Upregulation of DLX5 Promotes Ovarian Cancer Cell Proliferation by Enhancing IRS-2-AKT Signaling

Yinfei Tan¹, Mitchell Cheung¹, Jianming Pei¹, Craig W. Menges¹, Andrew K. Godwin², and Joseph R. Testa¹

Abstract

The distal-less homeobox gene (dlx) 5 encodes a transcription factor that controls jaw formation and appendage differentiation during embryonic development. We had previously found that Dlx5 is overexpressed in an Akt2 transgenic model of T-cell lymphoma. To investigate if DLX5 is involved in human cancer, we screened its expression in the NCI 60 cancer cell line panel. DLX5 was frequently upregulated in cell lines derived from several tumor types, including ovarian cancer. We next validated its upregulation in primary ovarian cancer specimens. Stable knockdown of DLX5 by lentivirus-mediated transduction of short hairpin RNA (shRNA) resulted in reduced proliferation of ovarian cancer cells due to inhibition of cell cycle progression in connection with the downregulation of cyclins A, B1, D1, D2, and E, and decreased phosphorylation of AKT. Cell proliferation resumed following introduction of a DLX5 cDNA harboring wobbled mutations at the shRNA-targeting sites. Cell proliferation was also rescued by transduction of a constitutively active form of AKT. Intriguingly, downregulation of IRS-2 and MET contributed to the suppression of AKT signaling. Moreover, DLX5 was found to directly bind to the IRS-2 promoter and augmented its transcription. Knockdown of DLX5 in xenografts of human ovarian cancer cells resulted in markedly diminished tumor size. In addition, DLX5 was found to cooperate with HRAS in the transformation of human ovarian surface epithelial cells. Together, these data suggest that DLX5 plays a significant role in the pathogenesis of some ovarian cancers.

Introduction

Cancer is often regarded as the consequence of developmental dysregulation underlaid by aberrant expression of transcription factors that are normally involved in embryonic development (1). The homeobox gene superfamily encodes transcription factors that act as master controllers of development by regulating a diverse range of target genes. For example, TAL1 and HOX11 are essential transcription factors involved in early hematopoiesis, but their misexpression in thymocytes causes T-cell acute lymphoblastic leukemia (T-ALL) by blocking thymocyte differentiation (2). In addition to HOX11, homeobox genes are widely implicated in various human cancers, by acting either as oncogenes or as tumor suppressors. For example, in rhabdomyosarcoma, an oncogenic chromosomal translocation results in the fusion of the DNA binding domain of the NKX3-1 transcription factor gene (3). The homeobox gene NKX3-1 plays an important role in normal differentiation of the prostatic epithelium, whereas its loss of function initiates prostate carcinogenesis (4). In breast cancer, HOXA5 expression is frequently lost due to gene deletion or promoter methylation (5).

We had previously identified a chromosomal abnormality in thymic tumor cells from a transgenic mouse model driven by a myristoylated (myr), constitutively active form of Akt2. Tumor cells from these mice often harbor an inversion of chromosome 6 that juxtaposes an evolutionally conserved homeobox bi-gene, Dlx5/6, to the enhancer of the Tcrb gene (6). Moreover, clonogenic assays revealed oncogenic cooperativity when both Dlx5 and activated Akt2 were coexpressed in mammalian cells. The Dlx gene family is related to the Drosophila distal-less (dll) gene and is mainly expressed in the developing forebrain and craniofacial structures. More recently, Dlx genes have also been found to play broader roles ranging from neurogenesis to hematopoiesis (7). Sonic hedgehog, BMP4, fibroblast growth factor (FGF), and Wnt, among others, can induce Dlx expression in a tissue-specific manner (8).

Dlx5 is expressed in adult bone marrow cells and fetal liver cells, but its expression is suppressed in Thy1-positive T cells (9). In thymic lymphoma cells observed in myr-Akt2 transgenic mice, Dlx5 protein levels are highly elevated, which may facilitate tumor development by interfering with T-cell differentiation (6). Another DLX family member, DLX7 (now known as DLX4) has also been implicated in human hematopoietic neoplasias. DLX4 is expressed in normal hematopoietic cells and leukemia cell lines with erythroid characteristics, and its...
knockdown induces apoptosis via downregulation of MYC and GATA1 (10). DLX4 was also found to be frequently overexpressed in acute myeloid leukemia and T-ALL (11), and expression of DLX4 has been implicated in breast cancer progression and choriocarcinoma cell survival (12). The role of DLX5 in tumor development, however, is only beginning to emerge. Kato and colleagues reported that overexpression of DLX5 in non–small cell lung cancer is linked to tumor size and is predictive of a poor prognosis (13). They also showed that knockdown of DLX5 suppresses lung cancer cell viability and colony formation. Here, we report that DLX5 is frequently overexpressed in human ovarian cancer cells, and that depletion of DLX5 by RNA interference (RNAi) inhibits cell proliferation, in part by attenuating AKT signaling.

