KLF4 as a rheostat of osteolysis and osteogenesis in prostate tumors in the bone

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Abstract

We previously showed that KLF4, a gene highly expressed in murine prostate stem cells, blocks the progression of indolent intraepithelial prostatic lesions into aggressive and rapidly growing tumors. Here we show that the anti-tumorigenic effect of KLF4 extends to PC3 human prostate cancer cells growing in the bone. We compared KLF4 null cells to cells transduced with a DOX-inducible KLF4 expression system, and find KLF4 function inhibits PC3 growth in monolayer and soft agar cultures. Furthermore, KLF4 null cells proliferate rapidly, forming large, invasive and osteolytic tumors when injected into mouse femurs whereas KLF4 re-expression immediately after their intra-femoral inoculation blocks tumor development and preserves a normal bone.

Competing interests

The authors declare no competing interests.
architecture. KLF4 re-expression in established KLF4 null bone tumors inhibits their osteolytic effects, preventing bone fractures and inducing an osteogenic response with new bone formation. In addition to these profound biological changes, KLF4 also induces a transcriptional shift from an osteolytic program in KLF4 null cells to an osteogenic program. Importantly, bioinformatic analysis shows that genes regulated by KLF4 overlap significantly with those expressed in metastatic prostate cancer patients and in three individual cohorts with bone metastases, strengthening the clinical relevance of the findings in our xenograft model.

Keywords
KLF4; prostate cancer; bone; osteolysis; osteogenesis; transcriptional program

Introduction
Prostate cancer is the second most frequently diagnosed tumor in American men (1). Bone metastases are present in 80% of patients with metastases, and these patients are faced with an incurable disease (2). A healthy bone is a continuously remodeling organ, characterized by cycles of bone resorption and formation (3). Disruption of this balance by cancer cells leads to pain, impaired mobility, fractures, spinal cord compression and hypercalcemia, all causes of morbidity. The metastatic cells divert the bone environment in their favor creating a “vicious cycle” fueled by tumor-secreted factors (4). Although some of these factors have been identified (5–8), and life-prolonging therapies have been devised (9), many men still succumb to metastatic disease.

We recently identified transcriptional networks that regulate prostate cancer progression (10). We proposed that overexpression of genes controlling the quiescence of adult prostate stem cells maintains prostate cancer in an indolent state, while their loss triggers tumor progression. We showed that decreased expression of one such gene, the cell fate defining transcription factor KLF4 (11), converts tiny prostatic intraepithelial neoplastic lesions into large and aggressive sarcomatoid cancers in a mouse model. Our findings are consistent with previous data showing decreased KLF4 expression in advanced primary prostate cancers (12) and inhibition of prostate tumor growth after its overexpression (13). KLF4 can have context-dependent oncogenic and tumor suppressive activities (14–16), and it may function differently in primary and metastatic tumors. We therefore aimed to define the function of KLF4 in prostate cancer bone metastases. Because most metastases are detected after hormone ablation therapy, a phase coinciding with the development of androgen resistance, we focused our in vivo work on a hormone-independent cell line, PC3, originally isolated from a bone metastasis (17). The choice of PC3 was further supported by a recent report (18) which documented a reciprocal upregulation of androgen receptor (AR) and KLF4. Such an interaction might suggest a mechanism for loss of KLF4 in advanced prostate cancer in which AR or its normal function is lost. Here we use PC3 and LNCaP cells to show KLF4 inhibits growth in 2D and 3D cultures. Importantly, we demonstrate KLF4 loss in PC3 cells triggers an invasive and osteolytic phenotype in bone, and KLF4 re-expression restrains tumor growth and stimulates new bone formation. We also uncovered KLF4-regulated transcriptional programs evoking osteolytic and osteogenic responses in the bones.
of our mouse model and in bone metastases of prostate cancer patients, providing a road map for future mechanistic exploration of the KLF4 effect.

