Forkhead Family Transcription Factor FKHRL1 Is Expressed in Human Megakaryocytes

REGULATION OF CELL CYCLING AS A DOWNSTREAM MOLECULE OF THROMBOPOIETIN SIGNALING

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FKHRL1, a member of the Forkhead transcription factor family, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt. This molecule is a mammalian homolog of DAF-16, which plays an important role in the longevity of Caenorhabditis elegans. In this study we found that Akt and FKHRL1 proteins were detectable in highly purified normal human megakaryocytes and that these molecules were actually phosphorylated by thrombopoietin (TPO). To clarify the functional role of FKHRL1 in TPO signaling, we established a tetracycline-inducible system in the human TPO-dependent leukemia cell line UT-7/TPO. Induced expression of active FKHRL1 led to cell cycle arrest at G0/G1 phase in this cell line. These results suggest that FKHRL1 plays an important role in the cell cycle of megakaryocytic cells as one of the downstream target molecules of phosphatidylinositol 3-kinase-Akt, presumably mediated through the activation or inactivation of cell cycle-associated gene(s).

FKHRL1, a mammalian homolog of DAF-16, belongs to the Forkhead transcription factor family (1). This family, characterized by the presence of a highly conserved forkhead domain having a winged-helix motif and DNA binding activity, is involved in embryogenesis, differentiation, and tumorigenesis (2). DAF-16 plays an important role in the longevity of Caenorhabditis elegans (3). In C. elegans, the dauer lava ensures its survival in adverse conditions by lowering its metabolism and changing its shape and can live up to ten times longer than a normal adult (4). DAF-16 induces dauer formation and its activity is negatively regulated by DAF-2. Indeed, in C. elegans, a loss-of-function mutation in the DAF-2 gene causes development arrest at the dauer stage via the enhancement of DAF-16 transcription activity, resulting in an extension of the life span (5). These observations strongly suggested that FKHRL1 plays an important role in mammalian biology. This notion is supported by the evidence that there is a highly conserved signaling pathway between C. elegans and human. DAF-2 is an insulin receptor-like protein, AGE-1 is a catalytic subunit of phosphatidylinositol 3-kinase (PI3K)-1-like protein, DAF-23 is a PI3K-like protein, AKT1/AKT2 is a serine/threonine kinase protein kinase B (PKB) (also known as Akt)-like protein, and DAF-16 has high homology to the mammalian Forkhead subfamily FKHRL1, AFX, and FKHR (4).

Akt has been identified as a downstream target of PI3K necessary for survival (6). Akt activated by growth factors phosphorylates apoptosis-associated molecules including Bad, caspase-9, IKKa, and GSK-3 ensuring cell survival (7–10). In addition, the constitutively active form of Akt blocks apoptosis induced by growth factor deprivation (11, 12). Thus, the PI3K-Akt activation pathway appears to be a prerequisite for cell survival. Recently, FKHRL1, AFX, and FKHR have been identified as substrates of Akt (13–19). Phosphorylation of these proteins by Akt regulates their nuclear translocation and targets gene transcription. There are two or three potential Akt phosphorylation sites (RRX(S/T)) on these three members. For example, FKHRL1 has three putative phosphorylation sites; Thr298 (RPRSCT298), Ser253 (RRYAS253), and Ser315 (RSRTNS315). When cells are stimulated with serum or growth factors, a phosphorylated form of FKHRL1 is retained in the cytoplasm and interacts with 14-3-3 proteins, resulting in inhibition of target gene transcription. By contrast, when cells are deprived of serum or growth factors, a nonphosphorylated form of FKHRL1 translocates into nucleus and activates the transcription of target genes. These findings indicate that FKHRL1, when not phosphorylated by Akt, is an activator of transcription. Thus, phosphorylation by Akt is essential for suppressing the transcription activity of FKHRL1. In other words, Akt negatively regulates the transcription activity of FKHRL1 by phosphorylation (13). This is also true for both AFX and FKHR (14–18).