Materials and Methods

Cell lines, tumor specimens, and reagents

Ovarian cancer cells lines IGROV1, OVCAR3, OVCAR4, OVCAR5, OVCAR8, OVCAR10, SKOV3, and A2780 were from Fox Chase Cancer Center and were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Primary and SV40-immortalized nontumorigenic human ovarian surface epithelial (HOSE) cells were maintained in 1:1 M199 and MCBD-105 media, respectively, supplemented with 5% fetal bovine serum and 0.2 IU/mL insulin, 100 μg/mL penicillin and streptomycin, and 2 mmol/L L-glutamine. Primary ovarian tumor specimens were obtained from patients who underwent surgery at Fox Chase Cancer Center. Antibodies against DLX5, DLX6, cyclin A, cyclin B1/2, cyclin D1/2/3, cyclin E1/2, insulin-like growth factor I receptor β (IGFIRβ), MET, IRS-1/2, phosphoinositide 3-kinase (PI3K) p110β, β-actin, and GAPDH were purchased from Santa Cruz Biotechnology. Antibodies against phosphorlated (p)-PKC, p-AKT/AKT, p-GSK3β, p-p70S6K/p70S6K, extracellular signal-regulated kinase (ERK)/p-ERK, human epidermal growth factor receptor 2 (HER2), and colony formation. Here, we report that DLX5 is frequently upregulated in cell lines derived from patients with leukemia or colorectal, prostate, and kidney cancers (Fig. 1). To delineate the involvement of various DLX family genes in ovarian oncogenesis, we next compared the mRNA expression of all six DLX members in ovarian cancers of various origins, including ovarian cancer.

Results

DLX5 is frequently upregulated in cell lines derived from human cancers of various origins, including ovarian cancer

To test whether DLX5 expression is deregulated in human cancers, we initially screened the NCI 60 cancer cell line panel for DLX5 transcript levels. Semiquantitative reverse transcriptase-PCR (RT-PCR) revealed that DLX5 mRNA is abundantly expressed in many cancer cell lines derived from malignant tissues of breast, brain, lung, skin, and ovary, but expression of DLX5 was low or undetectable in tumor cells from patients with leukemia or colorectal, prostate, and kidney cancers (Fig. 1). To delineate the involvement of various DLX family genes in ovarian oncogenesis, we next compared the mRNA expression of all six DLX members in ovarian cancers of various origins, including ovarian cancer.
cancer cells versus that observed in primary (pHOSE) and SV40-immortalized (iHOSE) ovarian epithelial cells. DLX1 and DLX2 were found to be equally expressed in primary and immortalized HOSE cells and malignant ovarian cells. DLX3 and DLX4 were upregulated in immortalized nontumorigenic and malignant cells. Interestingly, DLX5 and DLX6 were detected only in ovarian cancer cells, not in pHOSE or iHOSE cells (Supplementary Fig. S1A). These observations suggest that expression of DLX5/6 occurs only in fully transformed cells, which prompted us to determine if DLX5 and DLX6 have a role in ovarian tumor maintenance. The upregulation of DLX5/6 seems to be due to an epigenetic alteration, because high-level amplification was not observed in tumor cells, as shown by real-time PCR analysis (Supplementary Fig. S1B). We next performed immunoblot analysis and found that DLX5 is expressed in IGR-OV1, OVCAR3, OVCAR4, OVCAR8, and OVCAR10 cells at variable levels, but it was not detected in pHOSE and iHOSE cells (Fig. 2A). The predicted molecular weight of DLX5 protein is 33 kDa, which corresponds to the lower of two bands observed in the immunoblot analysis. The upper band (∼38 kDa) may be the result of a posttranslational modification, not an isoform resulting from alternatively spliced mRNA, because overexpression of DLX5 by transfecting 293T with a full-length human DLX5 cDNA showed the same pattern (Fig. 2A and B). Alternatively, it is possible that the faster migrating band could represent a partially cleaved form of the 38-kDa DLX5 protein. Fractionation experiments revealed that both forms of DLX5 are located in the nucleus (Supplementary Fig. S2A). Moreover, incubation of cells with the cell-permeable inhibitors of two major proteases, i.e., caspase or calpain, did not result in any obvious changes in the DLX5 immunoblot pattern, suggesting that the 33-kDa band is not a cleavage product of these proteases. Furthermore, global phosphatase inhibition by calyculin A resulted in increased phosphorylation of both forms (Supplementary Fig. S2B). Endogenous DLX6 protein could only be detected by immunoprecipitation/Western blot analysis using total cell lysates at multimilligram protein levels, suggesting a low expression level compared with that of DLX5, and thus may downplay its significance (Supplementary Fig. S3). Because of the abundance of DLX5 seen in malignant cells, we focused our subsequent studies on this protein. To rule out the possibility that DLX5 expression only occurs in cancer cell lines during in vitro culture, we next examined the expression of DLX5 in tumor samples. We found that DLX5 is not expressed in nonmalignant ovarian samples (Supplementary Fig. S4A), but is expressed in a subset of late-stage tumors (Fig. 2C, Supplementary Fig. S4B).