Results

KLF4 inhibits in vitro growth of PC3 and LNCaP cells

To explore the mechanism by which KLF4 exerts its effects on tumor cells, we genomically ablated KLF4 in PC3 cells using CRISPR/Cas9. The homozygote deletion disrupted all KLF4 splice variants and isoforms rendering KLF4 protein undetectable (Fig. 1a, top panel). KLF4 loss increased the anchorage-independent colony-forming ability of PC3 cells in soft agar (Fig. 1a, central and bottom panels). Treatment of null cells transduced with a Tet-ON KLF4 expression construct (KLF4-Tet) with increasing DOX concentrations increased KLF4 protein levels (Fig. 1b, top panel) while reducing, in a dose-dependent manner, their anchorage-dependent proliferation (Fig. 1b, bottom panel). Moreover, induction of KLF4 expression almost completely blocked PC3 growth in soft agar (Fig. 1c). Similar effects of KLF4 were observed in an androgen-sensitive cell line, LNCaP; an increase in KLF4 expression inhibited anchorage-dependent and independent growth (Supplementary Fig. S1).

KLF4 levels in PC3 cells regulate bone remodeling

Prostate cancer metastasizes predominantly to bone and lymph nodes (2). To study the role of KLF4 in bone tumors, we inoculated PC3 cells, expressing a constitutive GFP-luciferase transgene and different levels of KLF4, intra-femorally. KLF4 was induced in vivo by feeding the mice DOX-containing chow (1 g/kg) (Mice cohorts are described in Supplementary Table S1) and tumor growth was monitored periodically by bioluminescence imaging (BLI). At the experimental endpoint, mice were sacrificed and femurs analyzed by micro-CT and histology. Four weeks after cell inoculation and DOX induction, the majority of femurs injected with null cells had tumors (11/14), while no visible tumors were observed in femurs bearing KLF4-Tet cells (0/14) or in the PBS controls (0/4) (Fig. 2a, b). All mice cohorts were fed DOX-containing chow starting on the day of cell inoculation and continuing for the duration of the experiment (4 weeks).

X-ray radiography revealed fractures, the most extreme signs of bone damage, in two femurs injected with null cells (Supplementary Fig. S2b, c), necessitating the termination of the experiment. No fractures were found in other mice cohorts. Most of the femurs injected with null cells that were analyzed by micro-CT (7/10) showed signs of bone resorption with one or multiple lytic lesions in cortical bone and partial loss of trabecular bone (Fig. 3c, Supplementary Fig. S2h, k, l). These femurs had a decreased cortical area fraction and cortical thickness compared to femurs bearing KLF4-Tet cells fed DOX-containing chow from day 0 (Fig. 2c, d). Histological examination of tumors arising from null cells revealed areas of tumor growth with significant accumulation of osteoclasts (Fig. 3h); in femurs where tumor cells almost completely filled the bone marrow cavity, cortical and trabecular bone were damaged, and tumor cells invaded the bony collar (Fig. 3h, m). This was not observed in KLF4-expressing tumors (Fig. 3g, l).
In contrast, all femurs bearing KLF4-Tet cells, fed DOX-containing chow and analyzed by micro-CT had normal morphology and no bone resorption (0/8) (Fig. 3d), indicating that early induction of KLF4 prevents tumor growth and protects the bone from destruction. Cortical area fraction and cortical thickness appeared similar to those of femurs injected with wild-type cells (Fig. 2c, d), and their morphology (Fig. 3i, n) was indistinguishable from the PBS controls (Fig. 3a, f, k). Femurs injected with wild-type cells showed osteolytic lesions in only 2/11 femurs (Fig. 3b) and histological analysis confirmed the presence of small, localized tumors (Fig. 3g, l). To ascertain that the observed phenotype is due to KLF4 levels rather than tumor size, we compared a wild-type and a null-generated tumor of similar size; like the two cohorts, the null cell tumor invaded the cortical bone forming lytic lesions, while its equal size wild-type cell counterpart formed non-invasive tumors and only partially remodeled the bone (Supplementary Fig. S2e–g vs. h–j).