Very recently we identified FKHRL1 as one of the downstream molecules of the phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin (EPO) signal transduction (12), although the functional role of this molecule in erythropoiesis is still open to question. Based on several lines of evidence that there is a close relationship between erythropoiesis and megakaryopoiesis (20–22), we examined whether or not TPO, a major regulator of megakaryopoiesis, induces phosphorylation of Akt and FKHRL1 in a human TPO-dependent leukemia cell line UT-7/TPO (23) and highly purified normal human...
FKHRL1 and Cell Cycling in TPO Signaling

15083

man megakaryocytes. Moreover, to elucidate the function of FKHRL1 in TPO signaling, we established a tetracycline (Tet)-inducible expression system in UT-7/TPO cells. Our results suggest that FKHRL1 is phosphorylated by TPO stimulation via Akt activation and that unphosphorylated FKHRL1 negatively regulates the cell cycle, presumably via the activation or inactivation of cell cycle-associated genes.

**EXPERIMENTAL PROCEDURES**

**Hematopoietic Growth Factors and Reagents**—Recombinant TPO was kindly donated by Kirin Brewery Co. Ltd. (Tokyo, Japan) and reconstituted from its ezrin, CD42b (phycoerythrin conjugate (PE), PharMingen, San Diego, CA), and CD42a (fluorescein isothiocyanate conjugate (FITC), Nichirei, Tokyo, Japan) antibodies (27, 28). The cells were then cryopreserved and stored until use in a tank containing liquid nitrogen. The frozen peripheral blood CD34+ cells were thawed and suspended in IMDM containing 30% FCS and 100 units/ml DNase and then centrifuged at 400 × g for 5 min at 4 °C. The cells were washed with IMDM and reconstituted in liquid phase, as described elsewhere, with minor modifications (25). In brief, recombinant human granulocyte colony-stimulating factor (G-CSF; Chugai Pharmaceutical Co., and Kyowa Hakko Pharmaceutical Co., Tokyo, Japan) was administered to healthy volunteers (who had previously signed consent forms approved by the Hokkaido University School of Medicine and the Hokkaido Red Cross Blood Center committee for the Protection of Human Subjects), as described (26). The mobilized peripheral blood CD34+ cells were isolated using immunomagnetic beads (27, 28). The supernatants were boiled for 5 min and once with the Akt kinase buffer (20 mM Tris·HCl, pH 7.4, 10 mM MgCl2, 10 mM MnCl2). In vivo kinase experiments were performed with a commercial kit (Akt Kinase Assay Kit) using GS-K or GST-FKHRL1 fusion protein (13) as another substrate of Akt. Reaction products were resolved by SDS-PAGE and visualized by ECL.

**Tet-inducible Expression System**—To express FKHRL1-TM cDNA, we used a T-REX™ system (Invitrogen, Carlsbad, CA), in which transcription of a target cDNA is initiated by Tet treatment. In brief, UT-7/TPO cells initially were transfected with expression vectors for the Tet repressor, pcDNA6/TR, by lipofection. After culture with 0.5 μg/ml of blasticidin (Invitrogen), one clone (designated UT-7/TPO-pcDNA6/TR-2) was transfected further with a Tet-inducible expression vector, pcDNA4/T0, for FKHRL1-TM. After selection with Zeocin (Invitrogen), the cell line of 200 μg/ml of blasticidin (Invitrogen) was used for all experiments. The supernatants were incubated on ice, insoluble materials were removed by centrifugation at 10,000g. The supernatants were incubated with anti-Akt antibody (C-20) attached to protein G-Sepharose for 4 h at 4 °C in an Eppendorf shaker. Immunoprecipitates were collected by a brief centrifugation and washed four times with 1 ml of lysis buffer. The immunoprecipitated proteins were boiled for 5 min in SDS-PAGE sample buffer. After a brief centrifugation, the supernatants were resolved by SDS-PAGE and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The blots were blocked with 5% skim milk in Tris-buffered saline for 1 h and then incubated with the appropriate concentration of primary antibodies including anti-phosphoAkt(Ser473) (Cell Signaling Technology, Immunon, Pittsburgh, PA). Laser confocal scanning images were obtained using a TCS 4D confocal system (Leica Instruments, Wetzlar, Germany).

