculogenic mimicry (VM) that connect with endothelium-dependent vessels to obtain enough blood and a sufficient oxygen supply to provide for tumor growth, invasion, and metastasis [34,35]. However, in all above cases, O₂ are believed to be provided by the bone marrow-derived red blood cells.

We here provide direct evidence that the hypoxia-mimic agent CoCl₂, which is currently used clinically to treat anemia, can induce fibroblasts and epithelial cancer cells to generate their own red blood cells both *in vitro* and *in vivo*. Both fibroblasts and cancer cells treated with CoCl₂ showed a marked increase in size and formed polyploidy giant cells (PGCs) or polyploidy giant cancer cells (PGCCs) which can grow into spheroids. These large cells have the capability to form spheroid with activated expression of fetal and embryonic forms of hemoglobin and can generate the erythroid cells *in vitro* and *vivo*, suggesting that these large cells may have properties of stem-like cells. As these embryonic and fetal hemoglobins have stronger affinity for obtaining oxygen from the surrounding microenvironment compared with adult hemoglobin, their activation will increase ability of these cells to obtain the O₂ and facilitates the normal tissue and tumor growth [9,36–38]. Furthermore, different cell lines had different expression patterns of hemoglobin. Ovarian fibroblast cells immortalized with hTERT and/or p53 knockdown expressed only $\beta/\gamma/\delta/\epsilon$ hemoglobin. The BT-549 breast cancer cell and human ovarian carcinoma cells expressed more embryonic and fetal hemoglobin. Thirdly, C-Myc is an oncogene of four key pluripotency genes essential for the production of induced pluripotent stem cells [39]. c-Myc protein plays a critical role in controlling self-renewal versus differentiation in hematopoietic stem cells [5]. Our results confirmed that immortalized cells with c-Myc overexpression were positive for fetal and delta hemoglobin after CoCl₂ treatment, suggesting that c-Myc is a key regulator in the cellular reprogramming toward red blood cells.

The generation of erythroid cells from fibroblasts and tumor cells provides a rational explanation of how normal and cancer cells can obtain oxygen under hypoxia condition with or without angiogenesis. Our data may thus explain the ineffectiveness of conventional anti-angiogenic therapies for cancer, which are directed at endothelium-dependent vessels only, as tumor cells have the ability to directly generate erythroid cells and adapt to the hypoxic microenvironment. An understanding of the detailed mechanisms underlying the generation of erythroid cells by cancer cells may help in the design of effective novel therapeutic strategies for cancer. In addition, generation of erythroid cells from immortalized cell lines may provide an alternative unlimited source to generate red blood cells *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Generation of spheroids and erythroid cells after CoCl₂ in fibroblast lines. (A) CoCl₂-induced formation of spheroids in fibroblast lines. The morphology of NOF151 (a), NOF151hT (b), and NOF151p53ihT (c) cells is shown (×10). After CoCl₂ treatment, the regularly sized cells were killed, whereas a few PGCs of NOF151 (d), NOF151hT (e), and NOF151p53ihT (f) survived. These PGCs can form spheroids, which were shown to be NOF151 (g), NOF151hT (h), and NOF151p53ihT (i) (×10). (B) Comparison of the numbers of spheroids in NOF151, NOF151hT, and NOF151p53ihT cells. (C) SOX-2 staining of NOF151hT and NOFp53ihT spheroids. (a and c) NOF151hT and NOFp53ihT spheroids were positive for SOX-2 immunofluorescent stain (×20) (b and d) NOF151hT and NOFp53ihT spheroids were positive for SOX-2 immunochemical stain (×20). (D) Erythroid cell differentiation of NOF151hT and NOF151p53ihT cells induced by CoCl₂ treatment *in vitro*. H&E staining of NOF151hT and NOFp53ihT spheroid slides showed that erythroid cells generated in NOF151hT (a–c) and NOF151p53ihT (f–h) after the treatment (H&E staining, ×20); (b) and (g) are the high-power views of (a) and (f), respectively, to clearly show the erythroid cells budding from the spheroids (black arrowheads) (H&E staining, ×20). Spheroids were positive for hemoglobin-β/γ/δ/ε (d and i) and hemoglobin- δ (e and j) (immunohistochemical staining, ×20). (E) Western blot analysis of hemoglobin expression in NOF151hT and NOF151p53ihT cells with and without CoCl₂ treatment.

Fig. 2.