To mimic the clinical state of advanced bone metastasis, we allowed the bone tumors to grow before we induced KLF4. Twelve femurs were inoculated with KLF4-Tet cells and maintained on a regular diet for three weeks at which time (day 20) tumors were detectable in 7/12 femurs (Supplementary Fig. S2d); all mice cohorts were switched to DOX-containing chow and sacrificed two weeks later. Micro-CT analysis indicated a significant osteogenic response in 4/7 femurs in which KLF4 was re-expressed (Fig. 3e, Supplementary Fig. S2m, n). Only small and sparse areas of tumor cells were identified and these areas were surrounded by new bone (Fig. 3j, o), suggesting that KLF4 re-expression reduced tumor size and reversed the damage of advanced bone metastasis.

**KLF4-dependent transcriptional programs control osteolytic and osteogenic responses**

As most bone metastases go through an initial lytic phase (4), we chose bone metastasis-derived PC3 cells (17) which have an osteolytic phenotype (19) to gain insight into this phase of their growth. We analyzed their transcriptome by RNA-sequencing (RNA-seq) and identified 1542 differentially expressed genes between null and wild-type cells (adjusted P value < 0.05) (Fig. 4a). Because of the profound effects of KLF4 on established tumors, we also examined the transcriptome of null cells after KLF4 reexpression (10 ng/ml DOX) and identified 3959 differentially expressed genes between KLF4 re-expressing cells and null cells (adjusted P value < 0.05) (Fig. 4a). Most of these genes (2599) were upregulated in KLF4 re-expressing cells compared to null cells (Fig. 5b). KLF4 levels in KLF4 re-expressing cells were 20-fold higher than in PC3 wild-type cells, falling within the higher and lower range of KLF4 expression in primary prostate tumors (Supplementary Fig. S2a) with good and bad prognosis respectively (10).

In line with the observed phenotypes, Gene Ontology (GO) analysis uncovered pathways related to extracellular matrix (ECM) organization and disassembly, angiogenesis, proliferation, adhesion and migration (Fig. 4b, c). The expression of several membrane-bound proteins and proteins secreted in the ECM such as matrix metalloproteinases (MMPs) and Tenascin-C (TNC) were identified. MMP9, MMP14, MMP16, TNC and Formin Like 3 (FMNL3) expression was higher in null compared to wild-type cells (Fig. 5a). Diminished expression of MMP16 and TNC as well as increased expression of MMP16 inhibitors, TIMP1 and TIMP2, were observed in KLF4 re-expressing cells (Fig. 5b).
Genes associated with epithelial-mesenchymal transition (EMT) such as TWIST1, TCF4, CDH2, VIM and MMP9 were upregulated in null compared to wild-type cells (Fig. 5a), while the expression of the epithelial genes, ELF3 and RGS2, was decreased in null compared to wild-type cells (Fig. 5a). We conclude that although null cells retain some aspects of their epithelial identity, the increased expression of mesenchymal markers indicates they have undergone partial EMT. KLF4 re-expressing cells reverted to a more epithelial phenotype, as judged by their expression of cytokeratins (KRT6C, 16 and 17) not normally detected in PC3 cells. We also found increased expression of the epithelial genes RGS2, DSP, KRT6B and KRT8 and decreased expression of the mesenchymal markers TCF4 and CDH2 in KLF4 re-expressing cells compared to null cells (Fig. 5b). The decreased expression of VIM and MMP9 in KLF4 re-expressing cells, although not statistically significant, confirms the trend towards an epithelial phenotype. This data suggests that KLF4 re-expression in KLF4 deficient cells induces a mesenchymal-epithelial transition.

Remarkably, in support of our in vivo data, we identified numerous bone-related pathways, such as positive regulation of bone mineralization and osteoblast differentiation, skeletal system development, negative regulation of bone resorption, ossification and osteoblast development (Fig. 4b, c). The expression of several genes known to be important for osteolysis, (IL1B, IL6, MMP9, CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6) (20), was significantly higher in null compared to wild-type cells (Fig. 5a). Increased expression of these KLF4-dependent gene sets may account for the aggressive and osteolytic phenotype of null cells in bone. Additionally, KLF4 re-expressing cells had detectable WNT1, WNT3A, WNT11, BMP2, BMP6, NPPC, PRELP, IGF2 and PHOSPHO1 (not expressed in wild-type and null cells), and high expression of EDN1, PDGFA, BMP1 and COL1A1 (higher than in null cells) (Fig. 5b).