**Ex Vivo Generation of Megakaryocytic Cells**—Human megakaryocytic cells were generated ex vivo, as described, with minor modifications (25). In brief, recombinant human granulocyte colony-stimulating factor (G-CSF; Chugai Pharmaceutical Co., and Kyowa Hakko Pharmaceutical Co., Tokyo, Japan) was administered to healthy volunteers (who had previously signed consent forms approved by the Hokkaido University School of Medicine and the Hokkaido Red Cross Blood Center committee for the Protection of Human Subjects), as described (26). The mobilized peripheral blood CD34+ cells were isolated using immuno magnetic beads (27, 28). The supernatants were boiled for 5 min and once with the Akt kinase buffer (20 mM Tris·HCl, pH 7.4, 10 mM MgCl2, 10 mM MnCl2). In vivo kinase experiments were performed with a commercial kit (Akt Kinase Assay Kit) using GS-K or GST-FKHRL1 fusion protein (13) as another substrate of Akt. Reaction products were resolved by SDS-PAGE and visualized by ECL.
FKHRL1 and Cell Cycling in TPO Signaling

RESULTS

TPO Induced Phosphorylation of Akt Kinase in a Dose- and Time-dependent Manner—In the following experiments we mainly used the UT-7/TPO cell line (23). This cell line absolutely depends on TPO for growth and survival, and it has mature megakaryocytic properties such as a developed demarcation membrane in the cytoplasm, high expression of the megakaryocyte-specific markers, platelet factor-4 and glycoprotein Ib mRNAs, and high DNA content (23). Therefore, UT-7/TPO is a good model of megakaryocytic cells for elucidating the biological role of the PI3K-Akt-FKHRL1 activation pathway in TPO signaling. Based on the recent reports that Akt is activated by several cytokines including interleukin-3, granulocyte colony-stimulating factor and EPO (12, 30, 31), we initially examined whether or not TPO activates Akt in UT-7/TPO cells. Growth factor-deprived UT-7/TPO cells were exposed to increasing concentrations of TPO (0.1–100 ng/ml) for 10 min (B and D). After solubilization, cell lysates were immunoprecipitated with protein G-conjugated anti-Akt antibody (A and B). Immunoprecipitates were eluted with buffer containing SDS and were resolved by 10% SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane. Upper panel, immunoblotting with anti-phosphoAkt antibody. Lower panel, the blot was reprobed with anti-Akt serum to confirm equal loading of protein. In some experiments, after solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibodies directed against phospho-Thr32 (C and D, top panel, anti-phosphoT32) or phospho-Ser253 (C and D, middle panel, anti-phosphoS253). The blot was reprobed with anti-FKHRL1 antibody to confirm equal loading of protein (C and D, bottom panel). Anti-FKHRL1 antibody recognizes two bands; the upper band (*) is the phosphorylated form, the lower band (**) is the unphosphorylated form.

TPO-induced Phosphorylation of Akt Is Dependent on PI3K Activity—The growth factor-deprived UT-7/TPO cells were pre-treated with increasing concentrations of the PI3K-specific inhibitor LY294002 (1 μM-100 μM) for 45 min and then stimulated with TPO (100 ng/ml). Ten min later, the cells were harvested for cell extraction. Western blotting analysis was performed using anti-[phospho-Ser473]Akt antibody. As shown in Fig. 2A, the phosphorylation density of Akt was markedly diminished at 10 μM LY294002 and equal to the basal level at 50 μM LY294002. This result suggested that TPO-induced phosphorylation of Akt is mediated via PI3K activity.

TPO Indeed Induces Activation of Akt Kinase—To confirm the activation of Akt by TPO, we performed in vitro kinase assay using GSK-3 as substrate (Fig. 3A). After exposure to TPO (100 ng/ml) for given periods of up to 60 min, the cells were immunoprecipitated with anti-Akt antibody and in vitro kinase assay was performed according to the instructions of the AKT Kinase Assay Kit. The density of phosphorylated GSK-3 was enhanced after a 5-min exposure to TPO, and its enhancement continued until 60 min. This observation indicates that TPO indeed induced Akt kinase activation in UT-7/TPO cells.

