Title: CANCER BIOMARKERS AND USES THEREFOR

Abstract: Disclosed are methods and compositions for diagnosis, monitoring, treatment and management of cancer. More particularly, the present invention discloses the use of PITX2 as a diagnostic or prognostic marker of cancer including skeletal metastasis-associated, hormone-related cancers such as prostate cancer and breast cancer. The invention has practical use in diagnosing the presence, stage, degree or risk of development of cancer, in prognosing cancer, in monitoring clinically affected subjects, and in enabling better treatment and management decisions to be made in clinically and sub-clinically affected subjects.
TITLE OF THE INVENTION
"CANCER BIOMARKERS AND USES THEREFOR"

FIELD OF THE INVENTION

[0001] This invention relates generally to methods and compositions for diagnosis, monitoring, treatment and management of cancer. More particularly, the present invention relates to the use of PITX2 as a diagnostic or prognostic marker of cancer including skeletal metastasis-associated, hormone-related cancers such as prostate cancer and breast cancer. The invention has practical use in diagnosing the presence, stage, degree or risk of development of cancer, in prognosing cancer, in monitoring clinically affected subjects, and in enabling better treatment and management decisions to be made in clinically and sub-clinically affected subjects.

BACKGROUND OF THE INVENTION

[0002] Prostate cancer remains one of the most important male cancers in the Western World due to its prevalence as the most common lethal malignancy in men and the significant morbidity associated with its metastasis primarily to bone. While surgical and hormonal treatments are often effective for localized prostate cancer, advanced disease remains essentially incurable. Androgen ablation is the most common therapy for advanced prostate cancer, leading to massive apoptosis of androgen-dependent malignant cells and temporary tumor regression. In most cases, however, the tumor reemerges more aggressively and can proliferate in spite of androgen deprivation therapy.

[0003] Prostate cancer is currently detected by screening for prostate specific antigen (PSA) and a digital rectal exam (DRE). However, neither methodology can differentiate between benign disease and cancer. In addition, it is estimated that PSA testing misses 20%-30% of all individuals with cancer.

[0004] When PSA or digital tests indicate a strong likelihood that cancer is present, a transrectal ultrasound (TRUS) is used to map the prostate and show any suspicious areas. Biopsies of various sectors of the prostate are used to determine if prostate cancer is present. However, current imaging, pathologic grading systems and biomarkers are insufficient to provide prognostic information about the likelihood of disease behavior and to differentiate the aggressive, highly metastatic life threatening...
tumors, from the indolent, localised slow growing variants. The failure to distinguish between these disease states can result, for example, in exposure of patients with benign disease to treatments that are unnecessary and have side effects (e.g., impotency).

**SUMMARY OF THE INVENTION**

[0005] The present invention is predicated in part on the determination that PITX2 is overexpressed in primary (i.e., local) prostate cancer and even more so in secondary (i.e., metastatic) prostate cancer. In particular, it was discovered that expression $\alpha$PITX2 increases with increasing grade of disease, as assessed by Gleeson Score. It was also discovered that PITX2B-encoding transcripts were expressed in higher amounts than transcripts encoding PITX2A and C isoforms in PC3 derived mouse tumors as well as in clinical samples, with the levels in clinical samples rising incrementally from normal prostate tissue to primary cancer, to metastasis. The A- and C-encoding transcripts were detected in the malignant tissue samples at lower levels but also in an incrementally increasing pattern with the C transcript more highly expressed than the A species. Of interest, the PITX2A-encoding transcript was not detected in the normal prostate or in benign prostatic hypertrophy (BPH), whereas the C-encoding transcript was present at a low level. Significantly, it was also found that knocking down PITX2 expression in PC3 cells significantly reduced their proliferation rate as well as their cellular motility towards bone. These findings identify PITX2 as both a therapeutic target in the treatment or prevention of prostate cancer metastases and a biomarker to identify the aggressive, highly metastatic subgroup of prostate cancer patients from those with indolent "chronic" disease. These discoveries have been reduced to practice in the form of diagnostic, prognostic and treatment compositions and methods with application not only to prostate cancer but to other skeletal metastasis-associated, hormone-related cancers with similar pathophysologies such as breast cancer.

[0006] The present invention represents, therefore, a significant advance over current technologies for the management of cancer-affected subjects, especially those with skeletal metastasis-associated, hormone-related cancers. Certain embodiments relate to the identification and use of PITX2 gene expression profiles for diagnosing the presence or risk of development of cancer and for correlating cancer progression. Accordingly, these gene expression profiles may be applied to the diagnosis and/or prognosis of cancers, especially skeletal metastasis-associated, hormone-related cancers, and are particularly useful in predicting the progression to aggressive and/or metastatic
cancer in patients diagnosed with localized cancers. Compared to clinical parameters or biochemical markers used in existing prognosis methods, the PITX2 expression profiles disclosed herein constitute a useful signature of cancer progression and provide a non-subjective basis for selecting appropriate therapeutic regimens. The invention also relates to the screening of drugs that target PITX2 or its protein products, in particular for the development of therapeutics aimed at modulating cancer progression.

[0007] Accordingly, in one aspect, the present invention provides methods for diagnosing the presence or risk of development of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer and breast cancer) in a subject. These methods generally comprise detecting in the subject overexpression of PITX2, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer. In specific embodiments, the skeletal metastasis-associated, hormone-related cancer is selected from prostate cancer and breast cancer. Generally, the subject is one that is suspected of having cancer or at risk of having cancer.

[0008] In some embodiments, the methods comprise detecting overexpression of a PITX2 polynucleotide selected from the group consisting of PITX2A, PITX2B and PITX2C. Non-limiting examples of PITX2 polynucleotides can be selected from the group consisting of: (a) a polynucleotide comprising a nucleotide sequence that shares at least 80% (and at least 81% to at least 99% and all integer percentages in between) sequence identity with the sequence set forth in any one of SEQ ID NO: 1, 3, 5 or 7, or a complement thereof; (b) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence set forth in any one of SEQ ID NO: 2, 4, 6 or 8; (c) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide that shares at least 80% (and at least 81% to at least 99% and all integer percentages in between) sequence similarity with at least a portion of the sequence set forth in SEQ ID NO: 2, 4, 6 or 8, wherein the portion comprises at least 15 contiguous amino acid residues of that sequence; and (d) a polynucleotide comprising a nucleotide sequence that hybridizes to the sequence of (a), (b), (c) or a complement thereof, under at least medium or high stringency conditions. In illustrative examples of this type, the methods comprise detecting overexpression of at least 1, 2 or all PITX2 polynucleotide(s) selected from the group consisting of (a) a polynucleotide comprising a nucleotide sequence that shares at least 80% (and at least 81% to at least 99% and all integer percentages in between) sequence identity with the sequence set forth in any one
of SEQ ID NO: 1, 3, 5 or 7, or a complement thereof; (b) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence set forth in any one of SEQ ID NO: 2, 4, 6 or 8; (c) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide that shares at least 80% (and at least 81% to at least 99% and all integer percentages in between) sequence similarity with at least a portion of the sequence set forth in SEQ ID NO: 2, 4, 6 or 8, wherein the portion comprises at least 15 contiguous amino acid residues of that sequence; and (d) a polynucleotide comprising a nucleotide sequence that hybridizes to the sequence of (a), (b), (c) or a complement thereof, under at least medium or high stringency conditions.

[0009] In specific examples of this type, the methods comprise detecting overexpression of a PITX2A polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

[0010] In other specific examples, the methods comprise detecting overexpression of a PITX2B polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

[0011] In still other specific examples, the methods comprise detecting overexpression of a PITX2C polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

[0012] Typically, such overexpression is detected by: (1) providing a biological sample from the subject; (2) measuring in the biological sample the level or functional activity of at least one PITX2 expression product; and (3) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample obtained from one or more normal subjects or from one or more subjects lacking a skeletal metastasis-associated, hormone-related cancer, wherein a higher level or functional activity of the or each expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer in the subject. In some embodiments, the methods further comprise diagnosing the presence, stage or degree of the skeletal metastasis-associated, hormone-related cancer in the subject when the measured level or functional activity of the or each expression product is higher than the measured level or functional
activity of the corresponding expression product. In these embodiments, the higher expression typically represents an at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, or even an at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% increase in the level or functional activity of the expression product as compared to the level or functional activity of an individual corresponding expression product, which is hereafter referred to as "aberrant expression." In some embodiments in which the skeletal metastasis-associated, hormone-related cancer is prostate cancer, the biological sample largely comprises (i.e., at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) prostate cells from the peripheral zone of the prostate. In illustrative examples of this type, the biological sample largely lacks or does not comprise (i.e., no more than 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or 0%) prostate cells from the transition zone of the prostate.

[0013] In some embodiments, the methods further comprise diagnosing the absence of the skeletal metastasis-associated, hormone-related cancer when the measured level or functional activity of the or each expression product is the same as or similar to the measured level or functional activity of the corresponding expression product. In these embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%, which is hereafter referred to as "normal expression." In some embodiments in which the skeletal metastasis-associated, hormone-related cancer is prostate cancer, the biological sample largely comprises (i.e., at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) prostate cells from the peripheral zone of the prostate. In illustrative examples of this type, the biological sample largely lacks or does not comprise (i.e., no more than 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or 0%) prostate cells from the transition zone of the prostate.

[0014] Suitably, the biological sample comprises cells or cell lines, histological slides, biopsies, paraffin-embedded tissue, bodily fluids, ejaculate, urine, blood, sputum, bone, stool, tissue, prostate tissue, ovarian tissue, endometrial tissue, colon tissue, lung tissue, bladder tissue, testicular tissue and thyroid tissue. In specific embodiments, the biological sample comprises mammary cells or prostate cells, illustrative examples of which include mammary cells, ovarian cells, endometrial cells
or prostate cells, illustrative examples of which include mastectomy specimens, isolated breast cells, ovariectomy specimens, isolated ovary cells, endometrectomy specimen, menstrual fluid, breast milk, isolated endometrium cells, core needle biopsies, sentinel lymph node biopsies, bone marrow biopsies, bone marrow aspirations, prostatic excretions, semen, isolated prostate cells, prostatectomy specimens and TRUS biopsies. Suitably, the expression product is selected from a RNA molecule or a polypeptide. In some embodiments, the expression product is the same as the corresponding expression product. In other embodiments, the expression product is a variant (e.g., an allelic variant) of the corresponding expression product.

[0015] In certain embodiments, the expression product or corresponding expression product is a target RNA (e.g., mRNA) or a DNA copy of the target RNA whose level is measured using at least one nucleic acid probe that hybridizes under at least medium or high stringency conditions to the target RNA or to the DNA copy, wherein the nucleic acid probe comprises at least 15 contiguous nucleotides of a PITX2 polynucleotide. In these embodiments, the measured level or abundance of the target RNA or its DNA copy is normalized to the level or abundance of a reference RNA or a DNA copy of the reference RNA that is present in the same sample. Suitably, the nucleic acid probe is immobilized on a solid or semi-solid support. In illustrative examples of this type, the nucleic acid probe forms part of a spatial array of nucleic acid probes. In some embodiments, the level of nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by hybridization (e.g., using a nucleic acid array). In other embodiments, the level of nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by nucleic acid amplification (e.g., using a polymerase chain reaction (PCR)). In still other embodiments, the level of nucleic acid probe that is bound to the target RNA or to the DNA copy is measured by nuclease protection assay.

[0016] In other embodiments, the expression product or corresponding expression product is a PITX2 polypeptide (e.g., PITX2A, PITX2B or PITX2C) whose level is measured using at least one antigen-binding molecule that is immuno-interactive with the PITX2 polypeptide. In these embodiments, the measured level of the PITX2 polypeptide is normalized to the level of a reference PITX2 polypeptide that is present in the same sample. Suitably, the antigen-binding molecule is immobilized on a solid or semi-solid support. In illustrative examples of this type, the antigen-binding molecule
forms part of a spatial array of antigen-binding molecule. In some embodiments, the level of antigen-binding molecule that is bound to the target polypeptide is measured by immunoassay (e.g., using an ELISA or RIA).

[0017] In a related aspect, the present invention provides methods for providing a prognosis to a subject with a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer and breast cancer). These methods generally comprise detecting overexpression of PITX2 in the subject, as broadly described above, wherein the overexpression is characterized by higher expression of PITX2 in the subject than the expression of PITX2 in a subject having primary skeletal metastasis-associated, hormone-related cancer and wherein the overexpression is indicative of a negative prognosis. In some embodiments, this overexpression is detected by: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a primary skeletal metastasis-associated, hormone-related cancer (e.g., a primary prostate cancer and primary breast cancer) or from a healthy tissue sample or body fluid (e.g., from the subject or from another subject), wherein a higher level or functional activity of the or each expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the negative prognosis. In these embodiments, the higher level or functional activity typically represents an at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, or even an at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% increase in the level or functional activity of the expression product as compared to the level or functional activity of an individual corresponding expression product. In some embodiments in which the skeletal metastasis-associated, hormone-related cancer is prostate cancer, the biological sample largely comprises (i.e., at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) prostate cells from the peripheral zone of the prostate. In illustrative examples of this type, the biological sample largely lacks or does not comprise (i.e., no more than 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or 0%) prostate cells from the transition zone of the prostate.
In specific embodiments of the diagnostic / prognostic applications of the present invention, the methods comprise detecting overexpression of a PITX2A polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer or a negative prognosis. In related embodiments, the methods comprise detecting normal expression of a PITX2A polynucleotide, which indicates the absence of the skeletal metastasis-associated, hormone-related cancer or a positive prognosis.

In other embodiments, the overexpression is detected by: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a secondary skeletal metastasis associated, hormone-related cancer (e.g., a secondary prostate cancer or secondary breast cancer), wherein negative prognosis is determined when the level or functional activity of the or each expression product in the biological sample is the same as or similar to the level or functional activity of the corresponding expression product in the reference sample. In these embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%. In specific embodiments, the prognosis is determined with respect to any one or more of overall patient survival, disease- or relapse-free survival, tumor-related complications and rate of progression of tumor. In some embodiments in which the skeletal metastasis-associated, hormone-related cancer is prostate cancer, the biological sample largely comprises (i.e., at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) prostate cells from the peripheral zone of the prostate. In illustrative examples of this type, the biological sample largely lacks or does not comprise (i.e., no more than 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or 0%) prostate cells from the transition zone of the prostate.

In some embodiments, the biological sample is from a subject following surgical removal of a primary skeletal metastasis associated, hormone-related cancer-affected organ or tissue (e.g., mastectomy, prostatectomy etc) and PITX2
overexpression identifies recurrence of the skeletal metastasis associated, hormone-related cancer in the subject following the surgical removal.

[0021] In another related aspect, the present invention provides methods for diagnosing the presence of a primary skeletal metastasis associated, hormone-related cancer (e.g., primary prostate cancer or primary breast cancer). These methods generally comprise: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a primary skeletal metastasis associated, hormone-related cancer, wherein a positive diagnosis of the primary skeletal metastasis associated, hormone-related cancer is determined when the level or functional activity of the or each expression product in the biological sample is the same as or similar to the level or functional activity of the corresponding expression product in the reference sample. In some embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%. In some embodiments in which the skeletal metastasis-associated, hormone-related cancer is prostate cancer, the biological sample largely comprises (i.e., at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100%) prostate cells from the peripheral zone of the prostate. In illustrative examples of this type, the biological sample largely lacks or does not comprise (i.e., no more than 30%, 20%, 10%, 5%, 4%, 3%, 2%, 1% or 0%) prostate cells from the transition zone of the prostate. In specific embodiments, the methods comprise detecting overexpression of a PITX2A polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer or a negative prognosis. In related embodiments, the methods comprise detecting normal expression of a PITX2A polynucleotide, which indicates the absence of the skeletal metastasis-associated, hormone-related cancer or a positive prognosis.

[0022] In another related aspect, the present invention provides methods for providing a prognosis of prostate cancer in a subject comprising: (1) detecting overexpression of PITX2 (e.g., at least 1, 2 or all of PITX2A, PITX2B and PITX2Q) in the subject; (2) detecting or determining at least one factor selected from the group
consisting of: the subject's pre-treatment PSA; the subject's post-treatment PSA; primary Gleason grade in a biopsy specimen obtained from the subject; secondary Gleason grade in a biopsy specimen obtained from the subject; Gleason sum in a biopsy specimen obtained from the subject; pre-radical primary therapy of the subject; total length of cancer in biopsy cores obtained from the subject; number of positive biopsy cores obtained from the subject; percent of tumor biopsy in a multiple core biopsy set obtained from the subject; primary Gleason grade in a pathological specimen obtained from the subject; secondary Gleason grade in a pathological specimen obtained from the subject; Gleason sum in a pathological specimen obtained from the subject; the subject's pre-operative TGF-ssl level; the subject's prostatic capsular invasion level (PCI; also known as extracapsular invasion or extracapsular extension); the subject's surgical margin status; the subject's seminal vesicle involvement; the subject's lymph node status; the subject's pre-operative IL6sR level; the sensitivity of the subject's cancer to hormone therapy; the resistance of the subject's cancer to hormone therapy; the subject's prior therapy and/or clinical stage; and (3) correlating (1) and (2) with disease outcome. In some embodiments, the factor is selected from the group consisting of primary Gleason grade; secondary Gleason grade; Gleason sum. Suitably, the subject's clinical stage is selected from T3a, T3, T2c, T2b, T2a, T2, T1e, T1b, T1a or T1. In some embodiments, the subject's prior therapy is a primary therapy (e.g., surgical treatment, chemotherapy, cryotherapy, radiation therapy, brachytherapy and hormonal therapy).

[0023] In some embodiments, the diagnostic/prognostic methods of the present invention further comprise detecting expression of at least one other skeletal metastasis associated, hormone-related cancer marker gene, illustrative examples of which include PCA3 (Prostate Cancer Antigen 3), Claudin 4, Hepsin, PSMA (Prostate Specific Membrane Antigen), SPINK1 (Serine Peptidase Inhibitor, Kazal type 1), GOLPH2 (GOLgi PHosphoprotein 2), KLK2 (KaLUKrein 2), KLK4 (KaLHKrein 4), KLK11 (KaLHKrein 11), KLK14 (KaLUKrein 14), KLK15 (KaLUKrein 15), PBOVI (Prostate and Breast cancer OVerexpressed 1) / UROC28 , BCL2 (B-cell CLL/lymphoma 2), TMPRSS2.ERG, GalNAc-T3 (UDP-N-acetyl-alpha-D-GALactosamine:polypeptide N-ACetylgalactosaminylTransferase 3), MUC1 (Mucin 1), EGFR, mutantp53, cyclin D, PCNA, Ki67, uPA, PAI (Plasminogen), HER2 (Human Epidermal growth factor Receptor 2) /neu/ ERbB2, Cathepsin D, BRCAI (BReast
CAncer I), BRCA2 (Breast Cancer 2), ER (estrogen receptor), PR (Progesterone Receptor), AR (Androgen Receptor), MUCl (MUCin), EGFR (Epidermal Growth Factor Receptor), mutant p53, cyclin D, PCNA (Proliferating Cell Nuclear Antigen), Ki67, uPA (urokinase type Plaminogen Activator) and PAI (Plaminogen Activator Inhibitor).