Erythroid cells differentiation in NOF137p53ihTc-Myc and xenografted tissue. (A) Erythroid cells differentiation of NOF137p53ihTc-Myc treated with CoCl₂. (a) Control NOF137p53ihT ($\times 10$). (b) Suspension cells generated by NOF137p53ihTc-Myc after CoCl₂ treatment ($\times 10$). (c) H&E staining of paraffin-embedded suspension cells ($\times 20$). (d-f) Suspension cells were positive for hemoglobin- $\beta/\gamma/\delta/\epsilon$ (d), fetal (e), and - δ (f) ($\times 20$). (B) Flow cytometric analysis of suspension cells with CD71 (a), CD34 (b), and CD45 (c) after CoCl₂ treatment. The blue lines show PI staining only and the red lines are for PI-negative cells and CD71-positive (a), PI-negative cells and CD34-positive (b), and PI-negative cells and CD45-positive (c). (C) Western blot results of c-Myc expression. (D) Erythroid cell differentiation in xenografted CoCl₂-treated NOF151p53ihT and NOF137p53ihT spheroids (H&E staining, $\times 20$). Erythroid cells generated from NOF151p53ihT (a and b) and NOF137p53ihT (c and d) mouse xenografts (black arrowheads) (H&E staining, $\times 20$). (E) Immunohistochemical staining of mouse xenografts with human specific anti-mitochondrial antibodies. (a and b) Anti-mitochondrial antibody showed specific staining against mitochondrial in human tissue (a) and no staining in mouse tissue (b) ($\times 20$). (c and d) Human origin of the cells in mouse xenografts formed by NOF151p53ihT (c) and NOF137p53ihT (d) was confirmed using immunohistochemical staining with anti-mitochondrial antibodies (black arrowheads, $\times 20$). (F) Immunohistochemical staining with human specific anti-hemoglobin- δ antibody. The specificity of monoclonal antibody against human hemoglobin- δ was shown by its interaction with the erythroid cells of human origin (a) but not with erythroid cells of mouse origin (b). The erythroid cells generated by NOF151p53ihT (c) and

NOF137p53ihT (d) were positive for anti-hemoglobin- δ antibodies (black arrowheads, $\times 20$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Generation of erythroid cells in cancer cell lines. (A) Formation of BT-549 spheroids after CoCl_2 treatment. (a) Control BT-549 without CoCl_2 treatment ($\times 10$). (b) PGCCs of BT-549 (large arrowheads) survived CoCl_2 treatment and produced regularly sized cells (small arrowheads) ($\times 10$). (c) BT-549 spheroid formation after CoCl_2 treatment ($\times 10$). (d and e) Immunohistochemical stain for CD44 (c) and CD133 (d) ($\times 20$). (f) Immunofluorescent stain for SOX-2 ($\times 20$). (g) Immunohistochemical stain of spheroids for SOX-2 ($\times 20$). (B) Generation of erythroid cells by BT-549 cells *in vitro*. (a) H&E stain of spheroids showing erythroid cells generated by BT-549 cells ($\times 20$). (b) High-power view of erythroid cells (H&E stain, $\times 40$). (c) Erythroid cells budding from cancer cells (black arrow points; HE staining, $\times 20$). (d–h) Immunohistochemical staining of erythroid cells for hemoglobin- α -, β / γ / ϵ / δ -, ϵ -, fetal hemoglobin, and hemoglobin- ζ ($\times 20$). (C) Erythroid cells differentiation in mouse xenografted tumor from CoCl_2 -treated BT-549 injection. (a–c) H&E staining of tumor tissue. (a) Many erythroid cells around tumor cells. (b) Erythroid cells appearing in the cytoplasm of tumor cells. (c) Erythroid cells budding from tumor cells (black arrowheads) ($\times 20$). (d) Immunohistochemical staining with human specific anti-hemoglobin- δ antibody confirming the human origin of erythroid cells ($\times 20$).

Fig. 4.

Generation of erythroid cells and hemoglobin in cancer cell lines and tissue. (A) Generation of erythroid cells by cancer cell lines. Erythroid cells can be identified from spheroids generated by, MDA-MB-231 (a), FTE187SV40hT-Hras (b), and Phoenix (c) cells *in vitro* after CoCl_2 treatment (black arrowheads, $\times 20$). (B) Generation of erythroid cells and hemoglobin by human ovarian cancer cells. (a) Smear of cancer cells in the medium confirming the generation of erythroid cells by high-grade ovarian cancer cells *in vitro* (black arrowheads, $\times 20$). (b) Erythroid cells in the cytoplasm of high-grade ovarian carcinoma cells ($\times 20$). (c and d) Numerous round and red hemoglobin bodies surrounding and within human ovarian cancer cells. (e–h) Staining of round and red hemoglobin bodies for hemoglobin- α (e); hemoglobin- $\beta/\gamma/\delta/\epsilon$ (f); fetal hemoglobin (g); and hemoglobin- ζ (h) in the same fields shown in c (black arrowheads, $\times 20$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)