TPO Induces Phosphorylation of FKHRL1 in a Dose- and Time-dependent Manner—Recently it was reported that FKHRL1 is a substrate of Akt kinase in vitro (13). We also found that Akt kinase activated by EPO directly phosphorylated the FKHRL1 protein (12). To confirm that FKHRL1 lies downstream of Akt kinase in the TPO signaling pathway, we examined whether or not FKHRL1 is phosphorylated by TPO treatment. Growth factor-deprived UT-7/TPO cells were exposed to TPO (100 ng/ml) for given periods of up to 60 min or exposed to increasing concentrations of TPO (0.1–100 ng/ml) for 10 min, and then the cells were harvested for preparation of
FKHRL1 and Cell Cycling in TPO Signaling

**FKHRL1 Is One of the Target Molecules of AKT Kinase Activated by TPO**—To demonstrate that FKHRL1 is directly targeted by AKT kinase activated by TPO in vivo, we performed an in vitro kinase assay using GST-FKHRL1 fusion protein as a substrate. TPO-deprived UT-7/TPO cells were exposed to TPO for 10 or 20 min and then harvested for immunoprecipitation with anti-Akt antibody. Immunoprecipitates were incubated with GST-FKHRL1 fusion protein according to the instructions of the AKT Kinase Assay Kit with some modifications. As shown in Fig. 3B, a phosphorylated FKHRL1 band was obtained with antibody, which recognizes phosphorylated Thr32 and phosphorylated Ser253, respectively, indicating that Akt activated by TPO directly phosphorylated FKHRL1 at Thr32 and Ser253.

**FKHRL1 Protein Is Present in Normal Megakaryocytes**—We examined whether or not FKHRL1 is expressed in normal megakaryocytes. To obtain a large amount of megakaryocytes for Western blotting analysis, human CD34-positive cells were cultured in the presence of Interleukin-3, SCF, and TPO for 10 days. Isolated human megakaryocytic cells expressed the specific megakaryocytic markers CD41 at 93.7%, CD61 at 75.5%, and CD42b at 42.8%, but did not express the erythroid-specific marker GPA (Fig. 4, A–D). Virtually all CD42b-positive cells expressed CD41 antigens (Fig. 4C). Using these highly purified megakaryocytes, we examined whether or not Akt and its downstream molecule FKHRL1 are indeed phosphorylated in total cell lysates. Western blotting analysis was performed using anti-phospho-Thr28 antibody and anti-phospho-Ser253 that recognize a phosphorylated threonine 32 and a phosphorylated serine 253, respectively. FKHRL1 was phosphorylated at Thr32 and Ser253 at 1 min, the level reaching a plateau at 5–20 min and declining thereafter (Fig. 1C). Phosphorylated FKHRL1 was detectable at 0.1 ng/ml of TPO, and the level reached a plateau at 1 ng/ml of TPO (Fig. 1D). These findings indicate that TPO induced phosphorylation of FKHRL1 in a dose- and time-dependent manner.

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**FKHRL1 and Cell Cycling in TPO Signaling**—To examine the role of FKHRL1 in cell proliferation, we performed Western blotting analysis using anti-phospho-Thr28 antibody and anti-phospho-Ser253 that recognize phosphorylated Thr28 and phosphorylated Ser253, respectively. FKHRL1 was phosphorylated at Thr28 and Ser253 at 1 min, the level reaching a plateau at 5–20 min and declining thereafter (Fig. 1C). Phosphorylated FKHRL1 was detectable at 0.1 ng/ml of TPO, and the level reached a plateau at 1 ng/ml of TPO (Fig. 1D). These findings indicate that TPO induced phosphorylation of FKHRL1 in a dose- and time-dependent manner.