[0024] In representative examples of this type, the methods comprise: detecting in the biological sample: (a) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of PCA3 compared to normal expression of PCA3; (b) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of Claudin 4 compared to normal expression of Claudin 4; (c) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of Hepsin compared to normal expression of Hepsin; (d) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of PSMA compared to normal expression of PSMA; (e) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of SPINKl compared to normal expression of SPINKl; (f) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of GOLPHl compared to normal expression of GOLPHl; (g) overexpression of PITX2 compared to normal expression of PITX2 and presence of TMPRSS2.ERG; (h) overexpression of PITX2 compared to normal expression of PITX2 and overexpression of GalNAc-T3 compared to normal expression of GalNAc-T3; (i) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3 and overexpression of Claudin 4 compared to normal expression of Claudin 4; (j) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3 and overexpression of PSMA compared to normal expression of PSMA; (k) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3 and overexpression of PSMA compared to normal expression of PSMA; (l) overexpression of PITX2 compared to normal expression of PCA3 and overexpression of SPINKl compared to normal expression of SPINKl; (m) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3 and overexpression of GOLPHl compared to normal expression of GOLPHl; (n) overexpression of PITX2 compared to normal expression of PCA3 and overexpression of PSMA compared to normal expression of PSMA; (o) overexpression of PITX2 compared to normal expression of PCA3 and overexpression of GOLPHl compared to normal expression of GOLPHl; (p) overexpression of PITX2 compared to normal expression of PCA3 and overexpression of PSMA compared to normal expression of PSMA; (q) overexpression of PITX2 compared to normal expression of PCA3 and overexpression of GOLPHl compared to normal expression of GOLPHl.
normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3 and presence of TMPRSS2.ERG; (o) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCAS compared to normal expression of PCAi and overexpression of GalNAc-T3 compared to normal expression of GalNAc-T3; (p) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of Hepsin compared to normal expression of Hepsin; (q) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of GOLPH1 compared to normal expression of GOLPH1; (r) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Claudin 4 compared to normal expression of Claudin 4 and presence of TMPRSS2.ERG; (s) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of GalNAc-T3 compared to normal expression of GalNAc-T3; (t) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Hepsin compared to normal expression of Hepsin and overexpression of PSMA compared to normal expression of PSMA; (u) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Hepsin compared to normal expression of Hepsin and overexpression of SPINK1 compared to normal expression of SPINK1; (v) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Hepsin compared to normal expression of Hepsin and overexpression of SPINK1 compared to normal expression of SPINK1; (w) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Hepsin compared to normal expression of Hepsin and presence of TMPRSS2:ERG; (x) overexpression of PITX2 compared to normal expression of PITX2, overexpression of Hepsin compared to normal expression of Hepsin and overexpression of GalNAc-T3 compared to normal expression of GalNAc-T3; (aa) overexpression of PITX2 compared to normal expression of PITX2,
overexpression of PSMA compared to normal expression of PSMA and overexpression of SPINK1 compared to normal expression of SPINK1; (ab) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PSMA compared to normal expression of PSMA and overexpression of GOLPH1 compared to normal expression of GOLPH1; (ac) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PSMA compared to normal expression of PSMA and presence of TMPRSS2:ERG; (ad) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PSMA compared to normal expression of PSMA and overexpression of GaINAc-TS compared to normal expression of GalNAc-TS; (ae) overexpression of PITX2 compared to normal expression of PITX2, overexpression of SPINK1 compared to normal expression of SPINK1 and overexpression of GOLPH1 compared to normal expression of GOLPH1; (af) overexpression of PITX2 compared to normal expression of PITX2, overexpression of SPINK1 compared to normal expression of SPINK1 and presence of TMPRSS2:ERG; (ag) overexpression of PITX2 compared to normal expression of PITX2, overexpression of SPINK1 compared to normal expression of SPINK1 and overexpression of GaINAc-TS compared to normal expression of GaINAc-TS; (ah) overexpression of PITX2 compared to normal expression of PITX2, overexpression of GOLPH1 compared to normal expression of GOLPH1 and presence of TMPRSS2:ERG; (ai) overexpression of PITX2 compared to normal expression of PITX2, overexpression of GOLPH1 compared to normal expression of GOLPH1 and overexpression of GaINAc-TS compared to normal expression of GaINAc-TS; (aj) overexpression of PITX2 compared to normal expression of PITX2, presence of TMPRSS2:ERG and and overexpression of GaINAc-TS compared to normal expression of GaINAc-TS; (ak) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCAS compared to normal expression of PCAS, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of Hepsin compared to normal expression of Hepsin; (al) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCAS compared to normal expression of PCAS, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of PSMA compared to normal expression of PSMA; (am) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCAS compared to normal expression of PCAS, overexpression of Claudin 4 compared to normal expression of Claudin 4 and overexpression of GOLPH1 compared to normal expression of GOLPH1.
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and presence of TMPRSS2.ERG; (at) overexpression of PITX2 compared to normal
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of GalNAc-T3 compared to normal expression of GalNAc-T3; (au) overexpression of
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expression of Claudin 4, overexpression of Hepsin compared to normal expression of
Hepsin, overexpression of PSMA compared to normal expression of PSMA and
overexpression of SPINK1 compared to normal expression of SPINK1; (av)
overexpression of \textit{PITX2} compared to normal expression of \textit{PITX2}, overexpression of \textit{PCA3} compared to normal expression of \textit{PCAS}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA} and overexpression of \textit{GOLPH1} compared to normal expression of \textit{GOLPH1}; (aw) overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA} and presence of \textit{TMPRSS2:ERG}; (ax) overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA}, overexpression of \textit{SPINK1} compared to normal expression of \textit{SPINK1} and overexpression of \textit{GOLPH1} compared to normal expression of \textit{GOLPH1}; (az) overexpression of \textit{PITX2} compared to normal expression of \textit{PITX2}, overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA}, overexpression of \textit{SPINK1} compared to normal expression of \textit{SPINK1} and presence of \textit{TMPRSS2:ERG}; (aaa) overexpression of \textit{PITX2} compared to normal expression of \textit{PITX2}, overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA}, overexpression of \textit{SPINK1} compared to normal expression of \textit{SPINK1} and overexpression of \textit{GalNAC-T3} compared to normal expression of \textit{GalNAC-T3}; (aab) overexpression of \textit{PITX2} compared to normal expression of \textit{PITX2}, overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA}, overexpression of \textit{SPINK1} compared to normal expression of \textit{SPINK1} and overexpression of \textit{GalNAC-T3} compared to normal expression of \textit{GalNAC-T3}; (aab) overexpression of \textit{PITX2} compared to normal expression of \textit{PITX2}, overexpression of \textit{PCA3} compared to normal expression of \textit{PCA3}, overexpression of \textit{Claudin 4} compared to normal expression of \textit{Claudin 4}, overexpression of \textit{Hepsin} compared to normal expression of \textit{Hepsin}, overexpression of \textit{PSMA} compared to normal expression of \textit{PSMA}, overexpression of \textit{SPINK1} compared to normal expression of \textit{SPINK1} and overexpression of \textit{GalNAC-T3} compared to normal expression of \textit{GalNAC-T3};
Claudin 4, overexpression of Hepsin compared to normal expression of Hepsin, overexpression of PSMA compared to normal expression of PSMA, overexpression of SPINK1 compared to normal expression of SPINK1, overexpression of GOLPH1 compared to normal expression of GOLPH1 and presence of TMPRSS2.ERG; (aac) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCAS, overexpression of Claudin 4 compared to normal expression of Claudin 4, overexpression of Hepsin compared to normal expression of PSMA, overexpression of SPINK1 compared to normal expression of SPINK1, overexpression of GOLPH1 compared to normal expression of GOLPH1 and overexpression of GalNAc-TS compared to normal expression of GalNAc-TS; and (aad) overexpression of PITX2 compared to normal expression of PITX2, overexpression of PCA3 compared to normal expression of PCA3, overexpression of Claudin 4 compared to normal expression of Claudin 4, overexpression of Hepsin compared to normal expression of PSMA, overexpression of SPINK1 compared to normal expression of SPINK1, overexpression of GOLPH1 compared to normal expression of GOLPH1, presence of TMPRSS2.ERG and overexpression of GalNAc-TS compared to normal expression of GalNAc-T3, wherein detecting the overexpression and/or where stated the presence identifies the skeletal metastasis-associated, hormone-related cancer in the subject.

[0025] In some embodiments, the diagnostic/prognostic methods of the present invention further comprise comparing expression of a PITX2 polynucleotide to expression of at least one other PITX2 polynucleotide in the biological sample. In illustrative examples of this type, the methods comprise at least one comparison selected from the group consisting of: (1) comparing expression of a PITX2A polynucleotide to expression of a PITX2B polynucleotide; (2) comparing expression of a PITX2A polynucleotide to expression of a PITX2C polynucleotide; (3) comparing expression of a PITX2B polynucleotide to expression of a PITX2C polynucleotide; (4) comparing expression of a PITX2A polynucleotide to expression of all PITX2 polynucleotides; (5) comparing expression of a PITX2B polynucleotide to expression of PITX2A and PITX2C polynucleotides; (6) comparing expression of a PITX2C polynucleotide to expression of PITX2A and PITX2B polynucleotides; (7) comparing expression of a PITX2C polynucleotide to expression of all PITX2
polynucleotides; (8) comparing expression of a PITX2A polynucleotide to expression of all PITX2 polynucleotides (e.g., PITX2A, PITX2B and PITX2Q; (9) comparing expression of a PITX2B polynucleotide to expression of all PITX2 polynucleotides; and (10) comparing expression of a PITX2C polynucleotide to expression of all PITX2 polynucleotides.

[0026] In yet another aspect, the invention contemplates use of the methods broadly described above in the monitoring, treatment and management of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer). In these embodiments, the diagnostic/prognostic methods of the invention are typically used at a frequency that is effective to monitor the early development of a skeletal metastasis-associated, hormone-related cancer or to monitor the stage, degree or progression of the skeletal metastasis-associated, hormone-related cancer, to thereby enable early or more effective therapeutic intervention or treatment of the cancer. In illustrative examples, the diagnostic/prognostic methods are used at least at 1-, 2-, 4-, 6-, 8-, 12-, 16- or 18-hour intervals, or at least at 1-, 2-, 3-, 4-, 5- or 6-day intervals, or at least at weekly or monthly intervals.

[0027] In a related aspect, the present invention provides methods for treating, preventing or inhibiting the development or progression of a skeletal metastasis-associated, hormone-related cancer in a subject. These methods generally comprise detecting overexpression of PITX2 in the subject, and administering to the subject at least one therapy that treats or ameliorates the symptoms or reverses or inhibits the development or progression of the skeletal metastasis-associated, hormone-related cancer in the subject. Representative examples of such therapies include surgery, radiation therapy, chemotherapy, stem cell transplant; hormone therapy, anti-resorptive agent therapy and antibody therapy.

[0028] In still another aspect, the present invention provides probes for interrogating nucleic acid for the presence of a PITX2 polynucleotide as described for example above for use in the diagnostic/prognostic methods of the present invention. These probes generally comprise a nucleotide sequence that hybridizes under at least medium or high stringency conditions to a PITX2 polynucleotide. In some embodiments, the probes consist essentially of a nucleic acid sequence which corresponds or is complementary to at least a portion of a nucleotide sequence encoding
the amino acid sequence set forth in any one of SEQ ID NO: 2, 4, 6 or 8, wherein the
portion is at least 15 nucleotides in length. In other embodiments, the probes comprise a
nucleotide sequence which is capable of hybridizing to at least a portion of a nucleotide
sequence encoding the amino acid sequence set forth in any one of SEQ ID NO: 2, 4, 6,
or 8 under at least medium or high stringency conditions, wherein the portion is at least
15 nucleotides in length. In still other embodiment, the probes comprise a nucleotide
sequence that is capable of hybridizing to at least a portion of any one of SEQ ID NO:
1, 3, 5 or 7 under at least medium or high stringency conditions, wherein the portion is
at least 15 nucleotides in length. Representative probes for detecting the PITX2
polynucleotides are set forth in Table 4.

[0029] In a related aspect, the invention provides a solid or semi-solid support
for use the diagnostic/prognostic methods of the present invention, wherein the solid or
semi-solid support comprises at least one nucleic acid probe as broadly described above
immobilized thereon. In some embodiments, the solid or semi-solid support comprises a
spatial array of nucleic acid probes immobilized thereon.

[0030] Still a further aspect of the present invention provides an antigen-
binding molecule that is immuno-interactive with a PITX2 polypeptide as described for
example above for use the diagnostic/prognostic methods of the present invention.

[0031] In a related aspect, the invention provides a solid or semi-solid support
comprising at least one antigen-binding molecule as broadly described above
immobilized thereon. In some embodiments, the solid or semi-solid support comprises a
spatial array of antigen-binding molecules immobilized thereon.

[0032] Still another aspect of the invention provides the use of one or more
PITX2 polynucleotides as described for example above, or the use of one or more
probes as broadly described above, or the use of one or more PITX2 polypeptides as
described for example above, or the use of one or more antigen-binding molecules as
broadly described above, in the manufacture of a kit for diagnosing the presence, stage
or degree of a skeletal metastasis-associated, hormone-related cancer in a subject or for
providing a prognosis of a subject with a skeletal metastasis-associated, hormone-
related cancer.

[0033] In yet another aspect, the present invention provides methods for
treating or preventing a skeletal metastasis-associated, hormone-related cancer (e.g.,
prostate cancer or breast cancer) in a subject. These methods generally comprise administering to the subject an agent that modulates PITX2 expression or the level or functional activity of a PITX2 expression product, wherein the agent is administered in an amount that is effective to treat or prevent, or ameliorate the symptoms or reverse or inhibit the development or progression of the skeletal metastasis-associated, hormone-related cancer. Non-limiting examples of suitable agents include small molecules, such as nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon containing) or inorganic molecules.

[0034] In some embodiments, the agent is a PITX2 inhibitor that reduces the expression of PITX2 or the level or functional activity of a PITX2 expression product. In illustrative examples of this type, the PITX2 inhibitor reduces PITX2 expression or the level or functional activity of a PITX2 expression product to less than 9/10, 4/5, 7/10, 3/5, \( V_2 \), 2/5, 3/10, 1/5, 1/10, 1/20, 1/50, \( 10^{-1} \), \( 10^{-2} \), \( 10^{-3} \), \( 10^{-4} \), \( 10^{-5} \), \( 10^{-6} \), \( 10^{-8} \), \( 10^{-9} \), \( 10^{-10} \), \( 10^{-12} \), \( 10^{-13} \), \( 10^{-14} \) or about \( 10^{-15} \) of the expression, level or functional activity of a corresponding PITX2 expression product in the absence of the agent. In other embodiments, the PITX2 inhibitor antagonizes the function of a PITX2 polypeptide, including reducing or abrogating the interaction between the PITX2 polypeptide and a cognate DNA-binding site (e.g., PLOD-1 and PLOD-2 promoters).

[0035] In some embodiments, the agent is administered separately, sequentially or simultaneously with at least one other therapy that treats or ameliorates the symptoms or reverses or inhibits the development or progression of the skeletal metastasis-associated, hormone-related cancer in the subject. Representative examples of such therapies include surgery, radiation therapy, chemotherapy, stem cell transplant; hormone therapy, anti-resorptive agent therapy and antibody therapy.

[0036] In yet another aspect, the present invention provides methods for identifying agents that modulate the development or progression of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer). These methods typically comprise contacting a preparation with a test agent, wherein the preparation comprises (i) a polypeptide comprising an amino acid sequence corresponding to at least a biologically active fragment of a PITX2 polypeptide, or to a variant or derivative thereof; or (ii) a polynucleotide comprising at least a portion of a genetic sequence that regulates the expression of PITX2, which is operably linked to a
reporter gene. A detected reduction in the level and/or functional activity of the polypeptide, or an expression product of the reporter gene, relative to a normal or reference level and/or functional activity in the absence of the test agent, indicates that the agent modulates the development or progression of the skeletal metastasis-associated, hormone-related cancer.

[0037] In yet another aspect, the present invention resides in the use of an agent, which is optionally formulated with a pharmaceutically acceptable carrier or diluent, for treating or preventing the development of skeletal metastasis-associated, hormone-related cancer, wherein the agent modulates the expression of \( \text{PITX2} \) or the level or functional activity of a \( \text{PITX2} \) expression product. The agent used in these methods is characterized in that it binds to a \( \text{PITX2} \) expression product or to a genetic sequence (e.g., a transcriptional element) that modulates \( \text{PITX2} \) expression, as determined by: contacting a preparation comprising at least a portion of a \( \text{PITX2} \) expression product, or a variant or derivative of the expression product, or a genetic sequence that modulates the expression \( \text{oiPITX2} \), with the agent; and detecting a change in the level or functional activity of the at least a portion of the expression product, or the variant or derivative, or of a product expressed from the genetic sequence.

[0038] Still another aspect of the present invention provides methods of producing an agent for modulating the development or progression of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer). These methods generally comprise: testing an agent suspected of modulating \( \text{PITX2} \) as broadly described above; and synthesising the agent on the basis that it tests positive for the modulation. Suitably, the method further comprises derivatizing the agent, and optionally formulating the derivatized agent with a pharmaceutically acceptable carrier and/or diluent, to improve the efficacy of the agent for treating or preventing the development or progression of the skeletal metastasis-associated, hormone-related cancer.

[0039] Still another aspect of the present invention provides the use of a \( \text{P/LY2} \)-modulating agent as broadly described above in the manufacture of a composition for treating or preventing, or for ameliorating the symptoms or reversing or
inhibiting the development or progression of a skeletal metastasis-associated, hormone-related cancer.
BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Figure 1 is a graphical representation showing that (A) PC3 tumors were successfully formed in 100% of animals whereas LNCaP tumors formed in only 75% of animals injected. Panel (B) illustrates the average tumor volume produced by subcutaneous injection of cell lines with Matrigel.

[0041] Figure 2 is a photographic representation showing typical cellular morphology in subcutaneous tumors stained with H&E. Note larger cells and more structured architecture of LNCaP tumor compared to PC3 tumor. Muscle invasion and perineural invasion were seen in some tumors from each cell type (not shown). A high cellular proliferation rate as indicated by multiple mitotic bodies was noted in both PC3 and LNCaP tumors.

[0042] Figure 3 is a graphical representation showing mitotic index in PC3 and LNCaP cell line-derived tumors. Mean ± SD, n = 4 tumors per cell line.

[0043] Figure 4 is a graphical representation showing PITX2 expression (normalized to multiple housekeeping genes) in PC3 and LNCaP tumor samples by qPCR Wnt pathway array analysis (N = 4 tumors derived from each cell line.)

[0044] Figure 5 is a graphical representation confirming difference in PITX2 expression levels in tumors formed from PC3 and LNCaP cell lines. Real-time PCR was conducted in triplicate using independently prepared cDNA preparations from subcutaneous tumor RNA preparations, using independent specific PITX2 primers. ΔCT values were normalized to the cyclophilin house keeping gene (**P<0.0005)

[0045] Figure 6 is a graphical representation showing (A) qPCR array data illustrating normalized expression data for PITX2 in normal prostate, primary prostate cancer and prostate cancer bone metastasis compared to cell line expression levels. (B) Normalized data, omitting prostate cancer bone metastasis value to reveal expression levels in other samples.

[0046] Figure 7 is a graphical representation showing the trend of increasing PITX2 expression in primary prostate cancer specimens with increasing grade of disease as assessed by Gleason Score. One 3+3, four 3+4 and three 4+3 samples were analysed.
[0047] Figure 8 is a photographic representation showing (A) Western blot analysis to confirm detection of all three PITX2 protein isoforms by P2R10 antibody. Protein cell lysates of PC3 cells engineered to over-express Flag epitope-tagged individual isoforms of PITX2 (40μg protein per lane). (B) Parallel Western blots of PITX2 isoforms detected by Flag antibody in the same protein lysates. Migration of the 37 kDa protein size markers is indicated by red dashes on different filters. The P2R10 antibody was raised in rabbits against an epitope (DPSKKKR) N-terminal to the homeodomain and recognizes all known PITX2 protein isoforms except the PITX2D isoform (Lamba et al, 2008 BMC Molecular Biology 9(1):31).

[0048] Figure 9 is a photographic representation showing: (A) Section of paraffin-embedded LNCaP cells stained with P2R10 PITX2 antibody (1:500 dilution) showing negative result. (B) Section of paraffin-embedded PC3 cell subcutaneous tumor stained with P2R10 PITX2 antibody (1:350 dilution) showing widespread positive cellular staining. (C) A section paraffin-embedded normal liver, a negative control tissue, stained with P2R10 PITX2 antibody (1:350 dilution).

[0049] Figure 10 is a photographic representation showing a radical prostatectomy specimen stained with (A) H&E, (B) PSA antibody, (C) PITX2 antibody P2R10. Normal epithelial tissue is indicated by arrowhead and malignant epithelium by arrow. PSA antibody dilution 1:10000; P2R10 antibody dilution 1:100.

[0050] Figure 11 is a photographic representation showing (A) Normal epithelial tissue from radical prostatectomy specimen not adjacent to malignant epithelium stained for PITX2 protein (P2R10 antibody at 1:100 dilution). (B) TRUS biopsy specimen with malignant glands stained for PITX2 protein (1:200 dilution). Note very high proportion of nuclear staining. Quality and pattern of staining appears to be very dependent on the quality of tissue fixation. Underfixed tissues show significant cytoplasmic staining in addition to nuclear staining due to leakage of nuclear PITX2 into the cytoplasm. TRUS biopsy specimens are therefore excellent specimens to demonstrate the specific staining and pattern of staining as they have relatively small tissue volumes compared to the volume of fixative used.

[0051] Figure 12 is a photographic representation showing archival specimen of prostate cancer bone metastasis with new bone formation (arrowhead) and cancer cells (arrow) evident. (A) H&E staining. (B) PSA staining (antibody dilution 1:10000).
(C) PITX2 staining (P2R10 antibody dilution 1:100). Note very strong PITX2 positivity relative to PSA in a similar pattern to that in Figure 4.8 B and C.

[0052] Figure 13 is a photographic representation showing archival prostate cancer bone metastasis specimen was predominantly osteoblastic (A, black arrowheads) but also had areas of osteolytic activity with cortical bone destruction (B, black arrowheads). Intense staining for PITX2 was present in the osteolytic region (C) within the same section, PITX2 staining become lighter as the lesion became more osteoblastic (direction of arrow in D), until staining was negative (E). A, B stained with H&E; C, D, E stained with P2R10 antibody at 1:200 dilution.

[0053] Figure 14 is a photographic representation showing osteoblastic prostate cancer bone metastasis with evidence of high bone formation (A, H&E staining), uniform strong for PSA signal (B) and negative for PITX2 staining. PSA antibody dilution 1:10000; (C) P2R10 antibody dilution 1:100.

[0054] Figure 15 is a schematic representation showing (A) Alignment of original set PITX2 universal and isoform specific primers. (B) Alignment of the second set of universal and isoform specific primers (obtained from Dr J Martens, Erasmus MC) used for verification of results obtained with original primers.

[0055] Figure 16 is a graphical representation showing average PITX2 expression in PC3 and LNCaP cells grown alone or co-cultured with Saos-2 osteosarcoma cells. PITX2 expression by PC3 was significantly increased when co-cultured with the osteoblast like Saos-2 cells, compared to PC3 controls not exposed to bone cells (p= 0.01). This increase in expression was not observed in the LNCaP cells, whether alone or co-cultured with bone cells. PITX2 expression was normalized to the cyclophilin housekeeping gene.

[0056] Figure 17 is a graphical representation showing PITX2 and isoform specific expression in stably transfected LNCaP cultures. (A) PITX2 transcript levels in all stable cells lines, using universal and isoform-specific primer pairs to evaluate all stable cell lines. (B) Data from same analysis with value for B line omitted to allow visualization of low transcript levels for A and C lines. Note difference in Y axis values in the two panels. Values were normalized to the cyclophilin housekeeping gene.
Figure 18 is a graphical representation showing PITX2 expression in transiently transfected PC3 cells. Quantitative RT-PCR with universal primer pair was used to detect endogenous PITX2 transcripts in the native cell line (highlighted in the right panel omitting expression results from transiently overexpressing cultures), and isoform-specific primers were employed in the transiently over-expressing cultures. PITX2 values were normalized to the cyclophilin house keeping gene.