Gene Set Enrichment Analysis (GSEA) revealed that KLF4 ablation causes a global upregulation of genes associated with TNFa signaling (Fig. 6a). Conversely, KLF4 re-expression represses these genes (Fig. 6b). Thus, KLF4, or the transcriptional program it controls, represses TNFa signaling in prostate cancer cells. Because TNFa induces osteoclastogenesis (21), its repression by KLF4 may explain the osteogenic phenotype of KLF4 re-expressing cells in vivo. Moreover, GSEA confirmed the positive association between reduced KLF4 activity and EMT (Fig. 6c) and it highlighted its involvement in inflammatory responses (Fig. 6d). Taken together we identified KLF4-dependent transcriptional programs controlling either osteolytic or osteogenic responses in the bone.

**KLF4 and its targets have clinical significance in human metastatic prostate cancer**

To assess the significance of our xenograft data, we first interrogated the Taylor dataset (22) and found an inverse correlation between KLF4 levels and the metastatic potential of human prostate cancer (P < 0.0001, Fig. 6e), as previously noted (13). Next, we intersected the KLF4-regulated gene sets we identified in PC3 cells (Fig. 5) with gene sets which are deregulated in metastases of prostate cancer patients (23). We identified 84 transcripts common to 1360 genes significantly upregulated in null cells and to 943 genes upregulated in metastatic prostate cancer (P < 0.0001; Fig. 6f, Supplementary Table S2). We also
uncovered 164 genes common to 2599 genes significantly downregulated in null cells and to 1392 genes downregulated in metastatic prostate cancer patient specimens (P < 0.0001; Fig. 6g, Supplementary Table S2).

To establish clinical relevance for the PC3 findings, we examined whether the KLF4-regulated genes linked to osteolysis and osteogenesis are reflected in bone metastases of prostate cancer patients. Interrogation of three independent cohorts (24–26) showed a significant overlap between the 783 upregulated genes in bone metastases from the Kumar and Quigley datasets and the 691 upregulated genes in null cells (45 genes, P = 1.2E–03, Fig. 7a, Supplementary Table S2) as well as a significant overlap between the Kumar and Quigley datasets and the 2599 upregulated genes in null cells re-expressing KLF4 (135 genes, P = 1E–05, Fig. 7b, Supplementary Table S2). GO analysis on the common gene sets uncovered bone-related pathways, such as osteoblast differentiation and endodermal cell differentiation (Fig. 7c, d). A panel of 10 such genes (MMP9, MMP14, VIM, TWIST1, TNC, FMLN3, BMP1, BMP2, PHOSPHO1 and COL1A1) that were found elevated in the Kumar, Quigley and Robinson (24–26) cohorts underscores the commonality between KLF4-regulated genes in PC3 cells and bone metastases in prostate cancer patients (Fig. 7c–g), justifying the relevance of our PC3 xenograft model to improve the molecular and physiological understanding of bone metastasis in the future.

Discussion

Bone metastases present a significant clinical problem in prostate cancer patients as they cause severe bone damage, extensive pain, and poor survival (2). Still, how metastatic prostate cancer cells remodel the bone is poorly understood and therapies prolonging patient survival are urgently needed. Here we used PC3 cells, originally isolated from bone metastases of a prostate cancer patient (17), to investigate how KLF4 activity influences their transcriptome, behavior and pathology. We showed that KLF4 loss is responsible for their aggressive growth and osteolytic phenotype in vivo. Conversely, KLF4 re-expression in null cells, either immediately after bone inoculation or in already established tumors, was able to block tumor growth and protect the bone from resorption.