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**Fig. 2. Phosphorylation of Akt and FKHRL1 by TPO is dependent on PI3K activity.** TPO was removed from UT-7/TPO cells for 24 h. The cells were pretreated with increasing concentrations of LY294002 (1–100 µM) and then stimulated with TPO (100 ng/ml) for 10 min. After solubilization, cell lysates were immunoprecipitated with protein G-conjugated anti-Akt antibody (A). Immunoprecipitates were eluted with buffer containing SDS and resolved by 10% SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane. Upper panel, immunoblotting with anti-phospho-Akt antibody. Lower panel, the blot was reprobed with anti-Akt serum to confirm equal loading of protein. In some experiments, after solubilization, cell extracts were resolved by 7.5% SDS-PAGE and immunoblotted with the antibodies directed against phospho-Thr32 (**), phospho-Ser253 (**), or phospho-GSK-3 antibody (A) or the antibodies directed against phospho-Thr32 (**), phospho-Ser253 (**), or phospho-GSK-3 antibody (B). The blot was reprobed with anti-FKHRL1 antibody to confirm equal loading of protein (B, bottom panel). Anti-FKHRL1 antibody recognizes two bands; the upper band (*) is the phosphorylated form, the lower band (**) is the unphosphorylated form.
primary megakaryocytic cells. After a 2-h deprivation of growth factors, the cells were stimulated with TPO (100 ng/ml) for 20 min and then harvested for Western blotting analysis. As shown in Fig. 4, E and F, Akt and FKHRL1 proteins were indeed expressed in megakaryocytes. As expected, Akt at Ser\(^{473}\) and FKHRL1 at Thr\(^{32}\) and Ser\(^{253}\) were clearly phosphorylated by TPO treatment.

**FKHRL1 Blocks Cell Cycle Progression from G\(_0\)/G\(_1\) to S Phase in UT-7/TPO Cells**—Considering that FKHRL1 lies downstream of the PI3K-Akt activation pathway, these results raised the possibility that FKHRL1 is involved in the TPO-induced cell survival as a target molecule of the PI3K-Akt pathway. To test this possibility, we expressed the triple mutant of FKHRL1 (FKHRL1-TM) in UT-7/TPO by using a Tet-inducible system, in which expression of the target protein is inducible by Tet treatment. FKHRL1-TM in which Thr\(^{32}\), Ser\(^{253}\), and Ser\(^{315}\) are all converted to alanines is localized to the nucleus and activates the Fas ligand (FasL) gene promoter in vitro, indicating that FKHRL1-TM is an “active” form for activation of target gene(s). We transfected UT-7/TPO/pcDNA6/Tr-2 with pcDNA4/TO containing FKHRL1-TM cDNA and isolated several stable clones. Among them, UT-7/TPO/FKHRL1-TM clone 1 and clone 2 were used for the subsequent experiments because Tet treatment led to the strong induction of FKHRL1-TM protein in these clones. Western blotting analysis of the whole cell lysates revealed that the treatment with Tet resulted in rapid induction of FKHRL1-TM proteins in UT-7/TPO/FKHRL1-TM cells (Fig. 5A). Next, we investigated the nuclear localization of FKHRL1-TM in these transfectants. Confocal microscopic study revealed that induced FKHRL1-TM was localized to the nucleus after Tet treatment (Fig. 5B). Induced expression of FKHRL1-TM by Tet treatment led to the suppression of TPO-induced MTT incorporation into the cells (Fig. 6A), whereas Tet treatment had no effect on MTT incorporation into a control cell line that was transfected with an empty expression vector (data not shown). These results suggest that FKHRL1-TM is actually functional in our systems. To further elucidate the biological role of FKHRL1, using clone 1 we examined the effect of FKHRL1-TM on cell cycling of UT-7/TPO cells. After a 12-h exposure to Tet in the absence of TPO, the cells were in part harvested for cell cycle analysis (Fig. 6B, left panel). The remaining cells were sequentially cultured with TPO (10 ng/ml) for 24 h, and then harvested for cell cycle analysis (Fig. 6B, right panel). The ratio of cells at the G\(_0\)/G\(_1\) phase increased after Tet treatment, compared with the control cells (Tet\(^{-}\) versus Tet\(^{+}\); 0 h, 43.3% versus 44.9%; 24 h, 31.6% versus 48.0%). These results strongly suggested that FKHRL1 activates or inactivates the transcription of the cell cycle-associated gene(s). The subG\(_1\) population was not increased by Tet treatment compared with the control cells (Fig. 6B). Indeed, the ratio of annexin V-positive cells was not increased in the cells treated with Tet (data not shown). Similar results were obtained in clone 2 (data not shown). Therefore, active FKHRL1 induced cell cycle arrest at G\(_0\)/G\(_1\) phase but did not induce apoptosis in UT-7/TPO cells.