Figure 19 is a photographic representation showing western blots of whole cell lysates (80 µg protein per lane) from PC3 cultures transiently transfected with decreasing amounts of input plasmid (A) PITX2A, (B) PITX2B, or (C) PITX2C. Lanes in each panel, left to right: marker (M), input plasmid DNA at 2 µg, 1 µg, 0.5 µg and 0.25 µg per 6 well culture. Black outline indicates signal from blots using anti-Flag antibody at 1:1000 dilution; red outline, anti-β-tubulin at 1:1000. Intensity values for Flag signal are shown in Figure 20.

Figure 20 is a graphical representation showing flag signal intensity relative to input plasmid DNA, taken from immunoblot data in Figure 19. Bar labels (X-axis) indicate the PITX2 isoform specificity of the transfected plasmid and the amount of input plasmid (µg). Flag signal intensities were normalized to β-tubulin values.

Figure 21 is a photographic representation showing western blots of whole cell lysates (80 µg protein per lane) from PC3 cultures transiently cotransfected with isoform specific expression plasmids and 0.5 µg of each shRNA or NC. (A) PITX2A + shRNA, (B) PITX2B + shRNA, or (C) PITX2C + shRNA. Lanes in each panel, left to right: marker (M), input isoform specific expression plasmid DNA at 0.5 µg cotransfected with 0.5 µg of NC, shl, sh2, sh3 and sh4 per 6 well culture. Black outline indicates signal from blots using anti-Flag antibody at 1:1000 dilution; red outline, anti-β-tubulin at 1:1000. Intensity values for Flag signal are shown in Figure 22.

Figure 22 is a graphical representation showing PITX2 isoform specific protein expression data in Figure 21, as assessed by signal intensity for epitope-tagged PITX2 protein species using Flag antibody and normalized to β Tubulin signal. (A) PITX2A + shRNA, (B) PITX2B + shRNA, (C) PITX2C + shRNA. Knockdown efficacy is normalised to expression of each PITX2 isoform co-transfected with NC (i.e. zero value for each graph = PITX2 isoform + NC). Effective and consistent knockdown
of overexpression was seen in all three isoforms by sh3 and sh4 only. sh3 and sh4 therefore selected for use in all knockdown experiments.

[0062] Figure 23 is a photographic representation showing low power images of PC3 cells 4 days after transfection (original photograph at 10x magnification). (A) PC3 native cells, mock transfection. (B-D) PC3 cells transfected with expression vectors encoding (B) PITX2A, (C) PITX2B, (D) PITX2C.

[0063] Figure 24 is a graphical representation showing (A) Cell number 24 hr post transfection assessed by Syto 60 assay (B) Cell proliferation 24hr post transfection, assessed by BrdU incorporation. PC3 parental cells were mock transfected; labels A, B, C and V indicate cultures transiently over-expressing individual PITX2 isoforms and empty vector control, respectively. Bars represent average absorbance values from respective assays, mean +SE. Dashed line indicates mean absorbance value for mock transfected PC3 cells. *p<0.05 and **p<0.01 vs vector-transfected cultures.

[0064] Figure 25 is a graphical representation showing (A) Cell number 48 hr post transfection assessed by Syto 60 assay (B) Cell proliferation 48hr post transfection, assessed by BrdU incorporation. PC3 parental cells were mock transfected; labels A, B, C and V indicate cultures transiently over-expressing individual PITX2 isoforms and empty vector control, respectively. Bars represent average absorbance values from respective assays, mean +SE. Dashed line indicates mean absorbance value for mock transfected PC3 cells. *p<0.05; **p<0.01; ***p<0.0001 vs vector-transfected cultures.

[0065] Figure 26 is a graphical representation showing (A) Cell number 24 hr post transfection assessed by Syto 60 assay (B) Cell proliferation 24hr post transfection, assessed by BrdU incorporation. PC3 parental cells were mock transfected; labels sh3, sh4 and NC indicate cultures transiently transfected with individual shRNAs and scrambled control, respectively. Bars represent average absorbance values from respective assays, mean +SE. Dashed line indicates mean absorbance value for mock transfected PC3 cells. *p<0.05; **p<0.01; ***p<0.0001 vs vector-transfected cultures.

[0066] Figure 27 is a graphical representation showing (A) Cell number 48 hr post transfection assessed by Syto 60 assay (B) Cell proliferation 48hr post transfection, assessed by BrdU incorporation. PC3 parental cells were mock transfected; labels sh3, sh4 and NC indicate cultures transiently transfected with individual shRNAs and scrambled control, respectively. Bars represent average absorbance values from
respective assays, mean +SE. Dashed line indicates mean absorbance value for mock transfected PC3 cells.

[0067] Figure 28 is a graphical representation showing effects of *PITX2* isoform overexpression on cellular motility as assessed by scratch assay closure. Labels A, B, C and V indicate cultures transiently over-expressing individual *PITX2* isoforms and empty vector control, respectively. Bars represent average absorbance values from respective assays, mean ±SE.

[0068] Figure 29 is a graphical representation showing effect of co-transfection of *PITX2A* and shRNA on cellular motility. Labels Ash3, Ash4 and ANC indicate cultures transiently co-transfected with *PITX2A* expression vector and individual shRNAs and scrambled control, respectively. (*p < 0.05, **p < 0.005, ***p < 0.0005).

[0069] Figure 30 is a graphical representation showing effect of co-transfection of *PITX2B* and shRNA on cellular motility. Labels Bsh3, Bsh4 and BNC indicate cultures transiently co-transfected with *PITX2B* expression vector and individual shRNAs and scrambled control, respectively. (*p < 0.05).

[0070] Figure 31 is a graphical representation showing effect of co-transfection of *PITX2C* and shRNA on cellular motility. Labels Csh3, Csh4 and CNC indicate cultures transiently co-transfected with *PITX2C* expression vector and individual shRNAs and scrambled control, respectively. (*p < 0.05).

[0071] Figure 32 is a graphical representation showing effect of endogenous *PITX2* knockdown on cellular motility as assessed by scratch assay closure. Labels sh3, sh4 and NC indicate cultures transiently transfected with individual shRNAs and scrambled control, respectively. (*p < 0.05; **p < 0.001).

[0072] Figure 33 is a graphical representation showing percent change in motility towards different chemoattractants in transfected PC3 cells. Baseline absorbance was assessed in transwells with serum free media only (controls), absorbance from 10% FBS and 10% Saos CM transwells are expressed as a percentage change in motility from baseline. PC3 parental cells were mock transfected; labels sh3, sh4 and NC indicate cultures transiently transfected with individual shRNAs and scrambled control, respectively. Red and green dashed lines indicate mean mock
transfected values for 10% FBS and 10% Saos CM results respectively. Comparisons are made to the PC3 NC Saos CM motility result (**p<0.005, ***p<0.001, ****p<5E-05).

[0073] Figure 34 is a graphical representation showing attachment to ECM molecules by cells transiently transfected to overexpress PITX2 isoforms. PC3 parental cells were mock transfected; labels A, B, C and V indicate cultures transiently over-expressing individual PITX2 isoforms and empty vector control, respectively. (*p<0.05).

[0074] Figure 35 is a graphical representation showing attachment to ECM molecules by cells transiently transfected with shRNA. PC3 parental cells were mock transfected; labels sh3, sh4 and NC indicate cultures transiently transfected with individual shRNAs and scrambled control, respectively. (*p<0.05; **p<0.01).

[0075] Figure 36 is a schematic representation showing an alignment of shRNA target sequences to PITX2 transcripts encoding individual protein isoforms.

[0076] Figure 37 is a photographic representation showing a tissue microarray section of the breast cancer cell line SKBR3 stained with PITX2 antibody P2R10. This cell line shows strong positivity for PITX2.

[0077] Figure 38 is a photographic representation showing a tissue microarray section of breast cancer tissue stained with PITX2 antibody P2R10. PITX2 positivity is shown in a majority of the cancer cells.
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DETAILED DESCRIPTION OF THE INVENTION

/ Definitions

[0078] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[0079] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0080] The term "aberrant expression," as used herein to describe the expression of a PITX2 gene, refers to the overexpression of the PITX2 gene relative to a 'normal' level of expression of the PITX2 gene or allelic variant thereof in healthy or normal cells or in cells obtained from a healthy subject or from a subject lacking a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer), and/or to a level of a PITX2 gene product (e.g., transcript or polypeptide) in a healthy tissue sample or body fluid or in a tissue sample or body fluid obtained from a healthy subject or from a subject lacking the skeletal metastasis-associated, hormone-related cancer. In particular, a PITX2 gene is aberrantly- or over-expressed if the level of expression of the PITX2 gene is higher by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, or even an at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% than the level of expression of the PITX2 gene in healthy or normal cells or in cells obtained from a healthy subject or from a subject without a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer, breast cancer, endometrial cancer and ovarian cancer), and/or relative to a level of a PITX2 gene product in a healthy tissue sample or body fluid or in a tissue sample or body fluid obtained from a healthy subject or from a subject lacking the skeletal metastasis-associated, hormone-related cancer.

[0081] By "about" is meant a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15, 10,
9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[0082] The term "agent" or "modulatory agent" includes a compound that induces a desired pharmacological and/or physiological effect. The term also encompasses pharmaceutically acceptable and pharmacologically active ingredients of those compounds specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the above term is used, then it is to be understood that this includes the active agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs, etc. The term "agent" is not to be construed narrowly but extends to small molecules, proteinaceous molecules such as peptides, polypeptides and proteins as well as compositions comprising them and genetic molecules such as RNA, DNA and mimetics and chemical analogs thereof as well as cellular agents. The term "agent" includes a cell which is capable of producing and secreting the polypeptides referred to herein as well as a polynucleotide comprising a nucleotide sequence that encodes this polypeptide. Thus, the term "agent" extends to nucleic acid constructs including vectors such as viral or non-viral vectors, expression vectors and plasmids for expression in and secretion in a range of cells.

[0083] The term "amplicon" refers to a target sequence for amplification, and/or the amplification products of a target sequence for amplification. In certain other embodiments an "amplicon" may include the sequence of probes or primers used in amplification.

[0084] By "antigen-binding molecule" is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity.

[0085] As used herein, the term "binds specifically," "specifically immuno-interactive" and the like when referring to an antigen-binding molecule refers to a binding reaction which is determinative of the presence of an antigen in the presence of a heterogeneous population of proteins and other biologies. Thus, under designated immunoassay conditions, the specified antigen-binding molecules bind to a particular antigen and do not bind in a significant amount to other proteins or antigens present in
the sample. Specific binding to an antigen under such conditions may require an antigen-binding molecule that is selected for its specificity for a particular antigen. For example, antigen-binding molecules can be raised to a selected protein antigen, which bind to that antigen but not to other proteins present in a sample. A variety of immunoassay formats may be used to select antigen-binding molecules specifically immuno-interactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immuno-interactive with a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

[0086] Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

[0087] By "corresponds to" or "corresponding to" is meant a polynucleotide (a) having a nucleotide sequence that is substantially identical or complementary to all or a portion of a reference polynucleotide sequence or (b) encoding an amino acid sequence identical to an amino acid sequence in a peptide or protein. This phrase also includes within its scope a peptide or polypeptide having an amino acid sequence that is substantially identical to a sequence of amino acids in a reference peptide or protein.

[0088] As used herein the term "cytostatic agent" refers to a substance that can inhibit cell proliferation or cell division without necessarily killing the cell. Suitably, the cytostatic agent inhibits the proliferation of cancer cells.

[0089] The term "cytotoxic agent" or "cytotoxic therapy" as used herein refers to a substance or therapy that is harmful to cells and ultimately causes cell death. In some embodiments, the cytotoxic agent harms rapidly dividing cells such as cancer cells and causes cancer cell death, especially cancer cell death while not causing damage to or causing less damage to non-cancer cells. An example of a cytotoxic therapy is radiotherapy.

[0090] By "effective amount", in the context of treating or preventing a condition is meant the administration of that amount of active to an individual in need of such treatment or prophylaxis, either in a single dose or as part of a series, that is
effective for the prevention of incurring a symptom, holding in check such symptoms, and/or treating existing symptoms, of that condition. The effective amount will vary depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

[0091] The terms "expression" or "gene expression" refer to either production of RNA message or translation of RNA message into proteins or polypeptides. Detection of either types of gene expression in use of any of the methods described herein are part of the invention.

[0092] By "expression vector" is meant any autonomous genetic element capable of directing the transcription of a polynucleotide contained within the vector and suitably the synthesis of a peptide or polypeptide encoded by the polynucleotide. Such expression vectors are known to practitioners in the art.

[0093] As used herein, the term "functional activity" generally refers to the ability of a molecule (e.g., a transcript or polypeptide) to perform its designated function including a biological, enzymatic, or therapeutic function. In certain embodiments, the functional activity of a molecule corresponds to its specific activity as determined by any suitable assay known in the art. For example, PITX2 is a transcription factor and illustrative functional activities, which can be assayed include DNA-binding (e.g., binding to PLOD-1 and PLOD-2 promoters) and gene expression (e.g., using a suitable reporter assay).

[0094] The term "gene" as used herein refers to any and all discrete coding regions of the cell's genome, as well as associated non-coding and regulatory regions. The gene is also intended to mean the open reading frame encoding specific polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression. In this regard, the gene may further comprise control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals. The DNA sequences may be cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.
By "high density polynucleotide arrays" and the like is meant those arrays that contain at least 400 different features per cm².

The phrase "high discrimination hybridization conditions" refers to hybridization conditions in which single base mismatch may be determined.

By "housekeeping gene" is meant a gene that is expressed in virtually all cells since it is fundamental to the any cell's functions (e.g., essential proteins and RNA molecules).

As used herein the terms "drug resistant" and "refractory" refer to a cancer or tumor cell which is unresponsive or partially unresponsive to treatments normally used to treat the cancer or kill the tumor cell.

The terms "hormone ablation" and "hormone ablation therapy" refer to the deprivation of hormones that may be required for the survival and growth of cancer cells. Hormone ablation may be achieved by surgical removal of hormone-producing organs such as testes or ovaries or may be achieved chemically with compounds that interfere with hormone biosynthesis or secretion, compounds that antagonize or block hormone receptors or in some way prevent a hormone exerting its biological effect. For example, the conversion of testosterone to the more active dihydrotestosterone may be blocked by a 5-alpha reductase inhibitor such as finasteride. In other non-limiting examples, antiandrogens such as bicalutamide or the LHRH agonists such as goserelin may be used for hormone ablation.

"Hybridization" is used herein to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA, U pairs with A and C pairs with G. In this regard, the terms "match" and "mismatch" as used herein refer to the hybridization potential of paired nucleotides in complementary nucleic acid strands. Matched nucleotides hybridize efficiently, such as the classical A-T and G-C base pair mentioned above. Mismatches are other combinations of nucleotides that do not hybridize efficiently.

The phrase "hybridizing specifically to" and the like refer to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide
sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

[0102] Reference herein to "immuno-interactive" includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the molecules is, or mimics, a component of the immune system.

[0103] By "isolated" is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an "isolated polynucleotide", as used herein, refers to a polynucleotide, which has been purified from the sequences which flank it in a naturally-occurring state, e.g., a DNA fragment which has been removed from the sequences that are normally adjacent to the fragment. Alternatively, an "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to in vitro isolation and/or purification of a peptide or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, i.e., it is not associated with in vivo substances.

[0104] By "modulating" is meant increasing or decreasing, either directly or indirectly, the level or functional activity of a target molecule. For example, an agent may indirectly modulate the level/activity by interacting with a molecule other than the target molecule. In this regard, indirect modulation of a gene encoding a target polypeptide includes within its scope modulation of the expression of a first nucleic acid molecule, wherein an expression product of the first nucleic acid molecule modulates the expression of a nucleic acid molecule encoding the target polypeptide.

[0105] As used herein, a "naturally-occurring" nucleic acid molecule refers to a RNA or DNA molecule having a nucleotide sequence that occurs in nature. For example a naturally-occurring nucleic acid molecule can encode a protein that occurs in nature.

[0106] The term "oligonucleotide" as used herein refers to a polymer composed of a multiplicity of nucleotide residues (deoxyribonucleotides or ribonucleotides, or related structural variants or synthetic analogues thereof, including nucleotides with modified or substituted sugar groups and the like) linked via phosphodiester bonds (or related structural variants or synthetic analogues thereof). Thus, while the term "oligonucleotide" typically refers to a nucleotide polymer in which the nucleotide residues and linkages between them are naturally-occurring, it will be
understood that the term also includes within its scope various analogues including, but not restricted to, peptide nucleic acids (PNAs), phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate, phosphoroamidate, methyl phosphonates, 2-O-methyl ribonucleic acids, and the like.

The exact size of the molecule can vary depending on the particular application. Oligonucleotides are a polynucleotide subset with 200 bases or fewer in length. Preferably, oligonucleotides are 10 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g., for probes; although oligonucleotides may be double stranded, e.g., for use in the construction of a variant nucleic acid sequence. Oligonucleotides of the invention can be either sense or antisense oligonucleotides.

[0107] The term "oligonucleotide array" refers to a substrate having oligonucleotide probes with different known sequences deposited at discrete known locations associated with its surface. For example, the substrate can be in the form of a two dimensional substrate as described in U.S. Patent No. 5,424,186. Such substrate may be used to synthesize two-dimensional spatially addressed oligonucleotide (matrix) arrays. Alternatively, the substrate may be characterized in that it forms a tubular array in which a two dimensional planar sheet is rolled into a three-dimensional tubular configuration. The substrate may also be in the form of a microsphere or bead connected to the surface of an optic fiber as, for example, disclosed by Chee et al. in WO 00/39587. Oligonucleotide arrays have at least two different features and a density of at least 400 features per cm². In certain embodiments, the arrays can have a density of about 500, at least one thousand, at least 10 thousand, at least 100 thousand, at least one million or at least 10 million features per cm². For example, the substrate may be silicon or glass and can have the thickness of a glass microscope slide or a glass cover slip, or may be composed of other synthetic polymers. Substrates that are transparent to light are useful when the method of performing an assay on the substrate involves optical detection. The term also refers to a probe array and the substrate to which it is attached that form part of a wafer.

[0108] The term "operably connected" or "operably linked" as used herein means placing a structural gene under the regulatory control of a promoter, which then controls the transcription and optionally translation of the gene. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position
the genetic sequence or promoter at a distance from the gene transcription start site that is approximately the same as the distance between that genetic sequence or promoter and the gene it controls in its natural setting; i.e. the gene from which the genetic sequence or promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting; i.e., the genes from which it is derived.

[0109] The term "polynucleotide" or "nucleic acid" as used herein designates mRNA, RNA, cRNA, cDNA or DNA, synthetic forms and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog (such as the morpholine ring), internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendant moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators and modified linkages (e.g., α-anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen binding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule. RNA forms of the genetic molecules of the present invention are generally mRNA or iRNA including siRNAs. The genetic form may be in isolated form or integrated with other genetic molecules such as vector molecules and particularly expression vector molecules. The terms "nucleotide sequence," "polynucleotide" and "nucleic acid" used herein interchangeably and encompass polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides.

[0110] The terms "polynucleotide variant" and "variant" refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide sequence or polynucleotides that hybridize with a reference sequence under stringent
conditions that are defined hereinafter. These terms also encompass polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains a biological function or activity of the reference polynucleotide. The terms "polynucleotide variant" and "variant" also include naturally-occurring allelic variants.

[0111] "Polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally-occurring amino acid, such as a chemical analogue of a corresponding naturally-occurring amino acid, as well as to naturally-occurring amino acid polymers.

[0112] The term "polypeptide variant" refers to polypeptides which are distinguished from a reference polypeptide by the addition, deletion or substitution of at least one amino acid residue. In certain embodiments, one or more amino acid residues of a reference polypeptide are replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the polypeptide (conservative substitutions) as described hereinafter.

[0113] As used herein, the term "primary skeletal metastasis-associated, hormone-related cancer" refers to a tumor that originates on its own at a specific site in the body and that has potential for: (1) castrate (i.e., hormone ablation) sensitivity or castrate resistance; and (2) skeletal metastasis.

[0114] By "primer" is meant an oligonucleotide which, when paired with a strand of DNA, is capable of initiating the synthesis of a primer extension product in the presence of a suitable polymerizing agent. The primer is preferably single-stranded for maximum efficiency in amplification but can alternatively be double-stranded. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the polymerization agent. The length of the primer depends on many factors, including application, temperature to be employed, template reaction conditions, other reagents, and source of primers. For example, depending on the complexity of the target
sequence, the primer may be at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, 500, to one base shorter in length than the template sequence at the 3’ end of the primer to allow extension of a nucleic acid chain, though the 5’ end of the primer may extend in length beyond the 3’ end of the template sequence. In certain embodiments, primers can be large polynucleotides, such as from about 35 nucleotides to several kilobases or more. Primers can be selected to be "substantially complementary" to the sequence on the template to which it is designed to hybridize and serve as a site for the initiation of synthesis. By "substantially complementary", it is meant that the primer is sufficiently complementary to hybridize with a target polynucleotide. Desirably, the primer contains no mismatches with the template to which it is designed to hybridize but this is not essential. For example, non-complementary nucleotide residues can be attached to the 5’ end of the primer, with the remainder of the primer sequence being complementary to the template. Alternatively, non-complementary nucleotide residues or a stretch of non-complementary nucleotide residues can be interspersed into a primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize therewith and thereby form a template for synthesis of the extension product of the primer.

[0115] "Probe" refers to a molecule that binds to a specific sequence or subsequence or other moiety of another molecule. Unless otherwise indicated, the term "probe" typically refers to a polynucleotide probe that binds to another polynucleotide, often called the "target polynucleotide", through complementary base pairing. Probes can bind target polynucleotides lacking complete sequence complementarity with the probe, depending on the stringency of the hybridization conditions. Probes can be labeled directly or indirectly and include primers within their scope.