Our data suggest that KLF4 functions as a tumor suppressor in PC3 and LNCaP cells (Fig. 1, Supplementary Fig. S1), consistent with reports in prostate (13,27) and other cancers (28–31), and we uncover a critical function in bone metastasis that has not previously been reported. Most osteoblastic bone metastases in prostate cancer transit through an initial osteolytic period (4). However, with few exceptions (32–34), models of mixed bone lesions are still uncommon. That PC3 cells can induce osteolytic lesions in bone has been described (19), but the mechanisms by which this occurs remain elusive. We discovered that KLF4 controls this process by preventing the growth of aggressive bone tumors and by modifying pathways in established tumors that favor osteogenic responses (Fig. 3, Supplementary Fig. S2). This finding has potential therapeutic implications. Indeed, KLF4 has been shown to upregulate the transcription of osteogenic genes in murine smooth muscle cells (35), but inhibition of osteogenesis by KLF4 has also been described (36), suggesting that like the KLF4 context-dependent function in cancer (14), its role in osteogenesis might also require analysis in each specific tissue.

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To better understand tumor-related osteolysis and osteogenesis and its dependence on KLF4 levels, we analyzed the transcriptome of PC3 cells. GO analysis uncovered pathways related to bone biology, providing a molecular underpinning to the observed KLF4-dependent in vivo phenotype. Null cells express genes, such as MMPs, compatible with their aggressive, mesenchymal-like and osteolytic phenotype. Although EMT induction by KLF4 downregulation has been described in other cells (10,12), ours is the first report suggesting a direct link between KLF4-regulated transcription and the KLF4-dependent osteolytic activity of PC3 cells in bone.

Osteoclastogenesis and bone resorption are regulated by RANK, its ligand RANKL, OPG, cytokines and growth factors (37,38). RANK mediates cell migration to the bone and promotes the expression of metastatic genes (20). Null cells express higher levels of RANK and OPG than wild-type (not shown). While RANK increase is in line with the strongest osteoclastic activity of null cell-generated tumors, the concomitant OPG increase was unexpected, but possibly counterbalanced by the increased expression of ILs and CXCLs.

In agreement with our results indicating that KLF4 expression induced an osteoblastic phenotype, our RNA-seq analysis identified several genes (WNTs, BMPs, EDN1, PDGFA, NPPC, PRELP, IGF2 and PHOSPHO1) (39–44) that can explain the osteoblastic response. Wnt signaling promotes osteoblast differentiation, contributing to the formation of osteoblastic lesions (39), and this pathway is activated in metastatic castration-resistant prostate cancer (26). The fact that KLF4 reduces tumor burden and increases the level of osteoblast-promoting genes and new bone formation suggests a new approach to alleviation of bone metastasis and morbidity. However, before this approach can be tested clinically, further study of the quality of the newly deposited bone has to be undertaken to assure it is physiologically normal.

Another important and novel role for KLF4 we identified is the repression of TNFα signaling (Fig. 6). Although the function of TNFα in bone homeostasis is still debated (45), it is currently believed that TNFα induces osteoclastogenesis (21) and inhibits osteoblastogenesis (46). Thus, the KLF4-dependent repression of TNFα is likely to account for the osteogenic behavior of KLF4 re-expressing cells in vivo. The significant overlap between KLF4-regulated genes and metastatic genes (Fig. 6), together with the finding that expression of a number of these genes (MMP9, MMP14, VIM, TWIST1, TNC, FMNL3, BMP1, BMP2, PHOSPHO1 and COL1A1) specifically increases in bone metastases of patients in three independent cohorts (23–26) (Fig. 7), provides clinical significance to our xenograft model and data.

Using PC3 cells as a model of hormone-independent prostate cancer, we identified novel and important pathways, such as Wnt and TNFα, through which KLF4 switches tumor cell programs in favor of osteogenesis. We also uncovered a clinically-relevant network of KLF4-regulated genes that might serve as a basis for the development of new therapeutic targets for advanced prostate cancer. These findings provide a significant platform for future testing of specific mechanisms that govern the profound KLF4-regulated changes.
Material and Methods

Cell culture

PC3 and LNCaP cells (ATCC, Manassas, VA, USA) were cultured in RPMI with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mmol/l glutamine (Corning Cellgro, VA, USA), and maintained in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. Cells were authenticated by STR profiling and tested for mycoplasma. Two guide RNAs specific to human KLF4 (5’-CCCCGAATAACGTGAGTATCGCT-3’ and 5’-CCCACACTTGTGATTACGCGGGC-3’) were cloned into the pSpCas9(BB)-2A-GFP (PX458) vector (47) and transiently transfected into PC3 cells using Fugene6 (Promega, WI, USA). GFP positive cells were sorted and subcloned. KLF4 wild-type and null clones were sequenced and verified by Western blot. GFP loss indicated that neither Cas9 nor the sgRNAs were integrated in their genome.