**DLX5 knockdown diminishes the proliferation of ovarian cancer cells in vitro and inhibits tumor growth in vivo**

To assess the role of DLX5 in ovarian cancer, we used lentivirus-mediated RNAi knockdown to inhibit DLX5 expression in ovarian carcinoma cells. Two efficient knockdown constructs were selected for generating lentiviruses. WST-1 assays were performed to measure cell proliferation and viability 5 days after viral infection. We found that DLX5 shRNAs impaired the viability/proliferation of cell lines (OVCAR3, 4, 8, and 10, and IGR-OV1) overexpressing DLX5 but had little or no effect on DLX5-negative cell lines (A2780 and SKOV3; Fig. 3A). Cell proliferation curves for IGR-OV1, OVCAR4, and OVCAR8 cells documented that proliferation was inhibited, suggesting that cell cycle arrest, rather than apoptosis, results from downregulation of DLX5 (Fig. 3B). This notion was validated by results of a fluorescence activated cell sorting assay, which revealed that more cells are arrested in G1 and G2-M phases, with fewer cells in S phase (Fig. 3C). Similarly, DLX5 knockdown inhibited DNA
replication as shown by bromodeoxyuridine (BrdUrd) incorporation (Fig. 3D). Consistent with the reduced cell division rate, knockdown of DLX5 resulted in decreased expression of multiple cyclin family members, including cyclins A, B1, D2, and E1 in IGR-OV1, OVCAR4, and OVCAR8 cells (Fig. 3E). Knockdown of DLX6 did not consistently affect cell proliferation, which may be due to its low expression level (Supplementary Fig. S5A and B).

To evaluate the in vivo effect of DLX5 knockdown on tumor progression, OVCAR8 cells infected with control lentivirus or lentivirus expressing shRNA against DLX5 were injected s.c. into severe combined immunodeficient (SCID) mice. Mice were sacrificed 1 month after injection, and the xenograft tumors were weighted. These studies revealed that tumors expressing DLX5 RNAi were significantly smaller (Fig. 3F).

DLX5 knockdown attenuates AKT signaling by downregulating upstream signaling

To investigate the mechanism involved in cell cycle arrest following knockdown of DLX5, we next examined whether a specific signaling pathway is compromised upon DLX5 knockdown. Interestingly, AKT activity was found to be suppressed in IGR-OV1 and OVCAR4 cells after expression of DLX5 shRNA (Fig. 4A). The decreased p-AKT levels seemed to result from insufficient upstream signaling, because total AKT protein levels were not altered, whereas p-PDK1 levels were suppressed. The total protein levels of PDK1 and PI3K subunits were unaffected, suggesting that decreased AKT pathway activation arises from the upstream signal-initiating complex, rather than the protein levels of components of the PI3K-AKT pathway itself (Fig. 4A). In fact, AKT pathway inhibition by the inhibitors LY294002, GSK690693, and RAD001 seemed to mimic DLX5 knockdown in these cells, as shown by decreased cell proliferation and downregulation of cyclin levels (Supplementary Fig. S6A and B).

We thus decided to investigate the effects of DLX5 knockdown on major upstream tyrosine kinase receptors and their adaptor proteins. We found that knockdown of DLX5 in IGR-OV1 and OVCAR4 cells consistently resulted in decreased total protein levels of MET and IRS-2 but not EGFR, HER2, or IGFR (Fig. 4B). We also found that expression of MET, IRS-1/2, EGFR, and HER2 are upregulated in many ovarian cancer cell lines and that the cell lines that overexpress DLX5 consistently had increased IRS-2 protein levels (Supplementary Fig. S7). Moreover, DLX5 knockdown impaired IGF- or hepatocyte growth factor-induced AKT activation in IGR-OV1 and OVCAR4 cells (Fig. 4C). On the other hand, reintroduction of a myr-AKT2 cDNA antagonized the growth-inhibiting effect of DLX5 knockdown as shown by WST-1 assay (Fig. 4D). Phosphorylation of GSK3β and expression of various cyclins were also restored by expression of constitutively active AKT2 (Fig. 4E).