[0116] As used herein the term "prognosis" shall be taken to mean a prediction of the progression of the disease (illustrative examples of which include regression, stasis and metastasis), in particular aggressiveness and metastatic potential of a tumor. It is typically used to define patients with high, low and intermediate risks of death or recurrence after treatment that result from the inherent heterogeneity of the disease process. Prognosis may also be referred to in terms of 'aggressiveness' wherein an aggressive cancer is determined to have a high risk of negative outcome (i.e., negative prognosis) and wherein a non-aggressive cancer has a low risk of negative
outcome (i.e., positive prognosis). As used herein the term "aggressive" as used with respect to a tumor shall be taken to mean a cell proliferative disorder that has the biological capability to rapidly spread outside of its primary location or organ. Indicators of tumor aggressiveness standard in the art include but are not limited to tumor stage, tumor grade, Gleason grade, Gleason score, nodal status and survival. As used herein the term "survival" shall not be limited to mean survival until mortality (wherein said mortality may be either irrespective of cause or cell proliferative disorder related) but may be used in combination with other terms to define clinical terms, for example, to "recurrence-free survival" (wherein the term recurrence includes both localized and distant recurrence); metastasis free survival; disease free survival (wherein the term disease includes cancer and diseases associated therewith). The length of the survival may be calculated by reference to a defined start point (e.g., time of diagnosis or start of treatment) and a defined end point (e.g. death, recurrence or metastasis).

[0117] The term "radiotherapy" as used herein refers to the treatment or exposure of a cancer or cancer cells such as tumor cells to high energy radiation. The effectiveness of radiotherapy may be enhanced by selenate or its pharmaceutically acceptable salt. Furthermore, radiotherapy may be further enhanced by administration of radiosensitizing agent. Illustrative examples of radiosensitizing agents include but are not limited to efaproxir, etanidazole, fluosol, misonidazole, nimorazole, temoporfin and tirapazamine.

[0118] The term "recombinant polynucleotide" as used herein refers to a polynucleotide formed in vitro by the manipulation of nucleic acid into a form not normally found in nature. For example, the recombinant polynucleotide may be in the form of an expression vector. Generally, such expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleotide sequence.

[0119] By "recombinant polypeptide" is meant a polypeptide made using recombinant techniques, i.e., through the expression of a recombinant or synthetic polynucleotide.

[0120] By "regulatory element" or "regulatory sequence" is meant nucleic acid sequences (e.g., DNA) necessary for expression of an operably linked coding sequence in a particular host cell. The regulatory sequences that are suitable for prokaryotic cells for example, include a promoter, and optionally a cis-acting sequence
such as an operator sequence and a ribosome binding site. Control sequences that are suitable for eukaryotic cells include promoters, polyadenylation signals, transcriptional enhancers, translational enhancers, leader or trailing sequences that modulate mRNA stability, as well as targeting sequences that target a product encoded by a transcribed polynucleotide to an intracellular compartment within a cell or to the extracellular environment.

[0121] The term "RNA interference" or "RNAi" refers to the silencing or decreasing of gene expression by siRNAs. It is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by siRNA that is homologous in its duplex region to the sequence of the silenced gene. The gene may be endogenous or exogenous to the organism, present integrated into a chromosome or present in a transfection vector that is not integrated into the genome. The expression of the gene is either completely or partially inhibited. RNAi may also be considered to inhibit the function of a target RNA; the function of the target RNA may be complete or partial.

[0122] As used herein, the term "secondary skeletal metastasis-associated, hormone-related cancer" refers to a malignant tumor that has spread from the site of a primary skeletal metastasis-associated, hormone-related tumor to another part of the body (e.g., skeletal tissue, lymph nodes, liver etc). In some embodiments in which the malignant tumor has spread to skeletal or bone tissue, the tumor is selected from osteoblastic, osteolytic and mixed osteoblastic/osteolytic phenotypes.

[0123] The term "sequence identity" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, He, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be
understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software.

[0124] As used herein, the term "siRNAs" refers to small interfering RNAs. In some embodiments, siRNAs comprise a duplex, or double-stranded region, of about 18-25 nucleotides long; often siRNAs contain from about two to four unpaired nucleotides at the 3' end of each strand. At least one strand of the duplex or double-stranded region of a siRNA is substantially homologous to, or substantially complementary to, a target RNA molecule. The strand complementary to a target RNA molecule is the "antisense strand," the strand homologous to the target RNA molecule is the "sense strand," and is also complementary to the siRNA antisense strand. siRNAs may also contain additional sequences; non-limiting examples of such sequences include linking sequences, or loops, as well as stem and other folded structures. siRNAs appear to function as key intermediaries in triggering RNA interference in invertebrates and in vertebrates, and in triggering sequence-specific RNA degradation during posttranscriptional gene silencing in plants.

[0125] "Similarity" refers to the percentage number of amino acids that are identical or constitute conservative substitutions as defined in Table A infra. Similarity may be determined using sequence comparison programs such as GAP (Deveraux et al. 1984, Nucleic Acids Research 12, 387-395). In this way, sequences of a similar or substantially different length to those cited herein might be compared by insertion of gaps into the alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

[0126] As used herein a "small molecule" refers to a composition that has a molecular weight of less than 3 kilodaltons (kDa), and typically less than 1.5 kilodaltons, and more preferably less than about 1 kilodalton. Small molecules may be nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic (carbon-containing) or inorganic molecules. As those skilled in the art will appreciate, based on the present description, extensive libraries of chemical and/or biological mixtures, often fungal, bacterial, or algal extracts, may be screened with any of the assays of the invention to identify compounds that modulate a bioactivity. A
"small organic molecule" is an organic compound (or organic compound complexed with an inorganic compound (e.g., metal)) that has a molecular weight of less than 3 kilodaltons, less than 1.5 kilodaltons, or even less than about 1 kDa.

[0127] Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e., only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (i.e., gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e., resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul et al., 1997, *Nucl. Acids Res.* 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel et al., "Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998, Chapter 15.

[0128] As used herein, the term "stage of cancer" refers to a qualitative or quantitative assessment of the level of advancement of a cancer. Criteria used to determine the stage of a cancer include, but are not limited to, the size of the tumor,
whether the tumor has spread to other parts of the body and where the cancer has spread (e.g., within the same organ or region of the body or to another organ).

[0129] The terms "subject" or "individual" or "patient", used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, primates, avians, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes). However, it will be understood that the aforementioned terms do not imply that symptoms are present.

[0130] As used herein, the term "subject suspected of having cancer" refers to a subject that presents one or more symptoms indicative of a cancer (e.g., a noticeable lump or mass) or is being screened for a cancer (e.g., during a routine physical). A subject suspected of having cancer may also have one or more risk factors. A subject suspected of having cancer has generally not been tested for cancer. However, a "subject suspected of having cancer" encompasses an individual who has received an initial diagnosis (e.g., a CT scan showing a mass or increased PSA level) but for whom the stage of cancer is not known. The term further includes people who once had cancer (e.g., an individual in remission).

[0131] As used herein, the term "subject at risk for cancer" and the like refers to a subject with one or more risk factors for developing a specific cancer. Risk factors include, but are not limited to, gender, age, genetic predisposition, environmental exposure, previous incidents of cancer, preexisting non-cancer diseases, and lifestyle.

[0132] The phrase "substantially similar affinities" refers herein to target sequences having similar strengths of detectable hybridization to their complementary or substantially complementary oligonucleotide probes under a chosen set of stringent conditions.

[0133] The term "template" as used herein refers to a nucleic acid that is used in the creation of a complementary nucleic acid strand to the "template" strand. The template may be either RNA and/or DNA, and the complementary strand may also be RNA and/or DNA. In certain embodiments, the complementary strand may comprise all or part of the complementary sequence to the "template," and/or may include mutations.
so that it is not an exact, complementary strand to the "template". Strands that are not exactly complementary to the template strand may hybridize specifically to the template strand in detection assays described here, as well as other assays known in the art, and such complementary strands that can be used in detection assays are part of the invention.

[0134] The term "treat" is meant to include both therapeutic and prophylactic treatment.

[0135] By "vector" is meant a polynucleotide molecule, suitably a DNA molecule derived, for example, from a plasmid, bacteriophage, yeast, virus, mammal, avian, reptile or fish into which a polynucleotide can be inserted or cloned. A vector preferably contains one or more unique restriction sites and can be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector can be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a linear or closed circular plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector can contain any means for assuring self-replication. Alternatively, the vector can be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. A vector system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector can also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are known to those of skill in the art.

[0136] The terms "wild-type" and "normal" are used interchangeably to refer to the phenotype that is characteristic of most of the members of the species occurring naturally and contrast for example with the phenotype of a mutant.

[0137] As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its protein product, which is indicated by the name of
the gene in the absence of any underscoring or italicizing. For example, "PITX1" shall mean the PITX2 gene, whereas "PITX2" shall indicate the protein product or products generated from transcription and translation and alternative splicing of the "PITX2" gene.

2. **Abbreviations**

   [0138] The following abbreviations are used throughout the application:
   
   - nt = nucleotide
   - nts = nucleotides
   - aa = amino acid(s)
   - kb = kilobase(s) or kilobase pair(s)
   - kDa = kilodalton(s)
   - d = day
   - h = hour
   - s = seconds

3. **Cancer biomarkers and uses therefor**

   [0139] The present invention concerns the detection, diagnosis, or prognosis of a skeletal metastasis-associated, hormone-related cancer. In accordance with the present invention, it has been discovered that overexpression of PITX2 as compared to its expression in normal subjects or in subjects lacking a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer), is diagnostic for the presence, stage or degree of the skeletal metastasis-associated, hormone-related cancer in tested subjects. In particular, it has been found that expression of PITX2 increases from primary to metastatic disease, which indicates a role for this protein in tumor progression and thus serves as a prognostic factor for metastatic disease. Accordingly, in certain advantageous embodiments, it is possible to use PITX2 expression to discriminate between aggressive and indolent forms of disease. This is highly desirable as a large proportion of patients with indolent disease would avoid unnecessary treatment and the associated complications and side effects, whilst patients with metastatic disease would benefit from more aggressive adjuvant therapies along with primary therapy, leading to better long term outcomes.
Thus, characterization of a skeletal metastasis-associated, hormone-related cancer in terms of predicted outcome enables the physician to determine the risk of recurrence and/or death. This aids in treatment selection as the absolute reduction of risk of recurrence and death after treatments (e.g., adjuvant hormonal, chemo- and radiation therapy) can be determined based on the predicted negative outcome. The absolute reduction in risk attributable to treatment may then be compared to the treatment drawbacks (e.g. side effects, cost) in order to determine the suitability of the treatment for the patient. Accordingly, where a skeletal metastasis-associated, hormone-related cancer is characterized as aggressive (i.e., negative outcome with high risk of death or recurrence, or negative prognosis) the patient will derive benefit from more aggressive cancer treatments. Conversely, where a skeletal metastasis-associated, hormone-related cancer is characterized as non-aggressive (i.e., positive outcome with low risk of death and/or recurrence, or positive prognosis) the patient will derive low absolute benefit from adjuvant or other treatment and may be treated more appropriately through active surveillance or watchful waiting.

It will be apparent, therefore, that the PITX2 gene and its expression products will find utility in a variety of applications in detection, diagnosis, prognosis and treatment of skeletal metastasis-associated, hormone-related cancers. Examples of such applications within the scope of the present disclosure include amplification of PITX2 transcripts using specific primers, detection of PITX2 transcripts by hybridization with oligonucleotide probes, incorporation of PITX2 nucleic acids into vectors, expression of vector-incorporated nucleic acids as RNA and protein, and development of anti-PITX2 immunological reagents.

3.1 PITX2 nucleic acid molecules

In accordance with the present invention, PITX2 nucleic acid sequences find utility inter alia as hybridization probes or amplification primers. These nucleic acids may be used, for example, in diagnostic/prognostic evaluation of biological samples. In certain embodiments, these probes and primers represent oligonucleotides, which are of sufficient length to provide specific hybridization to a RNA or DNA sample extracted from the biological sample. The sequences typically will be about 10-20 nucleotides, but may be longer. Longer sequences, e.g., of about 30, 40, 50, 100, 500 and even up to full-length, are desirable for certain embodiments.
Nucleic acid molecules having contiguous stretches of about 10, 15, 17, 20, 30, 40, 50, 60, 75 or 100 or 500 nucleotides of a sequence set forth in any one of SEQ ID NO: 1, 3, 5 or 7 are contemplated. Molecules that are complementary to the above mentioned sequences and that bind to these sequences under at least medium or high stringency conditions are also contemplated. These probes are useful in a variety of hybridization embodiments, such as Southern and northern blotting. In some cases, it is contemplated that probes may be used that hybridize to multiple target sequences (e.g., allelic variants and/or single nucleotide polymorphisms) without compromising their ability to effectively diagnose the presence, stage, degree or risk of development of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer or breast cancer). In general, it is contemplated that the hybridization probes described herein are useful both as reagents in solution hybridization, as in PCR, for detection or quantification of PITX2 expression, as well as in embodiments employing a solid phase.

Various probes and primers may be designed around the disclosed nucleotide sequences. For example, in certain embodiments, the sequences used to design probes and primers may include repetitive stretches of adenine nucleotides (poly-A tails) normally attached at the ends of the RNA for the identified marker genes. In other embodiments, probes and primers may be specifically designed to not include these or other segments from the identified marker genes, as one of ordinary skilled in the art may deem certain segments more suitable for use in the detection methods disclosed. In any event, the choice of primer or probe sequences for a selected application is within the realm of the ordinary skilled practitioner. Illustrative probe sequences for detection of PITX2 nucleic acids are presented in Table 4 and are set forth in SEQ ID NO: 9 to 28.

Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is desirable. Probes, while perhaps capable of priming, are designed to bind to a target DNA or RNA and need not be used in an amplification process. In certain embodiments, the probes or primers are labeled with radioactive species $^{32}$P, $^{14}$C, $^{35}$S, $^3$H, or other label), with a fluorophore (e.g., rhodamine, fluorescein) or with a chemilluminiscent label (e.g., luciferase).

The invention also contemplates detection or quantification of naturally-occurring PITX2 nucleic acid sequences, inclusive of PITX2 allelic variants
(same locus), homologues (different locus), and orthologues (different organism).

*PITX2* nucleic acid sequences may therefore contain variations such as nucleotide
substitutions, deletions, inversions and insertions, relative to the sequences set forth in
SEQ ID NO: 1, 3, 5 or 7. Variation can occur in either or both the coding and non-
coding regions. The variations can produce both conservative and non-conservative
amino acid substitutions (as compared in the encoded product). For nucleotide
sequences, conservative variants include those sequences that, because of the
degeneracy of the genetic code, encode the same amino acid sequence. Generally,
*PITX2* polynucleotides will have at least about 80%, 85%, and usually at least about
90%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a reference
*PITX2* nucleotide sequence, as set forth for example in SEQ ID NO: 1, 3, 5, or 7, or to
their complements, as determined by sequence alignment programs described elsewhere
herein using default parameters.

[0147] *PITX2* polynucleotides will generally hybridize to a reference *PITX2*
polynucleotide, as set forth for example in SEQ ID NO: 1, 3, 5 or 7, or to a complement
thereof, under low stringency, medium stringency, high stringency, or very high
stringency conditions. As used herein, the term "hybridizes under low stringency,
medium stringency, high stringency, or very high stringency conditions" describes
conditions for hybridization and washing. Guidance for performing hybridization
reactions can be found in Ausubel *et al.* (1998, *supra*), Sections 6.3.1-6.3.6. Aqueous
and non-aqueous methods are described in that reference and either can be used.

[0148] Reference herein to low stringency conditions include and encompass
from at least about 1% *v/v* to at least about 15% *v/v* formamide and from at least about
1 M to at least about 2 M salt for hybridization at 42° C, and at least about 1 M to at
least about 2 M salt for washing at 42° C. Low stringency conditions also may include
1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS
for hybridization at 65° C, and (i) 2 x SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA,
40 mM NaHPO₄ (pH 7.2), 5% SDS for washing at room temperature. One embodiment
of low stringency conditions includes hybridization in 6 x sodium chloride/sodium
citrate (SSC) at about 45° C, followed by two washes in 0.2 x SSC, 0.1% SDS at least
at 50° C (the temperature of the washes can be increased to 55° C for low stringency
conditions). Another embodiment of low stringency conditions includes conditions
equivalent to binding or hybridization at 42° C in a solution consisting of 5 x SSPE (43.8 g/L NaCl, 6.9 g/L NaH$_2$PO$_4$·H$_2$O and 1.85 g/L EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5 x Denhardt’s reagent [50 x Denhardt’s contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 μg/mL denatured salmon sperm DNA followed by washing in a solution comprising 5 x SSPE, 0.1% SDS at 42° C when a probe of about 500 nucleotides in length is employed.

[0149] Medium stringency conditions include and encompass from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization at 42° C, and at least about 0.1 M to at least about 0.2 M salt for washing at 55° C. Medium stringency conditions also may include 1% Bovine Serum Albumin (BSA), 1 mM EDTA, 0.5 M NaHPO$_4$ (pH 7.2), 7% SDS for hybridization at 65° C, and (i) 2 x SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM NaHPO$_4$ (pH 7.2), 5% SDS for washing at 60-65° C. One embodiment of medium stringency conditions includes hybridizing in 6 x SSC at about 45° C, followed by one or more washes in 0.2 x SSC, 0.1% SDS at 60° C. Another embodiment of medium stringency conditions includes conditions equivalent to binding or hybridization at 42° C in a solution consisting of 5 x SSPE (43.8 g/L NaCl, 6.9 g/L NaH$_2$PO$_4$·H$_2$O and 1.85 g/L EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5 x Denhardt’s reagent and 100 μg/mL denatured salmon sperm DNA followed by washing in a solution comprising 1.0 x SSPE, 1.0% SDS at 42° C when a probe of about 500 nucleotides in length is employed.

[0150] High stringency conditions include and encompass from at least about 31% v/v to at least about 50% v/v formamide and from about 0.01 M to about 0.15 M salt for hybridization at 42° C, and about 0.01 M to about 0.02 M salt for washing at 55° C. High stringency conditions also may include 1% BSA, 1 mM EDTA, 0.5 M NaHPO$_4$ (pH 7.2), 7% SDS for hybridization at 65° C, and (i) 0.2 x SSC, 0.1% SDS; or (ii) 0.5% BSA, 1 mM EDTA, 40 mM NaHPO$_4$ (pH 7.2), 1% SDS for washing at a temperature in excess of 65° C. One embodiment of high stringency conditions includes hybridizing in 6 x SSC at about 45° C, followed by one or more washes in 0.2 x SSC, 0.1% SDS at 65° C. Another embodiment of high stringency conditions includes conditions equivalent to binding or hybridization at 42° C in a solution consisting of 5 x SSPE
(43.8 g/L NaCl, 6.9 g/L NaH$_2$PO$_4$ H$_2$O and 1.85 g/L EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5 x Denhardt's reagent and 100 µg/mL denatured salmon sperm DNA followed by washing in a solution comprising 0.1 x SSPE, 1.0% SDS at 42° C when a probe of about 500 nucleotides in length is employed.

[0151] In certain embodiments, a PITX2 polynucleotide hybridizes to a disclosed nucleotide sequence, as for example set forth in SEQ ID NO: 1, 3, 5 or 7, or to a complement thereof, under very high stringency conditions. One embodiment of very high stringency conditions includes hybridizing 0.5 M sodium phosphate, 7% SDS at 65° C, followed by one or more washes at 0.2 x SSC, 1% SDS at 65° C.

[0152] Other stringency conditions are well known in the art and a skilled addressee will recognize that various factors can be manipulated to optimize the specificity of the hybridization. Optimization of the stringency of the final washes can serve to ensure a high degree of hybridization. For detailed examples, see Ausubel et al., supra at pages 2.10.1 to 2.10.16 and Sambrook et al. (1989, supra) at sections 1.101 to 1.104.

[0153] While stringent washes are typically carried out at temperatures from about 42° C to 68° C, one skilled in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridization rate typically occurs at about 20° C to 25° C below the Tm for formation of a DNA-DNA hybrid. It is well known in the art that the Tm is the melting temperature, or temperature at which two complementary polynucleotide sequences dissociate. Methods for estimating Tm are well known in the art (see Ausubel et al., supra at page 2.10.8). In general, the Tm of a perfectly matched duplex of DNA may be predicted as an approximation by the formula:

\[ Tm = 81.5 + 16.6 \log_{10}(M) + 0.41 \times (\%G+C) - 0.63 \times (\%formamide) - \frac{(600/\text{length})}{100} \]

[0154] where: M is the concentration of Na+, preferably in the range of 0.01 molar to 0.4 molar; %G+C is the sum of guanosine and cytosine bases as a percentage of the total number of bases, within the range between 30% and 75% G+C; % formamide is the percent formamide concentration by volume; length is the number of base pairs in the DNA duplex. The Tm of a duplex DNA decreases by approximately
1\(^\circ\) C with every increase of 1\% in the number of randomly mismatched base pairs. Washing is generally carried out at Tm - 15\(^\circ\) C for high stringency, or Tm - 30\(^\circ\) C for moderate stringency.