Lentiviral constructs and infection

To re-express KLF4, we used a DOX-inducible Tet-ON lentiviral system. We modified the vector FUW-tetO-hKLF4 (48) by inserting the sequence of BFP-T2A-hKLF4 or BFP-T2A. To generate GFP-luciferase cells, we used the lentiviral construct BLIV513PA-1 (System Biosciences, CA, USA). Lentiviruses were produced in HEK293T cells as described (10). BFP-T2A-hKLF4 or BFP-T2A expression was induced by DOX (Sigma-Aldrich, MO, USA).

Western blot

Western blot was performed as described (10). Anti-KLF4 primary antibody (sc-20691; dilution 1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) was used with GAPDH (5174; dilution 1:10000; Cell Signaling Technologies, Danvers, MA, USA) or ACTIN (A5441; dilution 1:10000; Sigma-Aldrich) as a loading control. Membranes were incubated with rabbit or mouse HRP-conjugated secondary antibodies (HAF008 and HAF007; dilutions range 1:5000–1:20000; R&D Systems, Minneapolis, MN, USA).

Cell proliferation

PC3 null and LNCaP cells (2 × 10\textsuperscript{3} cells/well) were seeded in 96-well plates. Cells expressing the BFP-T2A-hKLF4 construct (KLF4-Tet) as well as the control vector BFP-T2A were incubated with or without DOX up to 4 (LNCaP) or 5 (PC3) days. DOX (1, 10 or 100 ng/ml) was added on day 0 and replaced at 48-hour intervals. Cell proliferation was evaluated using the MTS kit (PC3) or the CellTiter kit (LNCaP) (Promega). Experiments were repeated twice. Data are expressed as the mean of at least 3 technical replicates ± SD. No data were excluded.

Growth in soft agar

PC3 (2.5 × 10\textsuperscript{3} cells/well) and LNCaP (4 × 10\textsuperscript{3} cells/well) cells were mixed with 0.6% agarose in a 1:1 ratio and seeded into 24-well plates pre-coated with 0.5% agarose (49). KLF4-Tet and control cells were incubated with or without DOX up to 13 (PC3) or 23 (LNCaP) days. DOX (10 or 100 ng/ml for LNCaP or PC3, respectively) was added on day 0.
and replaced at 48-hour intervals. Colonies were imaged using the Thermo Cellomics ArrayScan VTI (Thermo Fisher Scientific) and images processed with JMP Pro v13. Colonies with a diameter greater than 50 μm were scored. Experiments were repeated twice. Data are expressed as the mean of at least 3 technical replicates ± SD. No data were excluded.

Animal studies

Wild-type, null and KLF4-Tet cells (10^5 cells) as well as PBS were injected into both femurs of 6-weeks old male athymic nude mice (strain code: 490; Charles River, MA, USA; n=6, 12, 13, 3 mice, respectively). No statistical method was used to predetermine sample size, which was based on similar studies and was sufficient to determine significance. Mice were randomly assigned to experimental groups; no particular method of randomization was used; investigators were not blinded. Mice were fed normal or DOX chow (1 g/kg, Bio Serv, NJ, USA) ad libitum starting at day 0 or 20. Bone lesions and tumor growth were monitored by X-ray and BLI with IVIS Lumina-III-XR (PerkinElmer, MA, USA). Images were analyzed by Living Image Software and plotted as average radiance (p/sec/cm²/sr). At the experimental end point, 8 mice (4 with null cells, 2 with KLF4-Tet cells + hKLF4 d0 and 2 with KLF4-Tet cells + hKLF4 d20) were used for other purposes. The remaining femurs were fixed in 4% PFA overnight at 4°C and scanned using a micro-CT scanner (SkyScan 1172, Bruker, Billerica, MA, USA). Images were reconstructed and analyzed in Dataviewer and Ctan. We quantified cortical area fraction (Bone Area/Total Area) and cortical thickness (50), excluding femurs with aberrant injections or bone fractures (Supplementary Table S1). All animal studies were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee at NYU School of Medicine.