Reintroduction of a wobbled DLX5 cDNA restores cell proliferation and cell signaling in DLX5 knockdown cells

To rule out a possible off-target effect of the DLX5 shRNA, we introduced three different wobble mutations into the...
Figure 3. DLX5 knockdown suppresses cell proliferation by inhibiting cell cycle progression. A, lentiviruses harboring shRNA against LacZ or DLX5 were generated and used to infect DLX5-positive (IGR-OV1, OVCAR4, OVCAR8 and OVCAR10) or DLX5-negative (SKOV3 and A2780) ovarian cancer cell lines. Cell viability/proliferation was determined by WST-1 assay 5 days after viral infection. B, cell proliferation curves of IGR-OV1, OVCAR4, and OVCAR8 cell lines after virus infection at the indicated times. C, cell cycle analysis was performed on IGR-OV1, OVCAR4, and OVCAR8 cells with control or DLX5 knockdown. D, rates of DNA synthesis were determined by counting cells staining with BrdUrd. E, DLX5 knockdown results in decreased expression of various cell cycle regulators, including cyclins A, B1, D2, and E1. F, DLX5 knockdown reduces growth of xenografted OVCAR8 cells in SCID mice. Tumors were recovered 1 month after s.c. injection and weighted (*, P < 0.05).
Figure 4. DLX5 knockdown attenuates AKT signaling pathway by downregulating upstream effectors. A, AKT signaling is compromised by DLX5 knockdown in IGR-OV1 and OVCAR4 cells. Expression of p-PDK1/PDK1, p-AKT/AKT, p-GSK3β, p-p70S6K, and p-S6 was analyzed by Western blotting. B, DLX5 knockdown reduces MET and IRS-2 expression. Levels of membrane receptor kinases MET, EGFR, HER2, and IGFR1, and adaptor proteins IRS-1 and IRS-2 were determined by immunoblotting. C, DLX5 knockdown reduces MET/HGF and IGFR signaling to AKT. IGR-OV1 and OVCAR4 cells with DLX5 knockdown were starved and treated with 10 ng/mL hepatocyte growth factor or 50 ng/mL IGF-I for 10 minutes. p-AKT/AKT levels were analyzed by immunoblotting. D, myr-AKT protects cells from DLX5 knockdown-induced inhibition of cell proliferation. Ovarian cancer cells were cotransduced by viruses containing DLX5 shRNA and myr-AKT, and cell viability/proliferation was analyzed by WST-1 assay 5 days after infection. E, cyclin levels and AKT signaling diminished by DLX5 knockdown are restored by expression of myr-AKT.
shRNA2-targeted sequence in a Flag-DLX5 cDNA. The wobbled DLX5 restored cell proliferation in cells expressing shRNA2 (Fig. 5A). The wobbled DLX5 was resistant to DLX5 shRNA2 in IGR-OV1 and OVCAR4 cells cotransduced with shRNA2, as shown by the expression of Flag-DLX5 protein (Fig. 5B). Thus, shRNA2 failed to diminish cyclin levels or to weaken AKT signaling in the wobbled DLX5-rescued cells likely due, at least in part, to unaffected IRS-2 and MET levels (Fig. 5B).

**DLX5 binds to the IRS-2 promoter and augments its transcription**

To determine if DLX5 is directly responsible for upregulation of IRS-2 in DLX5-positive cancer cells, the IRS-2 promoter was cloned into pGL3 vector, and luciferase assays were performed. DLX5 was found to augment IRS-2 promoter activity (Fig. 6A and B). The DLX5 binding consensus sequence of the IRS-2 promoter was synthesized, biotin labeled, and then used for gel shift assays. DLX5 protein caused a band shift in the DNA retardation gel, and addition of DLX5 antibody imposed a supershift (Fig. 6C). We next immunoprecipitated DLX5 protein from IGR-OV1 cells, and it produced the same band shift (Fig. 6D). Mutation in the DLX5 binding consensus of the IRS-2 promoter resulted in markedly diminished luciferase activity, as well as a gel shift (Fig. 6E and F). Moreover, chromatin immunoprecipitation assay showed that DLX5 binds to the IRS-2 promoter at the endogenous level in IGR-OV1 and OVCAR4 cells, but not in SKOV3 cells (Fig. 6G).