[0156] In an illustrative example of a hybridization procedure, a membrane (e.g., a nitrocellulose membrane or a nylon membrane) containing immobilized DNA is hybridized overnight at 42\(^\circ\) C in a hybridization buffer (50\% deionised formamide, 5 x SSC, 5 x Denhardt's solution (0.1\% ficoll, 0.1\% polyvinlypyrolidone and 0.1\% bovine serum albumin), 0.1\% SDS and 200 mg/mL denatured salmon sperm DNA) containing labeled probe. The membrane is then subjected to two sequential medium stringency washes (i.e., 2 x SSC, 0.1\% SDS for 15 min at 45\(^\circ\) C, followed by 2 x SSC, 0.1\% SDS for 15 min at 50\(^\circ\) C), followed by two sequential higher stringency washes (i.e., 0.2 x SSC, 0.1\% SDS for 12 min at 55\(^\circ\) C followed by 0.2 x SSC and 0.1\%SDS solution for 12 min at 65-68\(^\circ\) C.

3.2 PITX2 polypeptides

[0157] The present invention contemplates detection or quantification of naturally-occurring PITX2 polypeptides, inclusive of PITX2 allelic variants, homologues, and orthologues. PITX2 polypeptide sequences may therefore contain variations such as amino acid substitutions, deletions and insertions, relative to the sequences set forth in SEQ ID NO: 2, 4, 6 or 8. PITX2 polypeptides will generally have at least 80\%, 85\%, and usually at least about 90\%, 92\%, 93\%, 94\%, 95\%, 96\%, 97\%, 98\% or 99\% sequence similarity or identity with a reference PITX2 polypeptide, as set forth for example in SEQ ID NO: 2, 4, 6 or 8, as determined by sequence alignment programs described elsewhere herein using default parameters. A PITX2 polypeptide sequence may differ from a reference PITX2 polypeptide sequence generally by as much 60, 50, 40, 30 or 20 amino acid residues or suitably by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

3.3 Anti-PITX2 antigen-binding molecules

[0158] The present invention also contemplates the use of antigen-binding molecules that are specifically immuno-interactive with a PITX2 polypeptide for diagnosing the presence, stage or degree of a skeletal metastasis-associated, hormone-
related cancer. In some embodiment, the antigen-binding molecule is a whole polyclonal antibody. Such antibodies may be prepared, for example, by injecting a PITX2 polypeptide or portion thereof into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are described for example in Coligan et al., CURRENT PROTOCOLS IN IMMUNOLOGY, (John Wiley & Sons, Inc, 1991), and Ausubel et al., (1994-1998, supra), in particular Section III of Chapter 11.

[0159] In lieu of polyclonal antisera obtained in a production species, monoclonal antibodies may be produced using the standard method as described, for example, by Kohler and Milstein (1975, Nature 256, 495-497), or by more recent modifications thereof as described, for example, in Coligan et al, (1991, supra) by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of PITX2 polypeptides.

[0160] The invention also contemplates as antigen-binding molecules Fv, Fab, Fab' and F(ab')2 immunoglobulin fragments. Alternatively, the antigen-binding molecule may comprise a synthetic stabilized Fv fragment. Exemplary fragments of this type include single chain Fv fragments (sFv, frequently termed scFv) in which a peptide linker is used to bridge the N terminus or C terminus of a V\textsubscript{H} domain with the C terminus or N-terminus, respectively, of a V\textsubscript{L} domain. ScFv lack all constant parts of whole antibodies and are not able to activate complement. ScFvs may be prepared, for example, in accordance with methods outlined in Kreber et al (Kreber et al. 1997, J. Immunol Methods; 201(1): 35-55). Alternatively, they may be prepared by methods described in U.S. Patent No 5,091,513, European Patent No 239,400 or the articles by Winter and Milstein (1991, Nature 349:293) and Plückthun et al (1996, In Antibody engineering: A practical approach. 203-252). In another embodiment, the synthetic stabilized Fv fragment comprises a disulfide stabilized Fv (dsFv) in which cysteine residues are introduced into the V\textsubscript{H} and V\textsubscript{L} domains such that in the fully folded Fv molecule the two residues will form a disulfide bond between them. Suitable methods of producing dsFv are described for example in (Glockscuther et al. Biochem. 29: 1363-1367; Reiter et al. 1994, J. Biol. Chem. 269: 18327-18331; Reiter et al. 1994, Biochem. 33: 5451-5459; Reiter et al. 1994, Cancer Res. 54: 2714-2718; Webber et al 1995, Mol. Immunol 32: 249-258).

[0162] The antigen-binding molecule can be coupled to a compound, e.g., a label such as a radioactive nucleus, or imaging agent, e.g. a radioactive, enzymatic, or other, e.g., imaging agent, e.g., a NMR contrast agent. Labels which produce detectable radioactive emissions or fluorescence are preferred. An anti-PITX2 antigen-binding molecule (e.g., monoclonal antibody) can be used to detect PITX2 polypeptides (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the protein. In certain advantageous applications, such antigen-binding molecules can be used to monitor PITX2 polypeptides levels in biological samples (including tissues, cells and fluids) for diagnosing the presence, absence, degree, or stage of development of a skeletal metastasis-associated, hormone-related cancer.

Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance (i.e., antibody labeling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a
luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include $^{125}$I, $^{131}$I, $^{35}$S or $^3$H. The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu$^{3+}$), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

[0163] A large number of enzymes useful as labels is disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338. Enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme in solution.

4. Methods of detecting PITX2 overexpression

[0164] The present invention is predicated in part on the determination that PITX2 is overexpressed in primary skeletal metastasis-associated, hormone-related cancers and even more so in secondary skeletal metastasis-associated, hormone-related cancers. Accordingly, in certain embodiments, the invention features a method for diagnosing the presence, absence, degree or stage of a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer, breast cancer, endometrial cancer and ovarian cancer) or the risk of developing metastatic disease in a subject, by determining the expression status of PITX2 in a biological sample obtained from the subject and diagnosing the presence, absence, degree or stage of the skeletal metastasis-associated, hormone-related cancer or the risk of development of metastatic disease in the subject based on the expression status. It is desirable, therefore, to qualitatively or quantitatively determine the levels of PITX2 transcripts or the level or functional activity of PITX2 polypeptides in the subject in order to provide the diagnosis. In some embodiments, the presence, degree, or stage of development of the hormone related cancer or the risk of development of metastatic disease in the patient is diagnosed when a PITX2 gene product is expressed at a detectably higher level in the biological sample as compared to the level at which that gene is expressed in a reference sample obtained from normal
subjects or from subjects lacking the skeletal metastasis-associated, hormone-related cancer.

[0165] In other embodiments, the invention encompasses a method for providing a prognosis to a subject with a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer, breast cancer, endometrial cancer and ovarian cancer) by detecting overexpression of \( \text{PITX2} \) in the subject, wherein the overexpression is characterized by higher expression of \( \text{PITX2} \) than the expression of \( \text{PITX2} \) in a primary skeletal metastasis-associated, hormone-related cancer or in a benign organ hypertrophy (e.g., benign prostatic hypertrophy), which is indicative of a negative prognosis. In illustrative examples, this overexpression is detected by: (1) providing a biological sample from the subject; (2) measuring in the biological sample the level or functional activity of at least one \( \text{PITX2} \) expression product; and (3) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample obtained from one or more subjects with a primary skeletal metastasis-associated, hormone-related cancer (e.g., a primary prostate cancer, breast cancer, endometrial cancer and ovarian cancer) or with a benign organ hypertrophy (e.g., benign prostatic hypertrophy), wherein a higher level or functional activity of the or each expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the negative prognosis. In these examples, the higher level or functional activity typically represents an at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, or even an at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% increase in the level or functional activity of the expression product as compared to the level or functional activity of an individual corresponding expression product. In other illustrative examples, the methods comprise diagnosing the presence of a primary skeletal metastasis-associated, hormone-related cancer when the measured level or functional activity of the or each expression product is the same as or similar to the measured level or functional activity of the corresponding expression product. In these embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%.
In other illustrative examples, the overexpression is detected by: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a secondary skeletal metastasis-associated, hormone-related cancer (e.g., a primary prostate cancer, breast cancer, endometrial cancer and ovarian cancer), wherein negative prognosis is determined when the level or functional activity of the or each expression product in the biological sample is the same as or similar to the level or functional activity of the corresponding expression product in the reference sample. In these embodiments, the measured level or functional activity of an individual expression product varies from the measured level or functional activity of an individual corresponding expression product by no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%.

The corresponding expression product is generally selected from the same gene product, an alternate gene product including splice variants or expression products produced from alternate promoters of the gene, a gene product expressed from a variant gene (e.g., an homologous or orthologous gene) including an allelic variant, or protein products thereof. In some embodiments, the method comprises measuring the level or functional activity of two or more PITX2 expression products (e.g., at least two of PITX2A, PITX2B and PITX2Q).

In specific embodiments, expression is measured directly (e.g., at the RNA or protein level). In some embodiments, expression is detected in tissue samples such as but not limited to biopsy tissue and paraffin-embedded tissue, illustrative examples of which include: prostate tissue, ovarian tissue, endometrial tissue, colon tissue, bone tissue, lung tissue, bladder tissue, testicular tissue and thyroid tissue. In other embodiments, expression is detected in bodily fluids (e.g., including but not limited to, plasma, serum, whole blood, mucus, ejaculate, blood, sputum, and urine). In specific embodiments, the biological sample comprises mammary cells, ovarian cells, endometrial cells or prostate cells, illustrative examples of which include mastectomy specimens, breast milk, menstrual fluid, ovariectomy specimens, endometrectomy specimen, core needle biopsies, sentinel lymph node biopsies, bone marrow biopsies, bone marrow aspirations, prostatectomy specimens and TRUS biopsies.
[0169] PITX2 expression may be detected along with other cancer makers, especially skeletal metastasis-associated, hormone-related cancer makers, in a multiplex or panel format. Such markers are selected for their predictive value alone or in combination with PITX2. Thus, in some embodiments, the diagnostic/prognostic methods of the present invention further comprise detecting expression of at least one other skeletal metastasis-associated, hormone-related cancer marker gene, illustrative examples of which include PCA3, Claudin 4, Hepsin, PSMA, SPINK1, GOLPH2, TMPRSS2.ERG, GalNAc-T3, HER2/neu, ERbB2, Cathepsin D, BRCA1, BRCA2, ER, PR, AR, MUC1, EGFR, mutant p53, cyclin D, PCNA, Ki67, uPA and PAI.

4.1 Nucleic acid-based diagnostics

[0170] Nucleic acid used in polynucleotide-based assays can be isolated from cells contained in the biological sample, according to standard methodologies (Sambrook, et al., 1989, supra; and Ausubel et al., 1994, supra). The nucleic acid is typically fractionated (e.g., poly A+ RNA) or whole cell RNA. Where RNA is used as the subject of detection, it may be desired to convert the RNA to a complementary DNA. In some embodiments, the nucleic acid is amplified by a template-dependent nucleic acid amplification technique. A number of template dependent processes are available to amplify the PITX2 sequences present in a given template sample. An exemplary nucleic acid amplification technique is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159, Ausubel et al (supra), and in Innis et al., ("PCR Protocols," Academic Press, Inc., San Diego Calif, 1990). Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, e.g., Taq polymerase. If a cognate PITX2 sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated. A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook et al., 1989, supra. Alternative methods for reverse transcription
utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art.

[0171] In certain advantageous embodiments, the template-dependent amplification involves the quantification of transcripts in real-time. For example, RNA or DNA may be quantified using the Real-Time PCR technique (Higuchi, 1992, et al., Biotechnology 10: 413-417). By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundance of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundance is only true in the linear range of the PCR reaction. The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA.

[0172] Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPO No. 320 308. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Pat. No. 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

[0173] Qβ Replicase, described in PCT Application No. PCT/US7/00880, may also be used. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

[0174] An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-α-thio-triphosphates in one strand of a restriction site may also be

[0175] Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, i.e., nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

[0176] Still another amplification method described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, may be used. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (e.g., biotin) and/or a detector moiety (e.g., enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labelled probe signals the presence of the target sequence.

[0177] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh et al, 1989, Proc. Natl. Acad. Sci. U.S.A., 86: 1173; Gingeras et al., PCT Application WO 88/10315). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H
while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

[0178] Vincent and Kong disclose a method termed helicase-dependent isothermal DNA amplification (HDA) (Vincent and Kong, EMBO Reports, 5(8):795-800, 2004). This method uses DNA helicase to separate DNA strands and hence does not require thermal cycling. The entire reaction can be carried out at one temperature and this method should have broad application to point-of-care DNA diagnostics.

[0179] Davey et al., EPO No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of E. coli DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

[0181] Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used for amplifying target nucleic acid sequences. Wu et al., (1989, Genomics 4: 560).

[0182] Depending on the format, the PITX2 nucleic acid of interest (e.g., PITX2A, PITX2B or PITX2Q) is identified in the sample directly using a template-dependent amplification as described, for example, above, or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (e.g., ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994, J Macromol Sci. Pure, Appl Chem., A31(1): 1355-1376).

[0183] In some embodiments, amplification products or "amplicons" are visualized in order to confirm amplification of the PITX2 sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labelled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation. In some embodiments, visualization is achieved indirectly. Following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified PITX2 sequence. The probe is suitably conjugated to a chromophore but may be radiolabeled. Alternatively, the probe is conjugated to a binding partner, such as an antigen-binding molecule, or biotin, and the other member of the binding
pair carries a detectable moiety or reporter molecule. The techniques involved are well known to those of skill in the art and can be found in many standard texts on molecular protocols (e.g., see Sambrook et al., 1989, supra and Ausubel et al. 1994, supra). For example, chromophore or radiolabel probes or primers identify the target during or following amplification.

[0184] In certain embodiments, target nucleic acids are quantified using blotting techniques, which are well known to those of skill in the art. Southern blotting involves the use of DNA as a target, whereas Northern blotting involves the use of RNA as a target. Each provide different types of information, although cDNA blotting is analogous, in many aspects, to blotting or RNA species. Briefly, a probe is used to target a DNA or RNA species that has been immobilized on a suitable matrix, often a filter of nitrocellulose. The different species should be spatially separated to facilitate analysis. This often is accomplished by gel electrophoresis of nucleic acid species followed by "blotting" on to the filter. Subsequently, the blotted target is incubated with a probe (usually labeled) under conditions that promote denaturation and rehybridization. Because the probe is designed to base pair with the target, the probe will bind a portion of the target sequence under renaturing conditions. Unbound probe is then removed, and detection is accomplished as described above.

[0185] Following detection/quantification, one may compare the results seen in a given subject with a control reaction (e.g., a statistically significant reference group of normal subjects or of subjects lacking a skeletal metastasis-associated, hormone-related cancer; or a statistically significant reference group of subjects with a primary skeletal metastasis-associated, hormone-related cancer; or a or a statistically significant reference group of subjects with a secondary skeletal metastasis-associated, hormone-related cancer). In this way, it is possible to correlate the amount of }PITX2 nucleic acid detected with the presence of the skeletal metastasis-associated, hormone-related cancer or with progression of the skeletal metastasis-associated, hormone-related cancer or severity of the disease.

[0186] Also contemplated are genotyping methods and allelic discrimination methods and technologies such as those described by Kristensen et al. (Biotechniques 30(2): 318-322), including the use of single nucleotide polymorphism analysis, high
performance liquid chromatography, TaqMan®, liquid chromatography, and mass spectrometry.

[0187] Also contemplated are biochip-based technologies such as those described by Hacia et al. (1996, Nature Genetics 14: 441-447) and Shoemaker et al. (1996, Nature Genetics 14: 450-456). Briefly, these techniques involve quantitative methods for analysing large numbers of genes rapidly and accurately. By tagging genes with oligonucleotides or using fixed probe arrays, one can employ biochip technology to segregate target molecules as high density arrays and screen these molecules on the basis of hybridization. See also Pease et al. (1994, Proc. Natl. Acad. Sci. U.S.A. 91: 5022-5026); Fodor et al. (1991, Science 251: 767-773). Briefly, nucleic acid probes to PITX2 polynucleotides are made and attached to biochips to be used in screening and diagnostic methods, as outlined herein. The nucleic acid probes attached to the biochip are designed to be substantially complementary to specific expressed PITX2 nucleic acids, i.e., the target sequence (either the target sequence of the sample or to other probe sequences, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. This complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the nucleic acid probes of the present invention. However, if the number of mismatches is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. In certain embodiments, more than one probe per sequence is used, with either overlapping probes or probes to different sections of the target being used. That is, two, three, four or more probes, with three being desirable, are used to build in a redundancy for a particular target. The probes can be overlapping (i.e. have some sequence in common), or separate.

[0188] As will be appreciated by those of ordinary skill in the art, nucleic acids can be attached to or immobilized on a solid support in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one or more of either electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment
of a molecule, such as, streptavidin to the support and the non-covalent binding of the biotinylated probe to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the probe, are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds.

Covalent bonds can be formed directly between the probe and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the probe or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

[0189] In general, the probes are attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. As described herein, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

[0190] The biochip comprises a suitable solid or semi-solid substrate or solid support. By "substrate" or "solid support" is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of the nucleic acid probes and is amenable to at least one detection method. As will be appreciated by practitioners in the art, the number of possible substrates are very large, and include, but are not limited to, glass and modified or functionalised glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon™, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, etc. In general, the substrates allow optical detection and do not appreciably fluoresce.

[0191] Generally the substrate is planar, although as will be appreciated by those of skill in the art, other configurations of substrates may be used as well. For example, the probes may be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Similarly, the substrate may be flexible, such as a flexible foam, including closed cell foams made of particular plastics.

[0192] In certain embodiments, oligonucleotides probes are synthesized on the substrate, as is known in the art. For example, photoactivation techniques utilizing photopolymerization compounds and techniques can be used. In an illustrative example, the nucleic acids are synthesized in situ, using well known photolithographic
techniques, such as those described in WO 95/25116; WO 95/35505; U.S. Pat. Nos. 5,700,637 and 5,445,934; and references cited within; these methods of attachment form the basis of the Affymetrix GeneChip™ technology.

[0193] In an illustrative biochip analysis, oligonucleotide probes on the biochip are exposed to or contacted with a nucleic acid sample suspected of containing one or more PITX2 polynucleotides (e.g., PITX2A, PITX2B, PITX2C etc) under conditions favoring specific hybridization. Sample extracts of DNA or RNA, either single or double-stranded, may be prepared from fluid suspensions of biological materials, or by grinding biological materials, or following a cell lysis step which includes, but is not limited to, lysis effected by treatment with SDS (or other detergents), osmotic shock, guanidinium isothiocyanate and lysozyme. Suitable DNA, which may be used in the method of the invention, includes cDNA. Such DNA may be prepared by any one of a number of commonly used protocols as for example described in Ausubel, et al., 1994, supra, and Sambrook, et al., 1989, supra.

[0194] Suitable RNA, which may be used in the method of the invention, includes messenger RNA, complementary RNA transcribed from DNA (cRNA) or genomic or subgenomic RNA. Such RNA may be prepared using standard protocols as for example described in the relevant sections of Ausubel, et al. 1994, supra and Sambrook, et al. 1989, supra).

[0195] cDNA may be fragmented, for example, by sonication or by treatment with restriction endonucleases. Suitably, cDNA is fragmented such that resultant DNA fragments are of a length greater than the length of the immobilized oligonucleotide probe(s) but small enough to allow rapid access thereto under suitable hybridization conditions. Alternatively, fragments of cDNA may be selected and amplified using a suitable nucleotide amplification technique, as described for example above, involving appropriate random or specific primers.

[0196] Usually the target PITX2 polynucleotides are detectably labeled so that their hybridization to individual probes can be determined. The target polynucleotides are typically detectably labeled with a reporter molecule illustrative examples of which include chromogens, catalysts, enzymes, fluorochromes, chemiluminescent molecules, bioluminescent molecules, lanthanide ions (e.g., Eu²⁺), a radioisotope and a direct visual label. In the case of a direct visual label, use may be
made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like. Illustrative labels of this type include large colloids, for example, metal colloids such as those from gold, selenium, silver, tin and titanium oxide. In some embodiments in which an enzyme is used as a direct visual label, biotinylated bases are incorporated into a target polynucleotide. Hybridization is detected by incubation with streptavidin-reporter molecules.

[0197] Suitable fluorochromes include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red. Other exemplary fluorochromes include those discussed by Dower et al. (International Publication WO 93/06121). Reference also may be made to the fluorochromes described in U.S. Patents 5,573,909 (Singer et al), 5,326,692 (Brinkley et al). Alternatively, reference may be made to the fluorochromes described in U.S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218. Commercially available fluorescent labels include, for example, fluorescein phosphoramidites such as Fluoreprime™ (Pharmacia), Fluoredite™ (Millipore) and FAM (Applied Biosystems International)

[0198] Radioactive reporter molecules include, for example, ³²P, which can be detected by an X-ray or phosphoimager techniques.

[0199] The hybrid-forming step can be performed under suitable conditions for hybridizing oligonucleotide probes to test nucleic acid including DNA or RNA. In this regard, reference may be made, for example, to NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH (Homes and Higgins, eds.) (IRL press, Washington D.C., 1985). In general, whether hybridization takes place is influenced by the length of the oligonucleotide probe and the polynucleotide sequence under test, the pH, the temperature, the concentration of mono- and divalent cations, the proportion of G and C nucleotides in the hybrid-forming region, the viscosity of the medium and the possible presence of denaturants. Such variables also influence the time required for hybridization. The preferred conditions will therefore depend upon the particular application. Such empirical conditions, however, can be routinely determined without undue experimentation.
In certain embodiments, high discrimination hybridization conditions are used. For example, reference may be made to Wallace et al. (1979, Nucl. Acids Res. 6: 3543) who describe conditions that differentiate the hybridization of 11 to 17 base long oligonucleotide probes that match perfectly and are completely homologous to a target sequence as compared to similar oligonucleotide probes that contain a single internal base pair mismatch. Reference also may be made to Wood et al (1985, Proc. Natl. Acad. Sci. USA 82: 1585) who describe conditions for hybridization of 11 to 20 base long oligonucleotides using 3M tetramethyl ammonium chloride wherein the melting point of the hybrid depends only on the length of the oligonucleotide probe, regardless of its GC content. In addition, Drmanac et al. (supra) describe hybridization conditions that allow stringent hybridization of 6-10 nucleotide long oligomers, and similar conditions may be obtained most readily by using nucleotide analogues such as ‘locked’ nucleic acids (Christensen et al, 2001 Biochem J 354: 481-4).