Histological analysis

Fixed femurs were decalcified in 19% EDTA (pH 7.4) for 21 days, dehydrated in a graded ethanol series, embedded in paraffin and cut into 10-μm-thick sections. Pentachrome staining was used to detect bone (51). Tartrate-resistant acid phosphatase (TRAP) staining was performed with the Leukocyte Acid Phosphatase kit (Sigma-Aldrich). Pathological examination of histological sections was carried out by two independent investigators who were blind to experimental conditions.

RNA-sequencing and data processing

Differential gene expression between null and wild-type cells, and between cells reexpressing KLF4 and null cells was measured by RNA-seq. Uninfected null and wild-type cells as well as KLF4-Tet cells (null cells expressing the BFP-T2A-hKLF4 construct) and their control cells (null cells expressing the BFP-T2A construct) incubated with DOX (10 ng/ml) were harvested after 48 hours. Total RNA was extracted using the RNeasy kit (Qiagen, CA, USA). RNA samples were submitted in duplicate. RNA-seq libraries were prepared with the TruSeq sample preparation kit (Illumina, CA, USA). Sequencing reads were mapped to the human genome (GRCh37/hg19) using the STAR aligner (v2.5.0c) (52). Alignments were guided by a Gene Transfer Format file (Ensembl GTF version GRCh37.70). The mean read insert sizes and their standard deviations were calculated using Picard tools (v.1.126). The read count tables were generated using HTSeq (v0.6.0) (53),
normalized based on their library size factors using DESeq (v3.7) (54), and differential expression analysis was performed. The Read Per Million (RPM) normalized BigWig files were generated using BEDTools (v2.17.0) (55) and bedGraphToBigWig tool (v4). Statistical analyses were performed with R (v3.1.1), GO analysis with David Bioinformatics Resources 6.8 (56,57) and GSEA with the hallmarks (h.all.v6.1 symbols.gmt) gene sets of the Molecular Signature Database (MSigDB) v6.1 (58,59). KLF4 RNA expression levels in wild-type and KLF4 re-expressing cells were ascertained by RNA-seq and compared with a primary prostate adenocarcinoma cohort (TCGA, Provisional) of 499 patients on cBioPortal (60,61). Initial raw data analysis was blinded.

Analysis of KLF4 and its target genes in human prostate cancer cohorts

To test whether the KLF4-dependent genes that we identified are differentially expressed between primary and metastatic prostate cancers (23), we re-analyzed the Chandran dataset with GEO2R. Genes significantly upregulated and downregulated in KLF4 re-expressing cells compared to null cells were intersected with genes upregulated and downregulated in the dataset (23). Statistical significance was calculated with Fisher’s exact test. To determine the clinical relevance of KLF4 and its regulated genes in metastatic patients, we analyzed differences in gene expression between primary (n=109) and metastatic (n=19) prostate tumors (22), and between bone and lymph node metastases from three datasets: Kumar (n=20 vs. n=69) (24), Quigley (n=43 vs. n=35) (25) and Robinson (n=29 vs. n=50) (26). Genes significantly upregulated in bone compared to lymph node metastases from the Kumar and Quigley cohorts were intersected with genes upregulated in KLF4 null cells and KLF4 re-expressing cells. Monte Carlo simulations with 100,000 iterations indicate that the overlap is statistically significant. David Bioinformatics Resources revealed significantly enriched GO categories. Gene expression levels are illustrated as Box plots (Tukey), and statistical significance was determined by Wilcoxon non-parametric t-test (Taylor), GEO2R (Kumar), DEseq2 (Quigley) and Mann-Whitney non-parametric t-tests (Robinson).

Data deposition

Sequencing data are available under the accession number GSE117965.