**DISCUSSION**

In this study we showed that Akt and FKHRL1 were rapidly and transiently phosphorylated by TPO in a human leukemia
cell line UT-7/TPO. We found that PI3K inhibitor LY294002 completely blocked the TPO-induced phosphorylation of Akt and FKHRL1 in UT-7/TPO cells, indicating that the activation of Akt and FKHRL1 is completely regulated by PI3K activity. In addition, in vitro kinase assay revealed that Akt kinase activated by TPO directly phosphorylated FKHRL1 protein at threonine 32 and serine 253 residues. These findings indicate that FKHRL1 functions as a downstream molecule of the PI3K-Akt activation pathway, as is the case for EPO (12). Finally, we demonstrated that unphosphorylated active FKHRL1 induced cell cycle arrest at the G0/G1 phase in UT-7/TPO cells. Importantly, Akt and FKHRL1 were present and phosphorylated in highly purified normal human megakaryocytes. This strongly suggested that these molecules are involved in normal megakaryopoiesis and presumably in platelet production.

It is noteworthy that inducible expression of active FKHRL1 (FKHRL1-TM) induced cell cycle arrest at G0/G1 phase in UT-7/TPO. Very recently it was reported that AFX, a member of the Forkhead subfamily, blocked cell cycle progression in phase G1 via activation of the p27Kip1 gene at the transcriptional level (32). This observation prompted us to examine whether or not activation of p21WAF1/Cip1 and p27Kip1 is involved in FKHRL1-induced cell cycle arrest at the G0/G1 phase in UT-7/TPO cells. However, luciferase assay revealed that FKHRL1-TM did not
activate p21WAF1/Cip1 or p27Kip1 gene promoter in the Tet-inducible system (data not shown). In addition, neither p21WAF1/Cip1 nor p27Kip1 were enhanced at the protein level after Tet treatment (data not shown). These findings indicate that some cell cycle-associated molecule(s) other than p21WAF1/Cip1 and p27Kip1 influence the FKHR1-induced cell cycle arrest at G0/G1 phase in UT-7/TPO cells. There was a discrepancy in the dosage of LY294002 between the inhibition of phosphorylation of Akt-FKHR1 and induction of apoptosis in UT-7/TPO cells. Although a high dose of PI3K inhibitor (50 and 100 μg/ml of LY294002) induced apoptosis in more than 50% of UT-7/TPO cells (data not shown), phosphorylation of Akt and FKHR1 by TPO was significantly blocked at 10 μM LY294002 at which dosage apoptosis did not occur in UT-7/TPO cells (data not shown). Therefore, it is unlikely that phosphorylation of Akt and FKHR1 is closely involved in the anti-apoptotic effect of TPO. This notion is in part supported by the evidence that overexpression of different levels of FKHR1-TM did not induce apoptosis in UT-7/TPO cells (Fig. 6B and data not shown). Alternatively, our results may suggest that FKHR1 is in a dephosphorylated state is not sufficient to induce apoptosis in UT-7/TPO cells. If so, a third factor may be required for FKHR1-induced apoptosis in this cell line.