Generally, a hybridization reaction can be performed in the presence of a hybridization buffer that optionally includes a hybridization-optimizing agent, such as an isostabilizing agent, a denaturing agent and/or a renaturation accelerator. Examples of isostabilizing agents include, but are not restricted to, betaines and lower tetraalkyl ammonium salts. Denaturing agents are compositions that lower the melting temperature of double stranded nucleic acid molecules by interfering with hydrogen bonding between bases in a double stranded nucleic acid or the hydration of nucleic acid molecules. Denaturing agents include, but are not restricted to, formamide, formaldehyde, dimethylsulfoxide, tetraethyl acetate, urea, guanidium isothiocyanate, glycerol and chaotropic salts. Hybridization accelerants include heterogeneous nuclear ribonucleoprotein (hnRP) A1 and cationic detergents such as cetyltrimethylammonium bromide (CTAB) and dodecyl trimethylammonium bromide (DTAB), polylysine, spermine, spermidine, single stranded binding protein (SSB), phage T4 gene 32 protein and a mixture of ammonium acetate and ethanol. Hybridization buffers may include target polynucleotides at a concentration between about 0.005 nM and about 50 nM, preferably between about 0.5 nM and 5 nM, more preferably between about 1 nM and 2 nM.

A hybridization mixture containing the target PITX2 polynucleotides is placed in contact with the array of probes and incubated at a temperature and for a time appropriate to permit hybridization between the target sequences in the target
polynucleotides and any complementary probes. Contact can take place in any suitable container, for example, a dish or a cell designed to hold the solid support on which the probes are bound. Generally, incubation will be at temperatures normally used for hybridization of nucleic acids, for example, between about 20°C and about 75°C, example, about 25°C, about 30°C, about 35°C, about 40°C, about 45°C, about 50°C, about 55°C, about 60°C, or about 65°C. For probes longer than 14 nucleotides, 20°C to 50°C is desirable. For shorter probes, lower temperatures are preferred. A sample of target polynucleotides is incubated with the probes for a time sufficient to allow the desired level of hybridization between the target sequences in the target polynucleotides and any complementary probes. For example, the hybridization may be carried out at about 45°C +/- 10°C in formamide for 1-2 days.

[0203] After the hybrid-forming step, the probes are washed to remove any unbound nucleic acid with a hybridization buffer, which can typically comprise a hybridization optimizing agent in the same range of concentrations as for the hybridization step. This washing step leaves only bound target polynucleotides. The probes are then examined to identify which probes have hybridized to a target polynucleotide.

[0204] The hybridization reactions are then detected to determine which of the probes has hybridized to a corresponding target sequence. Depending on the nature of the reporter molecule associated with a target polynucleotide, a signal may be instrumentally detected by irradiating a fluorescent label with light and detecting fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be detected using a spectrophotometer; or detection of a dye particle or a colored colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label or chemiluminescent molecule employing a radiation counter or autoradiography. Accordingly, a detection means may be adapted to detect or scan light associated with the label which light may include fluorescent, luminescent, focussed beam or laser light. In such a case, a charge couple device (CCD) or a photocell can be used to scan for emission of light from a probe:target polynucleotide hybrid from each location in the micro-array and record the data directly in a digital computer. In some cases, electronic detection of the signal may not be necessary. For example, with enzymatically generated color spots associated with nucleic acid array
format, visual examination of the array will allow interpretation of the pattern on the array. In the case of a nucleic acid array, the detection means is suitably interfaced with pattern recognition software to convert the pattern of signals from the array into a plain language genetic profile. In certain embodiments, oligonucleotide probes specific for different PITX2 gene products (e.g., PITX2A, PITX2B, PITX2C etc) are in the form of a nucleic acid array and detection of a signal generated from a reporter molecule on the array is performed using a 'chip reader'. A detection system that can be used by a 'chip reader' is described for example by Pirrung et al (U.S. Patent No. 5,143,854). The chip reader will typically also incorporate some signal processing to determine whether the signal at a particular array position or feature is a true positive or maybe a spurious signal. Exemplary chip readers are described for example by Fodor et al (U.S. Patent No., 5,925,525). Alternatively, when the array is made using a mixture of individually addressable kinds of labeled microbeads, the reaction may be detected using flow cytometry.

4.2 Protein-based diagnostics

[0205] Consistent with the present invention, the overexpression of a PITX2 protein is indicative of the presence, degree, or stage of development of a skeletal metastasis-associated, hormone-related cancer. PITX2 protein levels in biological samples can be assayed using any suitable method known in the art. For example, antibody-based techniques may be employed, such as, for example, immunohistological and immunohistochemical methods for measuring the level of a protein of interest in a tissue sample. Specific recognition may be provided, for example, by a primary antibody (polyclonal or monoclonal) and a secondary detection system is used to detect presence (or binding) of the primary antibody. Detectable labels can be conjugated to the secondary antibody, such as a fluorescent label, a radiolabel, or an enzyme (e.g., alkaline phosphatase, horseradish peroxidase) which produces a quantifiable, e.g., coloured, product. In another suitable method, the primary antibody itself can be detectably labeled. As a result, immunohistological labeling of a tissue section is provided. In some embodiments, a protein extract is produced from a biological sample (e.g., tissue, cells) for analysis. Such an extract (e.g., a detergent extract) can be subjected to western-blot or dot/slot assay of the level of the protein of interest, using routine immunoblotting methods (Jalkanen et al., 1985, J Cell. Biol. 101: 976-985; Jalkanen et al., 1987, J. Cell. Biol. 105: 3087-3096).
Other useful antibody-based methods include immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, a protein-specific monoclonal antibody, can be used both as an immunoadsorbent and as an enzyme-labeled probe to detect and quantify a PITX2 protein (e.g., PITX2A, PITX2B, PITX2C etc). The amount of such protein present in a sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm (see Lacobilli et al., 1988, Breast Cancer Research and Treatment 11: 19-30). In other embodiments, two different monoclonal antibodies to the protein of interest can be employed, one as the immunoadsorbent and the other as an enzyme-labeled probe.

Additionally, recent developments in the field of protein capture arrays permit the simultaneous detection and/or quantification of a large number of proteins. For example, low-density protein arrays on filter membranes, such as the universal protein array system (Ge, 2000 Nucleic Acids Res. 28(2):e3) allow imaging of arrayed antigens using standard ELISA techniques and a scanning charge-coupled device (CCD) detector. Immuno-sensor arrays have also been developed that enable the simultaneous detection of clinical analytes. It is now possible using protein arrays, to profile protein expression in bodily fluids, such as in sera of healthy or diseased subjects, as well as in subjects pre- and post-drug treatment.

Protein capture arrays typically comprise a plurality of protein-capture agents each of which defines a spatially distinct feature of the array. The protein-capture agent can be any molecule or complex of molecules which has the ability to bind a protein and immobilize it to the site of the protein-capture agent on the array. The protein-capture agent may be a protein whose natural function in a cell is to specifically bind another protein, such as an antibody or a receptor. Alternatively, the protein-capture agent may instead be a partially or wholly synthetic or recombinant protein which specifically binds a protein. Alternatively, the protein-capture agent may be a protein which has been selected in vitro from a mutagenized, randomized, or completely random and synthetic library by its binding affinity to a specific protein or peptide target. The selection method used may optionally have been a display method such as ribosome display or phage display, as known in the art. Alternatively, the protein-capture agent obtained via in vitro selection may be a DNA or RNA aptamer which specifically binds a protein target (see, e.g., Potyrailo et al., 1998 Anal. Chem. 70:3419-
3425; Cohen et al., 1998, Proc. Natl. Acad. Sci. USA 95:14272-14277; Fukuda, et al., 1997 Nucleic Acids Symp. Ser. 37:237-238; available from SomaLogic). For example, aptamers are selected from libraries of oligonucleotides by the Selex™ process and their interaction with protein can be enhanced by covalent attachment, through incorporation of brominated deoxyuridine and UV-activated crosslinking (photoaptamers). Aptamers have the advantages of ease of production by automated oligonucleotide synthesis and the stability and robustness of DNA; universal fluorescent protein stains can be used to detect binding. Alternatively, the in vitro selected protein-capture agent may be a polypeptide (e.g., an antigen) (see, e.g., Roberts and Szostak, 1997 Proc. Natl. Acad. Sci. USA, 94:12297-12302).

[0209] An alternative to an array of capture molecules is one made through 'molecular imprinting' technology, in which peptides (e.g., from the C-terminal regions of proteins) are used as templates to generate structurally complementary, sequence-specific cavities in a polymerizable matrix; the cavities can then specifically capture (denatured) proteins which have the appropriate primary amino acid sequence (e.g., available from ProteinPrint™ and Aspira Biosystems).

[0210] Exemplary protein capture arrays include arrays comprising spatially addressed antigen-binding molecules, commonly referred to as antibody arrays, which can facilitate extensive parallel analysis of numerous proteins defining a proteome or subproteome. Antibody arrays have been shown to have the required properties of specificity and acceptable background, and some are available commercially (e.g., BD Biosciences, Clontech, BioRad and Sigma). Various methods for the preparation of antibody arrays have been reported (see, e.g., Lopez et al., 2003 J. Chromatogr. B 787:19-27; Cahill, 2000 Trends in Biotechnology 7:47-51; U.S. Pat. App. Pub. 2002/0055186; U.S. Pat. App. Pub. 2003/0003599; PCT publication WO 03/062444; PCT publication WO 03/077851; PCT publication WO 02/59601; PCT publication WO 02/39120; PCT publication WO 01/79849; PCT publication WO 99/39210). The antigen-binding molecules of such arrays may recognise at least a subset of proteins expressed by a cell or population of cells, illustrative examples of which include growth factor receptors, hormone receptors, neurotransmitter receptors, catecholamine receptors, amino acid derivative receptors, cytokine receptors, extracellular matrix receptors, antibodies, lectins, cytokines, serpins, proteases, kinases, phosphatases, rasi-
like GTPases, hydrolases, steroid hormone receptors, transcription factors, heat-shock transcription factors, DNA-binding proteins, zinc-finger proteins, leucine-zipper proteins, homeodomain proteins, intracellular signal transduction modulators and effectors, apoptosis-related factors, DNA synthesis factors, DNA repair factors, DNA recombination factors, cell-surface antigens, hepatitis C virus (HCV) proteases and HIV proteases.

[0211] Antigen-binding molecules for antibody arrays are made either by conventional immunization (e.g., polyclonal sera and hybridomas), or as recombinant fragments, usually expressed in *E. coli*, after selection from phage display or ribosome display libraries (e.g., available from Cambridge Antibody Technology, Biolnvent, Affitech and Biosite). Alternatively, 'combi bodies' comprising non-covalent associations of VH and VL domains, can be produced in a matrix format created from combinations of diabody-producing bacterial clones (e.g., available from Domantis). Exemplary antigen-binding molecules for use as protein-capture agents include monoclonal antibodies, polyclonal antibodies, Fv, Fab, Fab' and F(\(ab')_2\) immunoglobulin fragments, synthetic stabilized Fv fragments, e.g., single chain Fv fragments (scFv), disulfide stabilized Fv fragments (dsFv), single variable region domains (dAbs) minibodies, combi bodies and multivalent antibodies such as diabodies and multi-scFv, single domains from camels or engineered human equivalents.

[0212] Individual spatially distinct protein-capture agents are typically attached to a support surface, which is generally planar or contoured. Common physical supports include glass slides, silicon, microwells, nitrocellulose or PVDF membranes, and magnetic and other microbeads.

[0213] While microdrops of protein delivered onto planar surfaces are widely used, related alternative architectures include CD centrifugation devices based on developments in microfluidics (e.g., available from Gyros) and specialized chip designs, such as engineered microchannels in a plate (e.g., The Living Chip\textsuperscript{TM}, available from Biotrove) and tiny 3D posts on a silicon surface (e.g., available from Zyomyx).

[0214] Particles in suspension can also be used as the basis of arrays, providing they are coded for identification; systems include color coding for microbeads (e.g., available from Luminex, Bio-Rad and Nanomics Biosystems) and semiconductor nanocrystals (e.g., QDots\textsuperscript{TM}, available from Quantum Dots), and barcoding for beads.
(UltraPlex™, available from Smartbeads) and multimetal microrods (Nanobarcodes™ particles, available from Surromed). Beads can also be assembled into planar arrays on semiconductor chips (e.g., available from LEAPS technology and BioArray Solutions). Where particles are used, individual protein-capture agents are typically attached to an individual particle to provide the spatial definition or separation of the array. The particles may then be assayed separately, but in parallel, in a compartmentalized way, for example in the wells of a microtiter plate or in separate test tubes.

[0215] In operation, a protein sample, which is optionally fragmented to form peptide fragments (see, e.g., U.S. Pat. App. Pub. 2002/0055186), is delivered to a protein-capture array under conditions suitable for protein or peptide binding, and the array is washed to remove unbound or non-specifically bound components of the sample from the array. Next, the presence or amount of protein or peptide bound to each feature of the array is detected using a suitable detection system. The amount of protein bound to a feature of the array may be determined relative to the amount of a second protein bound to a second feature of the array. In certain embodiments, the amount of the second protein in the sample is already known or known to be invariant.

[0216] For analyzing differential expression of proteins between two cells or cell populations, a protein sample of a first cell or population of cells is delivered to the array under conditions suitable for protein binding. In an analogous manner, a protein sample of a second cell or population of cells to a second array, is delivered to a second array which is identical to the first array. Both arrays are then washed to remove unbound or non-specifically bound components of the sample from the arrays. In a final step, the amounts of protein remaining bound to the features of the first array are compared to the amounts of protein remaining bound to the corresponding features of the second array. To determine the differential protein expression pattern of the two cells or populations of cells, the amount of protein bound to individual features of the first array is subtracted from the amount of protein bound to the corresponding features of the second array.

[0217] In an illustrative example, fluorescence labeling can be used for detecting protein bound to the array. The same instrumentation as used for reading DNA microarrays is applicable to protein-capture arrays. For differential display, capture arrays (e.g. antibody arrays) can be probed with fluorescently labeled proteins
from two different cell states, in which cell lysates are labeled with different fluorophores (e.g., Cy-3 and Cy-5) and mixed, such that the color acts as a readout for changes in target abundance. Fluorescent readout sensitivity can be amplified 10-100 fold by tyramide signal amplification (TSA) (e.g., available from PerkinElmer Lifesciences). Planar waveguide technology (e.g., available from Zeptosens) enables ultrasensitive fluorescence detection, with the additional advantage of no washing procedures. High sensitivity can also be achieved with suspension beads and particles, using phycoerythrin as label (e.g., available from Luminex) or the properties of semiconductor nanocrystals (e.g., available from Quantum Dot). Fluorescence resonance energy transfer has been adapted to detect binding of unlabelled ligands, which may be useful on arrays (e.g., available from Affibody). Several alternative readouts have been developed, including adaptations of surface plasmon resonance (e.g., available from HTS Biosystems and Intrinsic Bioprobes), rolling circle DNA amplification (e.g., available from Molecular Staging), mass spectrometry (e.g., available from Sense Proteomic, Ciphergen, Intrinsic and Bioprobes), resonance light scattering (e.g., available from Genicon Sciences) and atomic force microscopy (e.g., available from BioForce Laboratories). A microfluidics system for automated sample incubation with arrays on glass slides and washing has been co-developed by NextGen and Perkin Elmer Life Sciences.

[0218] In certain embodiments, the techniques used for detection of PITX2 expression products will include internal or external standards to permit quantitative or semi-quantitative determination of those products, to thereby enable a valid comparison of the level or functional activity of these expression products in a biological sample with the corresponding expression products in a reference sample or samples. Such standards can be determined by the skilled practitioner using standard protocols. In specific examples, absolute values for the level or functional activity of individual expression products are determined.

4.3 In vivo Imaging

[0219] In some embodiments, in vivo imaging techniques are used to visualize the expression of PITX2 and optionally one or more other skeletal metastasis-associated, hormone-related cancer markers in a patient (e.g., a human or non-human mammal). For example, in some embodiments, skeletal metastasis-associated, hormone-
related cancer marker mRNA or protein is labeled using a labeled antigen-binding molecule (e.g., mAb) specific for the skeletal metastasis-associated, hormone-related cancer marker. A specifically bound and labeled antigen-binding molecule can be detected in an individual using an in vivo imaging method, including, but not limited to, radionuclide imaging, positron emission tomography, computerized axial tomography, X-ray or magnetic resonance imaging method, fluorescence detection, and chemiluminescent detection.

[0220] The in vivo imaging methods of the present invention are useful in the diagnosis of P/LY2-expressing skeletal metastasis-associated, hormone-related cancers (e.g., prostate cancer). In vivo imaging is used to visualize the presence and/or amount/level of a PITX2 expression product (e.g., PITX2 protein). Such techniques allow for diagnosis without the use of an unpleasant biopsy and are also useful for providing prognoses to skeletal metastasis-associated, hormone-related cancer patients. For example, PITX2 levels that are indicative of skeletal metastasis-associated, hormone-related cancers that likely to metastasize can be detected. The in vivo imaging methods of the present invention can further be used to detect metastatic skeletal metastasis-associated, hormone-related cancers in other parts of the body.

[0221] In illustrative examples, reagents (e.g., antibodies) specific for PITX2 and optionally one or more other skeletal metastasis-associated, hormone-related cancer markers are fluorescently labeled. The labeled reagents are introduced into a subject (e.g., orally or parenterally). Fluorescently labeled antibodies are detected using any suitable method (e.g., using the apparatus described in U.S. Pat. No. 6,198,107, herein incorporated by reference).

[0222] In other embodiments, antibodies are radiactively labeled. The use of antibodies for in vivo diagnosis is well known in the art. Sumerdon et al. (1990, Nucl. Med. Biol 17:247-254) have described an optimized antibody-chelator for the radioimmunoscintigraphy imaging of tumors using Indium-111 as the label. Griffin et al. (1991, J Clin One 9:631-640) have described the use of this agent in detecting tumors in patients suspected of having recurrent colorectal cancer. The use of similar agents with paramagnetic ions as labels for magnetic resonance imaging is known in the art (Lauffer, 1991, Magnetic Resonance in Medicine 22:339-342). The label used will depend on the imaging modality chosen. Radioactive labels such as Indium-111,
Technetium-99m, or Iodine-131 can be used for planar scans or single photon emission computed tomography (SPECT). Positron emitting labels such as Fluorine-19 can also be used for positron emission tomography (PET). For MRI, paramagnetic ions such as Gadolinium (III) or Manganese (II) can be used.

[0223] Radioactive metals with half-lives ranging from 1 hour to 3.5 days are available for conjugation to antibodies, such as scandium-47 (3.5 days) gallium-67 (2.8 days), gallium-68 (68 minutes), technetium-99m (6 hours), and indium-111 (3.2 days), of which gallium-67, technetium-99m, and indium-111 are desirable for gamma camera imaging, gallium-68 is desirable for positron emission tomography. An illustrative method of labeling antibodies with such radiometals is by means of a bifunctional chelating agent, such as diethylenetriaminepentaacetic acid (DTPA), as described, for example, by Khaw et al. (1980, Science 209:295) for In-111 and Tc-99m, and by Scheinberg et al. (1982, Science 215:1511). Other chelating agents may also be used, but the 1-(p-carboxymethoxybenzyl)EDTA and the carboxycarbonic anhydride of DTPA are advantageous because their use permits conjugation without affecting the antibody's immunoreactivity substantially. Another method for coupling DPTA to proteins is by use of the cyclic anhydride of DTPA, as described by Hnatowich et al. (1982, Int. J. Appl. Radiat. Isot. 33:327) for labeling of albumin with In-111, but which can be adapted for labeling of antibodies. A suitable method of labeling antibodies with Tc-99m which does not use chelation with DPTA is the pretinning method of Crockford et al. (U.S. Pat. No. 4,323,546). An exemplary method of labeling immunoglobulins with Tc-99m is that described by Wong et al. (1981, J. Nucl. Med., 23:229) for labeling antibodies.

[0224] In still further embodiments, in vivo biophotonic imaging (Xenogen, Almeda, Calif.) is utilized for in vivo imaging. This real-time in vivo imaging utilizes luciferase. The luciferase gene is incorporated into cells, microorganisms, and animals (e.g., as a fusion protein with a cancer marker of the present invention). When active, it leads to a reaction that emits light. A CCD camera and software is used to capture the image and analyze it.

[0225] All the essential materials and reagents required for detecting and/or quantifying PITX2 gene expression products, and optionally other skeletal metastasis-
associated, hormone-related cancer marker gene products may be assembled together in
a kit. The kits may also optionally include appropriate reagents for detection of labels,
positive and negative controls, washing solutions, blotting membranes, microtiter plates
dilution buffers and the like. For example, a nucleic acid-based detection kit may
include (i) a PITX2 polynucleotide, and optionally one or more other skeletal
metastasis-associated, hormone-related cancer marker polynucleotides, which may be
used as a positive control, (ii) a primer or probe that specifically hybridizes to a PITX2
polynucleotide, and optionally primers or probes that specifically hybridize to one or
more other skeletal metastasis-associated, hormone-related cancer marker
polynucleotides. Also included may be enzymes suitable for amplifying nucleic acids
including various polymerases (Reverse Transcriptase, Taq, Sequenase™ DNA ligase
etc depending on the nucleic acid amplification technique employed), deoxynucleotides
and buffers to provide the necessary reaction mixture for amplification. Such kits also
generally will comprise, in suitable means, distinct containers for each individual
reagent and enzyme as well as for each primer or probe. Alternatively, a protein-based
detection kit may include (i) a PITX2 polypeptide, and optionally one or more other
skeletal metastasis-associated, hormone-related cancer marker polypeptides, which may
be used as a positive control, (ii) an antigen-binding molecule that is immuno-
interactive with a PITX2 polypeptide and optionally antigen-binding molecules that are
immuno-interactive with one or more other skeletal metastasis-associated, hormone-
related cancer marker polypeptides. The kit can also feature various devices and
reagents for performing one of the assays described herein; and/or printed instructions
for using the kit to quantify the expression of a PITX2 gene and optionally the
expression of one or more other skeletal metastasis-associated, hormone-related cancer
marker polynucleotides.