**DLX5 promotes HRAS-induced HOSE transformation in vitro**

To investigate if DLX5 can facilitate HRAS-initiated ovarian epithelial cell transformation in vitro, SV40-immortalized HOSE cells were transduced with retroviral HRAS(G12V)/green fluorescent protein, retroviral HRAS(G12V)/DLX5, or retroviral Vector/DLX5. After double selection with hygromycin and puromycin, HOSE cells were seeded in soft agar plates, and colonies were counted four weeks later. DLX5 was found to enhance the transformation of HRAS-induced HOSE cells, when compared with oncogenic HRAS alone, suggesting oncogenic cooperativity between DLX5 and HRAS (Supplementary Fig. S8A–C).

**Discussion**

Homeodomain transcription factors play important roles in directing cellular proliferation and differentiation, and their dysregulation has been implicated in various human cancers. In some ovarian cancers, HOXB7 is expressed at high levels, and overexpression of HOXB7 in normal HOSE cells enhances cell proliferation (15). Other homeobox proteins such as MEIS, PBX, and PAX8 are also frequently upregulated.
in ovarian carcinomas (16, 17). Moreover, upregulation of DLX4 has been observed in high-grade ovarian cancers; overexpression of DLX4 promotes ovarian cancer cell proliferation and increases clonogenicity in vitro, and it induces vascular endothelial growth factor transcription and enhances tumor vascularization in vivo (18).

Our findings suggest that overexpression of DLX5 promotes the proliferation of ovarian cancer cells and that knockdown of DLX5 causes cell cycle arrest in vitro and diminished tumor size in xenografts of ovarian cancer cells in SCID mice. The arrest seems to occur in both G1 and G2-M. Moreover, downregulation of cyclin proteins was shown to underlie the reduced cell proliferation rate seen in tumor cells following knockdown of DLX5. Thus, the primary consequence of DLX5 knockdown may be the inhibition of DNA synthesis and S-phase entry, which may be related to the downregulation of cyclin A and cyclin E that we observed. At the same time, downregulation of cyclin D2 as well as cyclin B may cause the arrest of cells at G1 phase and G2-M phase, respectively.

We also found that overexpression of DLX5 increases HRAS-induced colony formation in HOSE cells. It has been reported that activated HRAS can transform SV40-imortalized HOSE in vitro (19). Our study implies that DLX5 is not only important in maintaining ovarian cancer cell proliferation, but it also participates in the transformation of SV40-imortalized HOSE cells by cooperating with activated HRAS, similar to the oncogenic cooperativity we observed between Dlx5 and activated Akt2 in Rat-1 fibroblasts (20).
The receptor kinases EGFR, HER2, MET, and IGFIR are often overexpressed in ovarian cancers and may contribute to tumor progression (21, 22). Inhibition of IGFIR function induces apoptosis in ovarian cancer cells (23–25). Its adaptor proteins IRS-1 and IRS-2 have also been shown to be oncogenic. IRS proteins have been shown to be overexpressed in hepatic and pancreatic cancers and to possess constitutive activity in breast cancer, myosarcoma, and multiple endocrine neoplasia, among others (26). Furthermore, transgenic mice overexpressing IRS-1 or IRS-2 develop breast cancer (27). Interestingly, IRS-2 has been implicated in positive feedback regulation of IGFR specifically through the mTOR pathway (28). The elevated IRS levels observed in certain cancers are related to activation of the AKT pathway, and dephosphorylation of IRS protein has been shown to inhibit AKT signaling (29–31). Similarly, inhibition of MET represses AKT signaling and abolishes tumor cell invasion (22). Importantly, our findings indicate that knockdown of DLX5 reduces the expression of IRS-2 and MET in ovarian cancer cells overexpressing DLX5, which in turn results in decreased AKT activity.

The basal activity of IRS-2 promoter is maintained by the transcription factor AP1 but is susceptible to certain oncogenic stimuli. In breast cancer cells, IRS-2 transcription can be augmented by EGFR signaling through the JNK-AP1 pathway (32). Moreover, in some prostate cancer cells, the steroid receptor coactivator-3 binds to the IRS-2 promoter and enhances IRS-2 transcription (33). Our studies have revealed that IRS-2 protein levels are upregulated in ovarian cancer cells overexpressing DLX5 and that DLX5 can bind to the IRS-2 promoter and augment its activity and downstream AKT signaling. Interestingly, the truncated DLX5 forms also retained partial activity on IRS-2 promoter, possibly because shorter mRNAs have a higher translation rate than the full-length mRNA and/or truncated DLX5 proteins are more stable. Identification of other potential targets of DLX5 participating in oncogenic signaling is worthy of investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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