FKHR1 was originally identified as a novel partner of the MLL gene, in undifferentiated acute leukemia with t(6;11)(q21; q23) translocation (33). Interestingly, it has been reported that AFM-MLL and FKHR-PAX3 fusion genes are detectable in acute leukemias with t(X;11)(q13;q23) and alveolar rhabdomyosarcoma, respectively (34, 35). Therefore, aberrant expression of FKHR1 in human malignancies strongly suggests that FKHR1 is a housekeeping molecule (13). In conclusion, considering our results that inducible expression of FKHR1-TM inhibited TPO-induced proliferation of UT-7/TPO cells (see Fig. 6A), FKHR1 may function as a tumor suppressor in normal cells, although this is speculation.

There is a close relationship between cell differentiation and cell cycle arrest. To examine whether or not FKHR1 affects TPO-induced megakaryocytic differentiation of UT-7/TPO cells, we cultured UT-7/TPO-FKHR1-TM cells in the presence of TPO and Tet for 5 days. However, inducible expression of FKHR1-TM by Tet did not enhance the expression of CD41 and CD61 antigens, which are specific for the megakaryocytic lineage (data not shown). This finding suggests that FKHR1-TM-induced cell cycle arrest at the G0/G1 phase is not sufficient for megakaryocytic differentiation.

There was a discrepancy between Akt and FKHR1 in the dosage of TPO needed for their phosphorylation. Phosphorylation of Akt by TPO was induced at 1 ng/ml of TPO, whereas FKHR1 was phosphorylated by TPO at 0.1 ng/ml. In addition, the in vitro kinase assay revealed that the phosphorylation level of FKHR1 by Akt was much weaker than that in vivo. Although we cannot completely exclude the possibility that the discrepancy in phosphorylation level between in vitro and in vivo is because of technical problems, the discrepancy may be because of the different sensitivity of antibody used in this experiment, or that not only Akt but other protein kinase(s) are involved in the phosphorylation of FKHR1 protein.

FKHR1 has three putative Akt consensus phosphorylation sites, Thr32, Ser253, and Ser315. Among them, Thr32 and Ser253 were in vitro phosphorylated by TPO stimulation in UT-7/TPO cells. Indeed, an in vitro kinase assay revealed that both sites were directly phosphorylated by Akt activated by TPO. However, we could not demonstrate the phosphorylation of Ser315 induced by TPO because adequate anti-phospho-Ser315 antibody was not available. To overcome this obstacle, we took advantage of the finding that phosphorylation of FKHR1 at Ser315 but not Thr32 or Ser253 had a significant effect on the mobility of FKHR1 on an SDS gel (13). As shown in Figs. 1C, 1D, 2B, and 4F, TPO induced a shift up in the mobility of FKHR1. This result strongly suggested that FKHR1 at Ser315 was phosphorylated by TPO stimulation, although it is still unknown whether or not Akt directly phosphorylated Ser315.

Because the FasL promoter contains three Forkhead-responsive elements that bind FKHR1 (13), it was predicted that FasL is one of the target molecules of FKHR1 in UT-7/TPO cells. Indeed, the nonphosphorylated active form of FKHR1 can activate the FasL promoter in vitro and induce apoptosis in cerebellar neurons, fibroblasts, and Jurkat T lymphoma cells (13). Based on these observations, we examined whether or not FKHR1 can activate the FasL gene using a Tet-inducible system in UT-7/TPO cells. However, unexpectedly, reverse transcriptase-polymerase chain reaction revealed that FasL mRNA was undetectable after the addition of Tet (data not shown), suggesting that the FasL gene is not a target molecule for FKHR1 at least in UT-7/TPO cells.

To our knowledge, this is the first report that shows that Akt and FKHR1 are present in normal megakaryocytes and that these molecules are actually phosphorylated by TPO. The evidence that FKHR1 is ubiquitously expressed in all tissues strongly suggests that FKHR1 is a housekeeping molecule (13). In conclusion, considering our results that FKHR1 induced cell cycle arrest at G0/G1 phase but had no effect on differentiation and apoptosis, FKHR1 may play an important role in keeping the cells in the quiescent state via the activation or inactivation of cell cycle-associated gene(s).

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