5. Cancer therapies

[0226] The PITX2 expression profiles disclosed herein constitute a useful
signature of cancer progression and provide a non-subjective basis for selecting
appropriate therapeutic regimens. For example, where a skeletal metastasis-associated,
hormone-related cancer is characterized as aggressive (i.e., negative outcome with high
risk of death or recurrence) and the patient is found to be at risk of metastatic disease,
additional or more aggressive cancer therapies can be administered or started at an
earlier point when they are more likely to be effective (e.g., before metastasis).
Alternatively, where a skeletal metastasis-associated, hormone-related cancer is characterized as non-aggressive and the patient is found to have indolent disease, the patient will derive more benefit from watchful waiting.

[0227] In some embodiments, a patient's PITX2 expression profile is assessed in combination with at least one ancillary skeletal metastasis-associated, hormone-related cancer factor to correlate disease outcome for the patient. Non-limiting examples of ancillary skeletal metastasis-associated, hormone-related cancer factors include the subject's pre-treatment PSA; the subject's post-treatment PSA; primary Gleason grade in a biopsy specimen obtained from the subject; secondary Gleason grade in a biopsy specimen obtained from the subject; Gleason sum in a biopsy specimen obtained from the subject; pre-radical primary therapy of the subject; total length of cancer in biopsy cores obtained from the subject; number of positive biopsy cores obtained from the subject; percent of tumor biopsy in a multiple core biopsy set obtained from the subject; primary Gleason grade in a pathological specimen obtained from the subject; secondary Gleason grade in a pathological specimen obtained from the subject; Gleason sum in a pathological specimen obtained from the subject; the subject's pre-operative TGF-ssl level; the subject's prostatic capsular invasion level (PCI); the subject's surgical margin status; the subject's seminal vesicle involvement; the subject's lymph node status; the subject's pre-operative IL6sR level; the sensitivity of the subject's cancer to hormone therapy; the resistance of the subject's cancer to hormone therapy; the subject's prior therapy and/or clinical stage. Other non-limiting ancillary hormone-related cancer factors may be selected from expression profiles of hormone-related cancer maker genes, illustrative examples of which include PCAS, Claudin 4, Hepsin, PSMA, SPINK1, GOLPH2, TMPRSS2.ERG, GalNAc-T3, HER2/ neu / ERbB2, Cathepsin D, BRCA1, BRCA2, ER, PR, AR, MUC1, EGFR, mutant p53, cyclin D, PCNA, Ki67, uPA and PAI.

[0228] Disease outcome may be defined according to at least one of: probability of disease recurrence or metastasis at one or a plurality of time points between one and twenty years e.g., five, ten or fifteen years following a primary treatment; estimated disease or metastasis free survival; organ confined disease; extracapsular extension; seminal vesical involvement and lymph node status in the patient following radical prostatectomy.
The functional correlation between disease outcome, PITX2 expression profiles and the one or more ancillary skeletal metastasis-associated, hormone-related cancer factors may be generated by any means known in the art. For example, the functional correlation may be generated by a means selected from the group consisting of a neural network, Cox proportional hazards regression model and support vector machine. In some embodiments, the correlation is generated by computer and/or software means.

Thus, in operation, when a PITX2 expression profile, either alone or in combination with one or more ancillary skeletal metastasis-associated, hormone-related cancer factors, correlates with a negative disease outcome for a patient, the patient may be administered additional or more aggressive cancer therapies, non-limiting examples of which include radiotherapy, surgery, chemotherapy, hormone ablation therapy, pro-apoptosis therapy and immunotherapy.

5.1 Radiotherapy
Radiotherapies include radiation and waves that induce DNA damage for example, γ-irradiation, X rays, UV irradiation, microwaves, electronic emissions, radioisotopes, and the like. Therapy may be achieved by irradiating the localized tumor site with the above described forms of radiations. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes.

Dosage ranges for X rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

Non-limiting examples of radiotherapies include conformal external beam radiotherapy (50-100 Grey given as fractions over 4-8 weeks), either single shot or fractionated, high dose rate brachytherapy, permanent interstitial brachytherapy, systemic radio-isotopes (e.g., Strontium 89). In some embodiments the radiotherapy may be administered in combination with a radiosensitizing agent. Illustrative examples of radiosensitizing agents include but are not limited to efaproxiral, etanidazole, fluosol, misonidazole, nimorazole, temoporfin and tirapazamine.
5.2 Surgery

Surgical treatment for removal of a cancerous growth is generally a standard procedure for the treatment of tumors and cancers. This attempts to remove the entire cancerous growth. However, surgery is generally combined with chemotherapy and/or radiotherapy to ensure the destruction of any remaining neoplastic or malignant cells.

5.3 Chemotherapy

Chemotherapeutic agents may be selected from any one or more of the following categories:

(i) antiproliferative/antineoplastic drugs and combinations thereof, as used in medical oncology, such as alkylating agents (for example cis-platin, carboplatin, cyclophosphamide, nitrogen mustard, melphalan, chlorambucil, busulphan and nitrosoureas); antimetabolites (for example antifolates such as fluoropyridines like 5-fluorouracil and tegafur, raltitrexed, methotrexate, cytosine arabinoside and hydroxyurea; anti-tumor antibiotics (for example anthracyclines like adriamycin, bleomycin, doxorubicin, daunomycin, epirubicin, idarubicin, mitomycin-C, dactinomycin and mithramycin); antimitotic agents (for example vinca alkaloids like vincristine, vinblastine, vindesine and vinorelbine and taxoids like paclitaxel and docetaxel; and topoisomerase inhibitors (for example epipodophyllotoxins like etoposide and teniposide, amsacrine, topotecan and camptothecin);

(ii) cytostatic agents such as antioestrogens (for example tamoxifen, toremifene, raloxifene, droloxifene and idoxyfene), oestrogen receptor down regulators (for example fulvestrant), antiandrogens (for example bicalutamide, flutamide, nilutamide and ciproterone acetate), UH antagonists or LHRH agonists (for example goserelin, leuprorelin and buserelin), progestogens (for example megestrol acetate), aromatase inhibitors (for example as anastrozole, letrozole, vorazole and exemestane) and inhibitors of 5α-reductase such as finasteride;

(iii) agents which inhibit cancer cell invasion (for example metalloproteinase inhibitors like marimastat and inhibitors of urokinase plasminogen activator receptor function);
(iv) inhibitors of growth factor function, for example such inhibitors include growth factor antibodies, growth factor receptor antibodies (for example the anti-erbb2 antibody trastuzumab [Herceptin™] and the anti-erbbl antibody cetuximab [C225]), farnesyl transferase inhibitors, MEK inhibitors, tyrosine kinase inhibitors and serine/threonine kinase inhibitors, for example other inhibitors of the epidermal growth factor family (for example other EGFR family tyrosine kinase inhibitors such as N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholinopropoxy)quinazolin-4-amine (gefitinib, AZD1 839), N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine (erlotinib, OSI-774) and 6-acrylamido-N-(3-chloro-4-fluorophenyl)-7-(3-morpholinopropoxy)quinazol- n-4-amine (CI 1033)), for example inhibitors of the platelet-derived growth factor family and for example inhibitors of the hepatocyte growth factor family;

(v) anti-angiogenic agents such as those which inhibit the effects of vascular endothelial growth factor, (for example the anti-vascular endothelial cell growth factor antibody bevacizumab [Avastin™], compounds such as those disclosed in International Patent Applications WO 97/22596, WO 97/30035, WO 97/32856 and WO 98/13354) and compounds that work by other mechanisms (for example linomide, inhibitors of integrin .alpha.v.beta.3 function and angiostatin);

(vi) vascular damaging agents such as Combretastatin A4 and compounds disclosed in International Patent Applications WO 99/02166, WO00/40529, WO 00/41669, WOO 1/92224, WO02/04434 and WO02/08213;

(vii) antisense therapies, for example those which are directed to the targets listed above, such as ISIS 2503, an anti-ras antisense; and

(viii) gene therapy approaches, including for example approaches to replace aberrant genes such as aberrant p53 or aberrant BRCA1 or BRCA2, GDEPT (gene-directed enzyme pro-drug therapy) approaches such as those using cytosine deaminase, thymidine kinase or a bacterial nitroreductase enzyme and approaches to increase patient tolerance to chemotherapy or radiotherapy such as multi-drug resistance gene therapy.

5.4 Immunotherapy

Immunotherapy approaches, include for example ex-vivo and in-vivo approaches to increase the immunogenicity of patient tumor cells, such as transfection.
with cytokines such as interleukin 2, interleukin 4 or granulocyte-macrophage colony stimulating factor, approaches to decrease T-cell anergy, approaches using transfected immune cells such as cytokine-transfected dendritic cells, approaches using cytokine-transfected tumor cell lines and approaches using anti-idiotypic antibodies. These approaches generally rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

In targeting embodiments, the tumor cell generally bears some marker that is amenable to targeting, i.e., is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common skeletal metastasis-associated, hormone-related cancer markers include PSA and other Kallikrein-related proteases, PSMA, PR, AR, ER, laminin receptor, erb B and pI 55.

5.5 Hormone ablation therapy

In accordance with the present invention, it is proposed that PITX2 plays a role in development of castrate resistance and it can thus be targeted as a cotherapy with hormone ablation in hormone sensitive disease to prevent development of hormone independence or as monotherapy once castrate resistance develops.

Accordingly, in some embodiments, the methods further comprise separately, sequentially or simultaneously exposing the subject to hormone ablation therapy.

5.6 Anti-resorptive agents

Anti-resorptive agents or ARAs have several properties including selective uptake at active bone sites, suppression of osteoblast and osteoclast mediated bone resorption, reduction in the number of osteoclasts and long skeletal retention, which have been shown to minimize the destructive consequences of bone metastases and to exert a profound effect on tumor-induced osteolysis and tumor growth in bone.
The most common classes of anti-resorptive drugs include estrogen, selective estrogen receptor modulators (SERMs), biphosphonates, calcitonin, osteoprotegrin (OPG), cathespin K and statins. Current products include FOSAMAX™ (alendronate) in the U.S., Biphosphonate DIDRONEL™ (etidronate) and ACTONEL™ (risedronate)

5.7 Other Cancer Therapies

[0248] Examples of other cancer therapies include phototherapy, cryotherapy, toxin therapy or pro-apoptosis therapy. One of skill in the art would know that this list is not exhaustive of the types of treatment modalities available for cancer and other hyperplastic lesions.

6. PITX2 modulators

[0249] The present invention further provides agents that are useful for treating or preventing a skeletal metastasis-associated, hormone-related cancer (e.g., prostate cancer, breast cancer, endometrial cancer and ovarian cancer), wherein the agents modulates PITX2 expression or modulates the level or functional activity of a PITX2 polypeptide (e.g., PITX2A, PITX2B, PITX2C, etc). In specific embodiments, the agent is a PITX2 inhibitor, which reduces or abrogates PITX2 expression or the level or functional activity of a PITX2 polypeptide. Suitable agents for reducing or abrogating gene expression include, but are not restricted to, oligoribonucleotide sequences, including anti-sense RNA and DNA molecules, RNAi molecules and ribozymes, that function to inhibit the translation, for example, of PITX2-encoding mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions are preferred.

[0250] Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyse endonucleolytic cleavage of target sequences. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and...
GUC. Once identified, short RNA sequences of between 15 and 25 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

[0251] Both anti-sense RNA and DNA molecules and ribozymes may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesising oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

[0252] Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo-or deoxy-nucleotides to the 5' or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

[0253] Alternatively, RNA molecules that mediate RNA interference (RNAi) of a target gene or gene transcript can be used to reduce or abrogate gene expression. RNAi refers to interference with or destruction of the product of a target gene by introducing a single stranded, and typically a double stranded RNA (dsRNA) that is homologous to the transcript of a target gene. Thus, in some embodiments, dsRNA per se and especially dsRNA-producing constructs corresponding to at least a portion of a target gene may be used to reduce or abrogate its expression. RNAi-mediated inhibition of gene expression may be accomplished using any of the techniques reported in the art, for instance by transfecting a nucleic acid construct encoding a stem-loop or hairpin RNA structure into the genome of the target cell, or by expressing a transfected nucleic acid construct having homology for a target gene from between convergent promoters,
or as a head to head or tail to tail duplication from behind a single promoter. Any
similar construct may be used so long as it produces a single RNA having the ability to
fold back on itself and produce a dsRNA, or so long as it produces two separate RNA
transcripts which then anneal to form a dsRNA having homology to a target gene.

[0254] Absolute homology is not required for RNAi, with a lower threshold
being described at about 85% homology for a dsRNA of about 200 base pairs (Plasterk
and Ketting, 2000, Current Opinion in Genetics and Dev. 10: 562-67). Therefore,
depending on the length of the dsRNA, the RNAi-encoding nucleic acids can vary in the
level of homology they contain toward the target gene transcript, i.e., with dsRNAs of
100 to 200 base pairs having at least about 85% homology with the target gene, and
longer dsRNAs, i.e., 300 to 100 base pairs, having at least about 75% homology to the
target gene. RNA-encoding constructs that express a single RNA transcript designed to
anneal to a separately expressed RNA, or single constructs expressing separate
transcripts from convergent promoters, are suitably at least about 100 nucleotides in
length. RNA-encoding constructs that express a single RNA designed to form a dsRNA
via internal folding are usually at least about 200 nucleotides in length.

[0255] The promoter used to express the dsRNA-forming construct may be
any type of promoter if the resulting dsRNA is specific for a gene product in the cell
lineage targeted for destruction. Alternatively, the promoter may be lineage specific in
that it is only expressed in cells of a particular development lineage. This might be
advantageous where some overlap in homology is observed with a gene that is
expressed in a non-targeted cell lineage. The promoter may also be inducible by
externally controlled factors, or by intracellular environmental factors.

[0256] In other embodiments, small inhibitory RNA (siRNA) molecules of
about 18 to about 25 nucleotides, which direct cleavage of specific mRNA to which
they correspond, as for example described by Tuschl et al. in U.S. Patent Application
Publication No. 20020086356, can be utilised for mediating RNAi. Such 18-25 nt RNA
molecules can comprise a 3’ hydroxyl group, can be single-stranded or double stranded
(as two 18-25 nt RNAs) wherein the dsRNA molecules can be blunt ended or comprise
overhanging ends (e.g., 5′, 3′).

[0257] The invention also features methods of screening for agents that
modulate PITX2 expression or the level or function activity of a PITX2 polypeptide
(e.g., PITX2A, PITX2B, PITX2c, etc). In specific examples, the agents inhibit PITX2 expression or reduce or abrogate the level of functional activity of a PITX2 polypeptide. In some embodiments, the methods comprise: (1) contacting a preparation with a test agent, wherein the preparation contains (i) a polypeptide comprising an amino acid sequence corresponding in whole or in part to the amino acid sequence of a PITX2 polypeptide; or (ii) a polynucleotide comprising a genetic sequence that regulates PITX2 expression, wherein the genetic sequence is operably linked to a reporter gene; and (2) detecting a decrease in the level and/or functional activity of the PITX2 polypeptide, or of an expression product of the reporter gene, relative to a normal or reference level and/or functional activity in the absence of the test agent, which indicates that the agent inhibits PITX2 expression or reduces the level or functional activity of the PITX2 polypeptide.

[0258] In other embodiments, candidate agents are evaluated for their ability to alter PITX2 expression by contacting a candidate agent with a V7X2-expressing cell and then assaying for the effect of the candidate agent on expression. In illustrative examples of this type, the effect of candidate agents on PITX2 expression is assayed for by detecting the level of a PITX2 transcript (i.e., mRNA) expressed by the cell. PITX2 transcripts can be detected by any suitable method.

[0259] In still other embodiments, the effect of candidate agents on PITX2 expression is assayed by measuring the level of a PITX2 polypeptide (e.g., PITX2A, PITX2B, PITX2c, etc). The level of polypeptide expressed can be measured using any suitable method, including but not limited to, ELISA, cell-based ELISA, inhibition ELISA, Western blots, immunoprecipitation, slot or dot blot assays, immunostaining, RIA, scintillation proximity assays, fluorescent immunoassays using antigen-binding molecule conjugates or antigen conjugates of fluorescent substances such as fluorescein or rhodamine, Ouchterlony double diffusion analysis, immunoassays employing an avidin-biotin or a streptavidin-biotin detection system, and nucleic acid detection assays including reverse transcriptase polymerase chain reaction (RT-PCR).

[0260] Modulators falling within the scope of the present invention include inhibitors of PITX2 expression or PITX2 activity, including antagonistic antigen-binding molecules, and inhibitor peptide fragments, antisense molecules, ribozymes, RNAi molecules, siRNA molecules and co-suppression molecules. Candidate agents
encompass numerous chemical classes, though typically they are organic molecules, suitably small organic compounds having a molecular weight of more than 50 and less than about 2,500 Dalton. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agent often comprises cyclical carbon or heterocyclic structures or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogues or combinations thereof.

[0261] Small (non-peptide) molecule modulators of a PITX2 polypeptide are particularly advantageous. In this regard, small molecules are desirable because such molecules are more readily absorbed after oral administration, have fewer potential antigenic determinants, or are more likely to cross the cell membrane than larger, protein-based pharmaceuticals. Small organic molecules may also have the ability to gain entry into an appropriate cell and affect the expression of a gene (e.g., by interacting with the regulatory region or transcription factors involved in gene expression); or affect the activity of a gene by inhibiting or enhancing the binding of accessory molecules.

[0262] Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc to produce structural analogues.

[0263] Screening may also be directed to known pharmacologically active compounds and chemical analogues thereof.

[0264] The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone, which are resistant
to enzymatic degradation but which nevertheless remain bioactive; see, e.g.,


[0266] In a series of embodiments, the present invention provides assays for identifying small molecules or other compounds (i.e., modulatory agents) which are capable of modulating the level or functional activity of a PITX2 polypeptide for treating, preventing or inhibiting the development or progression of a skeletal metastasis-associated, hormone-related cancer in a subject. The assays may be performed in vitro using non-transformed cells, immortalized cell lines, or recombinant cell lines. In addition, the assays may detect the presence of decreased expression of genes or production of proteins on the basis of decreased mRNA expression (using, for example, nucleic acid probes that hybridise to a PITX2 gene or coding sequence thereof), decreased levels of a PITX2 polypeptide (using, for example, antigen binding molecules that are immuno-interactive with a PITX2 polypeptide), or decreased levels of expression of a reporter gene (e.g., GFP, β-galactosidase or luciferase) operably
linked to a *PITX2* regulatory region (e.g., a promoter or enhancer) in a recombinant construct.

[0267] Thus, for example, one may culture cells which produce a *PITX2* polypeptide and add to the culture medium one or more test compounds. After allowing a sufficient period of time (e.g., 6-72 hours) for the compound to modulate the level or functional activity of the *PITX2* polypeptide, any change in the level from an established baseline may be detected using, for example, any of the techniques described herein or known in the art. Using suitable nucleic acid probes or antigen-binding molecules, detection of changes in the level and or functional activity of a *PITX2* expression product, and thus identification of the compound as a *PITX2* inhibitor or antagonist, requires only routine experimentation.

[0268] In other embodiments, recombinant assays are employed in which a reporter gene encoding, for example, GFP, β-galactosidase or luciferase is operably linked to the 5" regulatory regions of a *PITX2* gene. Such regulatory regions may be easily isolated and cloned by one of ordinary skill in the art. The reporter gene and regulatory regions are joined in-frame (or in each of the three possible reading frames) so that transcription and translation of the reporter gene may proceed under the control of the *PITX2* regulatory elements. The recombinant construct may then be introduced into any appropriate cell type although mammalian cells are desirable, and human cells are more desirable. The transformed cells may be grown in culture and, after establishing the baseline level of expression of the reporter gene, test compounds may be added to the medium. The ease of detection of the expression of the reporter gene provides for a rapid, high throughput assay for the identification of *PITX2* inhibitors or antagonists of the invention.

[0269] In other embodiments, methods of identifying agents that modulate *PITX2* activity are provided in which a purified preparation of a *PITX2* polypeptide is incubated in the presence and absence of a candidate agent under conditions in which the *PITX2* is active, and the functional activity of *PITX2* is measured by a suitable assay. For example, a *PITX2* inhibitor can be identified by measuring binding of the *PITX2* polypeptide to a cognate promoter sequence (e.g., *PLOD-1* and *PLOD-2* promoters).
In still other embodiments, random peptide libraries consisting of a large number of possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to a PITX2 polypeptide or to a functional domain thereof. Identification of molecules that are able to bind to a PITX2 polypeptide may be accomplished by screening a peptide library with a recombinant soluble PITX2 polypeptide. The PITX2 polypeptide may be purified, recombinantly expressed or synthesized by any suitable technique. Such polypeptides may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook, et al, (1989, supra) in particular Sections 16 and 17; Ausubel et al, ("Current Protocols in Molecular Biology", John Wiley & Sons Inc, 1994-1998), in particular Chapters 10 and 16; and Coligan et al, ("Current Protocols in Immunology", (John Wiley & Sons, Inc, 1995-1997), in particular Chapters 1, 5 and 6. Alternatively, a PITX2 polypeptide may be synthesised using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 of Atherton and Shephard (supra) and in Roberge et al (1995, Science 269: 202).