Statistical analysis

Statistical differences were measured using the 2-tailed unpaired Student’s t-test, unless otherwise specified. All tests were done in GraphPad Prism7 or R. A P value of < 0.05 is considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
KLF4 decreases PC3 cell growth. 

**a** Western blot showing KLF4 absence in null cells (top panel); KLF4 ablation increases 3D growth in soft agar (center and bottom panels). **b** Increasing DOX concentrations in KLF4-Tet cells increases KLF4 protein levels (top panel) and inhibits 2D proliferation (bottom panel) and **c** 3D growth in soft agar. 

Representative fields and quantification of colonies grown in soft agar are shown. Experiments were repeated twice. Data represent the mean of technical replicates ± SD. Scale bar = 200 μm.
Fig. 2.
KLF4 re-expression in PC3 null cells prevents tumor growth in bone. a BLI four weeks after intra-femoral injection of null cells (n=14), KLF4-Tet cells (n=14) or PBS (n=4). To induce KLF4, mice were fed DOX-containing chow starting on day 0 (d0). Two representative mice per group are shown. b Quantitative analysis of luciferase signal as a measure of tumor growth. Femurs were isolated and analyzed by micro-CT. Two cortical bone parameters were examined: c Cortical area fraction and d cortical thickness. Data represent the mean of biological replicates (femurs) ± SD. Femurs were inoculated with: PBS (n=5), *KLF4*+/+ cells (n=11), *KLF4*−/− cells (n=10), KLF4-Tet cells induced at day 0 (n=8) and day 20 (n=7).
Fig. 3.
KLF4 determines osteolytic and osteogenic phenotypes. Micro-CT images of femurs inoculated with a PBS, b KLF4+/+ cells, c KLF4−/− cells, d KLF4-Tet cells induced at day 0 and e day 20. Small boxed images depict axial sections. L, lytic lesion; NB, new bone; yellow dashed lines, lytic lesions; black dashed line, new bone. f-j TRAP and k-o pentachrome staining of invasive tumor (IT) and tumor (T), with overt g, h bone destruction and o new bone (NB) formation. B, cortical bone; BM, bone marrow; black dashed lines, tumor areas; black arrows, osteoclasts. Scale bar = 200 μm.
Fig. 4.
RNA-seq reveals bone-related pathways. a Venn Diagram of differentially expressed genes between PC3 null and wild-type cells, and between cells re-expressing KLF4 (by 10 ng/ml DOX) and null cells. b, c GO analysis indicates KLF4-regulated pathways. Green bars, bone-related pathways. Adjusted P value < 0.05.
Fig. 5.
RNA-seq defines KLF4-dependent transcriptional programs. Volcano plot showing distribution of differentially expressed genes between a PC3 null and wild-type cells, and between b cells re-expressing KLF4 (by 10 ng/ml DOX) and null cells. Adjusted P value < 0.05.
Fig. 6.
KLF4 regulates genes and pathways and inversely correlates with the metastatic potential of human prostate cancer. GSEA shows a positive enrichment of the TNFα pathway after KLF4 ablation and b a negative enrichment with KLF4 re-expression. GSEA also reveals a positive enrichment of c EMT and d inflammatory response after KLF4 ablation. e Box plot illustrating KLF4 mRNA expression in primary and metastatic prostate cancer specimens (22). Venn diagrams showing significant overlap between f upregulated genes in PC3 null cells and Chandran’s (23) dataset (84 genes; P < 0.0001) and between g downregulated genes in null cells and Chandran’s dataset (164 genes; P < 0.0001).
Fig. 7. KLF4-regulated genes are overexpressed in patients with bone metastasis. Venn diagrams showing significant overlap between upregulated genes in Kumar (24) and Quigley (25) bone metastases and upregulated genes in a PC3 null cells (45 genes; P = 1.2E-03) and b cells re-expressing KLF4 (10 ng/ml DOX; 135 genes; P = 1E-05). c, d GO analyses indicate KLF4-regulated pathways. Green bars, bone-related pathways. e-g Box plots illustrating gene expression data of candidate genes in bone and lymph node metastases of three independent datasets (24–26).