To identify and isolate the peptide/solid phase support that interacts and forms a complex with the PITX2 polypeptide it is generally desirable to label or "tag" the PITX2 polypeptide. In this regard, the PITX2 polypeptide can be conjugated to any suitable reporter molecule, including enzymes such as alkaline phosphatase and horseradish peroxidase and fluorescent reporter molecules such as fluorescein isothiocyanate (FITC), phycoerythrin (PE) and rhodamine. Conjugation of any given reporter molecule, with a PITX2 polypeptide, may be performed using techniques that are routine in the art. Alternatively, PITX2 expression vectors may be engineered to express a chimeric PITX2 polypeptide containing an epitope for which a commercially available antigen-binding molecule exists. The epitope specific antigen-binding molecule may be tagged using methods known in the art including labelling with enzymes, fluorescent dyes or coloured or magnetic beads.

For example, the "tagged" PITX2 polypeptide conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between PITX2 polypeptide and peptide species within the library. The library is then washed to remove any unbound PITX2 polypeptide. If the PITX2 polypeptide has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrate for either alkaline
phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase- PITX2 polypeptide complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescently tagged PITX2 polypeptide has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric PITX2 polypeptide having a heterologous epitope has been used, detection of the peptide/ PITX2 polypeptide complex may be accomplished by using a labeled epitope specific antigen-binding molecule. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

[0273] Compounds identified by these methods will have potential utility in reducing the expression of PITX2 in vivo. These compounds may be further tested in the animal models or ex vivo assays to identify those compounds having the most potent in vivo effects. For example, putative PITX2 inhibitors can be further screened for an in vivo functional activity such as, but not limited to, inhibition of cell proliferation or cell motility. In illustrative examples of this type, a PC3 cell is exposed to, or cultured in the presence and absence of, a putative PITX2 inhibitor under conditions in which the PITX2 polypeptide is active in the PC3 cell, and an activity relating to PC3 cell proliferation or motility of the PC3 cell towards bone is detected. An agent tests positive if it inhibits any of these activities.

[0274] In addition, these molecules may serve as "lead compounds" for the further development of pharmaceuticals by, for example, subjecting the compounds to sequential modifications, molecular modeling, and other routine procedures employed in rational drug design.

7. Pharmaceutical compositions

[0275] In accordance with the present invention, it is proposed that agents that inhibit or antagonize PITX2 activity are useful as drugs for treating or preventing, or ameliorating the symptoms or reversing or inhibiting the development or progression of skeletal metastasis-associated, hormone-related cancers. Such drugs can be administered to a patient either by themselves, or in pharmaceutical compositions where they are mixed with a suitable pharmaceutically acceptable carrier.
[0276] The PITX2 inhibitors or antagonists of the present invention may be conjugated with biological targeting agents which enable their activity to be restricted to particular cell types. Such biological-targeting agents include substances which are immuno-interactive with cell-specific surface antigens (e.g., PSMA).

[0277] Depending on the specific conditions being treated, the drugs may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition. Suitable routes may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. For injection, the drugs of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

[0278] The drugs can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated in dosage forms such as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. These carriers may be selected from sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulphate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

[0279] Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. The dose of drug administered to a patient should be sufficient to effect a beneficial response in the patient over time such as inhibiting the growth and/or proliferation of cancer or tumor cells or for causing cancer or tumor cell death. The quantity of the drug(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition
thereof. In this regard, precise amounts of the drug(s) for administration will depend on the judgement of the practitioner. In determining the effective amount of the drug to be administered in the treatment of a skeletal metastasis-associated, hormone-related cancer, the physician may evaluate tissue levels of PITX2 expression products, and stage or degree of cancer progression. In any event, those of skill in the art may readily determine suitable dosages of the drugs of the invention.

[0280] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilisers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[0281] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatine, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more drugs as described above with the carrier which constitutes one or more necessary ingredients. In general, the pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.
Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterise different combinations of active compound doses.

Pharmaceutical which can be used orally include push-fit capsules made of gelatine, as well as soft, sealed capsules made of gelatine and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, or lubricants such as talc or magnesium stearate and, optionally, stabilisers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilisers may be added.

Dosage forms of the drugs of the invention may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of an agent of the invention may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactide and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, controlled release may be effected by using other polymer matrices, liposomes or microspheres.

PITX2-modulating drugs may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulphuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 as determined in cell culture (e.g., the concentration of a test agent, which achieves a half-maximal inhibition in an activity of a PITX2 polypeptide, illustrative examples of which include, DNA-binding,
transcriptional, cell proliferation and cell motility activities). Such information can be used to more accurately determine useful doses in humans.

[0287] Toxicity and therapeutic efficacy of such drugs can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilised. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See for example Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 pi).

[0288] Dosage amount and interval may be adjusted individually to provide plasma levels of the active agent which are sufficient to maintain PITX2-inhibitory effects. Usual patient dosages for systemic administration range from 1-2000 mg/day, commonly from 1-250 mg/day, and typically from 10-150 mg/day. Stated in terms of patient body weight, usual dosages range from 0.02-25 mg/kg/day, commonly from 0.02-3 mg/kg/day, typically from 0.2-1.5 mg/kg/day. Stated in terms of patient body surface areas, usual dosages range from 0.5-1200 mg/m²/day, commonly from 0.5-150 mg/m²/day, typically from 5-100 mg/m²/day.

[0289] Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a tissue, which is preferably subcutaneous or omental tissue, often in a depot or sustained release formulation.

[0290] Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the tissue.
In cases of local administration or selective uptake, the effective local concentration of the agent may not be related to plasma concentration.

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

EXAMPLE 1

ABERRANT EXPRESSION OF PITX2 IN PROSTATE CANCER

Subcutaneous Tumor Formation

Subcutaneous tumors were formed by both LNCaP and PC3 cell lines using the Matrigel extracellular matrix substrate, with the PC3 cell line more tumorigenic (Figure 1). Histologic analysis of the subcutaneous tumors was performed by an experienced anatomical pathologist (Figure 2). Initial histological examination indicated that tumors formed from PC3 cells had high proliferation rate evident by multiple mitotic bodies. Muscle invasion and perineural invasion were seen in some tumors from each cell type (not shown). Tumors formed from the LNCaP cell line showed a more structured architecture with less cellular pleomorphism and a high mitotic rate.

A detailed assessment of the mitotic index of each tumor was performed to investigate a possible cause of the variation in gross histology and tumor volume between groups. No significant difference between the PC3 and LNCaP mitotic indices was observed (Figure 3).

Analysis of Gene Expression in Subcutaneous Tumors

The Wnt pathway expression profiles of PC3 and LNCaP tumors were obtained using real time PCR arrays which allow simultaneous analysis of 83 Wnt pathway associated genes relative to five inbuilt housekeeping genes. Significant but relatively modest (1 - 3 fold) variations between the cell lines in Wnt gene expression were detected between the PC3 and LNCaP tumor types (data not shown). One gene however, PITX2, was over-expressed 38 fold in the PC3 tumors compared to the LNCaP tumors (P=0.0002) (Figure 4).
Array results for PITX2 were confirmed by further qPCR using independent gene specific primers (normalized to the housekeeping gene cyclophilin) (Figure 5).

Materials and Methods

Cell Culture

The cell lines PC3 and LNCaP were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were incubated at 37°C in 5% CO2. Cell lines were grown in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin [P/S] (Invitrogen).

RNA Extraction from Cell Culture

RNA preparations from all cell cultures were made using 1 mL of Trizol reagent (Invitrogen) and then processed as per manufacturer's instructions. Some experiments required the resulting RNA pellet cleaned up using the RNeasy Minikit (Qiagen Inc, CA USA) as per manufacturer's instructions including the optional on-column DNase I steps. All samples were then assessed for concentration using the NanoDrop ND-1000 spectrophotometer (Biolab; Scoresby, VIC, Australia), requiring 260/280 >2.0 and 260/240 >1.7 and RNA quality by assessing 18S and 28S band intensities on agarose gel electrophoresis. If the RNeasy Minikit was not used, the resulting total RNA was DNase treated using RNase Free DNase I kit (Ambion/Applied Biosystems Tx, USA) as per the manufacturer's instructions and the RNA concentration re-assessed.

Preparation of cDNA

First strand synthesis was done using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. The RT reaction mix was 1×First-Strand Buffer, 200 U/µL Superscript III, 40 U/µL RNaseOUT, 5 M DTT, 500 µM of each dNTP (Roche), and 250 ng random primers (Promega). DNase I treated RNA (1 µg) was incubated with dNTPs and random primers in a reaction volume of 13 µL at 65°C for 5 min and then cooled on ice for at least 1 min. Remaining reagents of the RT reaction mix were added to a final volume of 20 µL and samples incubated at 25°C for 5 min, 50°C for 1 h, and 70°C for 15 min. All reactions were carried out in the PCR Express thermal cycler (Hybaid Ltd, Middlesex, UK). cDNA was aliquoted and stored at -20°C.
Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Specific transcript levels were analyzed by quantitative RT-PCR using gene specific primers on the Rotor-gene RG-3000 or Rotor-gene RG-6000 thermocyclers (Corbett Research). Reactions contained 1xSYBR premix (Takara) and 0.2 µM of each primer in a volume of 15 µL. PCR conditions were held at 95°C for 10 s, followed by 45 cycles of denaturation and extension at 95°C for 5 s and 60°C for 20 s respectively. Amplification of specific targets was validated using melt curve analysis using the Rotorgene version 6 software. Target gene expression was quantified relative to the expression of cyclophilin housekeeping gene, using the ΔΔCT calculation method.

Protein Harvesting from Cell Culture

Whole cell lysate protein preparations from all cell lines were similarly prepared using lysis buffer containing 1 M TRIS pH 8.0, 10% Triton x-100, 0.5M EDTA, protease inhibitor cocktail (Roche, Ind, USA) and H2O. After rinsing cells in cold PBS, 250 µL of cold lysis buffer was placed directly onto cells which were scraped into solution. The solution was placed on ice for 15 minutes with intermittent passing of lysate through a 26G needle to shear DNA. The lysate was then spun for 5min @ 10000 RPM in a centrifuge at 4°C. The resulting supernatant was then aliquoted into 50 µL volumes and stored at -80°C for later immunoblot analysis.

Protein Concentration Determination

Concentration of protein whole cell lysates was determined relative to a BSA (Pierce) standard curve using the bicinchoninic acid (BCA) protein assay reagents (Pierce Biotechnology Inc, Rockford, IL, USA) according to the manufacturer's instructions. Lysates were incubated with 50:1 ratio of assay reagents A:B at 37°C for 1 h and quantitated on the Multiskan plate reader (Labsystems) at 595 run.

Immunoblot Analysis

Lysates were boiled in loading dye at 100°C C for 10 min and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-FL (for Odyssey detection) or Immobilon-P (for ECL detection) PVDF membrane (Millipore, Bedford, Massachusetts, USA). Immobilon-FL membranes were blocked...
with Odyssey blocking solution [a 1:1 solution of PBS and Odyssey blocking buffer (LI-COR Biosciences)] for 1 h at room temperature, and incubated with primary antibody diluted in fresh PBS:Odyssey blocking solution at 4°C overnight. Membranes were washed with four changes of 0.1% Tween-20/PBS (PBS-T) for a total of 20 min, and incubated in fluorescent conjugated secondary antibodies diluted in fresh PBS:Odyssey blocking buffer for 1 h. Membranes were washed in PBS-T as above and scanned and analysed using the LI-COR Odyssey Infrared Imaging System and software. Immobilon-P membranes were blocked in 5% skim milk/0.05% Tween-20/PBS for 1 h at room temperature, and incubated in primary antibody diluted in 2% skim milk/0.05% Tween-20/PBS at 4°C overnight. Membranes were washed in PBS-T four times and incubated in horse-radish peroxidise conjugated secondary antibody in 2% skim milk/0.05% Tween-20/PBS for 1 h at room temperature and washed four times. Blots were incubated with the Supersignal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer's instruction. Blots were exposed to film (Fujifilm) or detected using the CHEMI 5000 chemiluminescence documentation system (Viber Lourmat, Marne-la- Vallee Cedex, France), and signal quantitated using ImageQuant version 5.2 software.

Subcutaneous Tumor Formation in NOD SCID Mice

[0304] Cell lines prepared and grown as above were used in the generation of subcutaneous tumors in NOD SCID mice.

NOD SCID Mice

[0305] All animals were treated and maintained according to University of Queensland animal ethics guidelines and ethical approvals were obtained before any experimentation. Animals were maintained under specific-pathogen-free temperature controlled conditions. Cages, bedding food and water were autoclaved. 5 week old male NOD SCID mice were obtained from the supplier (Animal Resource Centre [ARC], Perth, WA) and allowed to settle for a period of one week prior to the commencement of any experimentation.

Subcutaneous Injection Of Tumor Cells In NOD SCID Mice

[0306] Cell lines were injected subcutaneously into the right flank of NOD SCID mice in Growth Factor Reduced Matrigel Matrix (BD Biosciences) as per manufacturers' instructions to facilitate tumor formation. Cells were suspended and
injected subcutaneously at $1 \times 10^5$ PC3 cells or $1 \times 10^6$ LNCaP cells. Individual mice in each treatment group were identified by ear punch and recorded. Mice were then observed on a weekly basis for six weeks using the NOD SCID mouse score sheet, taking note of weight and tumor dimensions which were recorded using callipers. If a score of 3 in any category, a total score >10 or weight loss >20% body weight was recorded, the animal was immediately euthanized and tumor collected as below.

**Euthanasia and Subcutaneous Tumor Collection**

[0307] At 6 weeks post injection of prostate cancer cell lines animals were euthanased by carbon dioxide asphyxiation as per University of Queensland standard operating procedure. Subcutaneous tumors were immediately excised post-mortem. Tumor dimensions and weight were recorded for subsequent evaluation. Tumor volume was estimated by the formula: $a \times b^2/2$, where $a$ is the longest dimension and $b$ is the width (Nemeth et al, 1999, Cancer Res 59(8): 1987-93). Half of each tumor was placed in cold neutral buffered formalin [NBF] (Sigma) and fixed under vacuum at room temperature over night, then transferred to 70% ETOH for storage prior to paraffin embedding and sectioning. The remaining half of the tumor mass was snap frozen in liquid nitrogen and then stored at -70° C prior to RNA preparation.

**Statistics**

[0308] Data analysis was performed using Microsoft Excel 2007 (Microsoft Corp, Redmond, WA, USA) and R version 2.7.2, pgirmess library (Team RDC, Vienna, Austria). Data were analysed using ANOVA and the Student's $t$ test to evaluate the significance of the difference in mean values between cell lines, treatments and specimens. P values <0.05 were considered to indicate statistically significant differences. Data are represented as mean ± standard error (SE) unless otherwise stated.

**EXAMPLE 2**

INVESTIGATION OF PITX2 IN CLINICAL SAMPLES AND ISOFORM EXPRESSION

**Prostate Cancer Clinical Sample Array Analysis**

[0309] Multiple gene expression differences between normal prostate, primary prostate cancer and metastatic prostate cancer samples were noted (data not shown). The most significant differences, in terms of both fold change and P value were those between PITX2 expression levels in the various samples (Table 1).
TABLE 1: Normalized PITX2 expression levels in mouse subcutaneous tumors derived from human prostate cancer cell lines, and in human clinical specimens.

<table>
<thead>
<tr>
<th>Gene</th>
<th>LNCaP (ΔΔCt)</th>
<th>MDA (ΔΔCt)</th>
<th>PC3 (ΔΔCt)</th>
<th>Normal Prostate (ΔΔCt)</th>
<th>Benign Prostatic Hypertrophy (BPH) (ΔΔCt)</th>
<th>Primary Prostate Cancer (ΔΔCt)</th>
<th>Metastatic Prostate Cancer (Bone) (ΔΔCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PITX2</td>
<td>2.8E-05</td>
<td>2.6E-05</td>
<td>1.1E-03</td>
<td>1.5E-04</td>
<td>2.9E-02</td>
<td>3.7E-03</td>
<td>3.5E-01</td>
</tr>
</tbody>
</table>

PITX2 expression in the bone metastasis sample was 324, 12327 and 13367 fold higher than in subcutaneous tumors generated from the PC3 (p=0.0003), LNCaP (p=0.00004) and MDA (p=0.00002) cell lines respectively. This high level of expression in the bone metastasis also exceeded the levels in normal prostate (2252 fold, p=0.0005), primary prostate cancer (227 fold, p=0.0008) and BPH samples (13 fold, p=0.012) [Figures 6 A and B].

To confirm the array results, an independent qPCR analysis was conducted using a pair of universal PITX2 primers designed to amplify all transcripts encoding the three major PITX2 isoforms (Figure 7 A and B).

To test whether PITX2 expression increased with disease progression, a subgroup analysis of available primary cancer samples taking into account Gleason score was conducted. The results of this analysis indicated a trend to higher PITX2 expression with increasing grade (Figure 8).

Investigation of PITX2 Isoform Expression in Cell Lines and Clinical Samples

Expression levels of total PITX2 transcripts as well as individual PITX2 A, B and C transcript species were assessed in the cell line derived subcutaneous mouse tumors and in clinical specimens using the Universal and isoform specific primer pairs (Table 2 and 3).
**TABLE 2:** Normalized expression of transcripts encoding individual PITX2 isoforms in subcutaneous mouse tumors generated from human cell lines assessed by quantitative PCR using isoform-specific primer pairs. The PITX2B- and 2C-encoding transcripts appear to be the major RNA species present in PC3 tumors, at higher levels than any PITX2 species in either LNCaP or MDA PCa.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PITX2A Normalised Expression (Universal Primers)</th>
<th>PITX2A Normalised Expression (Specific Primers)</th>
<th>PITX2B Normalised Expression (Specific Primers)</th>
<th>PITX2C Normalised Expression (Specific Primers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>1.6E-04</td>
<td>1.5E-07</td>
<td>5.3E-05</td>
<td>2.6E-05</td>
</tr>
<tr>
<td>LnCaP</td>
<td>1.4E-05</td>
<td>5.5E-07</td>
<td>2.3E-08</td>
<td>4.0E-07</td>
</tr>
<tr>
<td>MDA PCa 2b</td>
<td>9.2E-06</td>
<td>1.9E-06</td>
<td>9.9E-08</td>
<td>1.1E-07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>PITX2A Normalised Expression (Universal Primers)</th>
<th>PITX2A Normalised Expression (Specific Primers)</th>
<th>PITX2B Normalised Expression (Specific Primers)</th>
<th>PITX2C Normalised Expression (Specific Primers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Prostate (n=2)</td>
<td>8.4E-04</td>
<td>0</td>
<td>2.7E-05</td>
<td>1.6E-06</td>
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<tr>
<td>Primary CaP (n=9)</td>
<td>3.4E-03</td>
<td>2.2E-05</td>
<td>6.8E-05</td>
<td>2.0E-05</td>
</tr>
<tr>
<td>Human Met (n=1)</td>
<td>9.6E-01</td>
<td>3.5E-02</td>
<td>1.7E-01</td>
<td>8.4E-02</td>
</tr>
</tbody>
</table>
TABLE 3: Normalized levels of PITX2 encoding transcripts in clinical tissue samples as assessed using quantitative PCR with Universal and isoform-specific primers. The PITX2B-expressing transcript appears to represent the major RNA species present in both primary prostate cancer and metastatic prostate cancer, but with RNA species encoding all three PITX2 isoforms expressed at much higher levels in the metastatic deposit.

The expression data in Tables 2 and 3 indicate that the PITX2B-encoding transcript was the most highly expressed RNA species in PC3 derived mouse tumors as well as in the clinical samples, with the levels in clinical samples rising incrementally from normal prostate tissue to primary cancer, to metastasis. The A and C isoform encoding transcripts were detected in the malignant tissue samples at lower levels but also in an incrementally increasing pattern with the C transcript more highly expressed than the A species. The PITX2A transcript was not detected in the normal prostate or in BPH, whereas the C isoform encoding transcript was present at a low level. Expression patterns and levels in the primary cancer specimens were almost identical to those in the PC3 based mouse tumors.

**Protein Expression of PITXHn Clinical Samples**

To determine whether PITX2 protein was also produced at varying levels in normal and malignant prostate tissues a variety of clinical specimens were assessed by immunohistochemical staining using the P2R10 antibody which is designed to detect all three major isoforms of PITX2 protein.

Antibody specificity was initially confirmed by Western immunoblot analysis of protein lysates from PC3 cells transiently over-expressing individual PITX2 protein isoforms, each also epitope-tagged with the FLAG peptide (Figure 9) and
staining of control cell blocks of LNCaP (negative) and PC3 (positive) tumors (Figure 10).

The specificity and sensitivity of PITX2 protein detection by immunohistochemistry using the P2R10 antibody was then assessed. No signal was observed on negative control sections of paraffin embedded LNCaP cells (Figure 9A) and liver tissue (Figure 9C), whereas robust nuclear and cytoplasmic signals were detected on sections of a PC3-derived subcutaneous tumor (Figure 9B). These results indicate that the P2R10 immunohistochemistry protocol for detection of PITX2 is both specific and sensitive.

PITX2 protein expression in clinical specimens was then investigated using these validated immunohistochemistry conditions. Sections of radical prostatectomy specimens were stained using the P2R10 antibody, along with matched H&E and PSA stained sections from the same specimens (Figure 10). In sections with areas of primary cancer and adjacent normal prostate (panel A), there was strong PSA staining of normal epithelial cells and light cellular staining of malignant epithelium (panel B). In contrast, PITX2 immunohistochemistry on the same specimen yielded light staining of normal epithelial tissue and strong staining of malignant glands. The relative levels of PSA and PITX2 staining in normal and malignant epithelial regions of the same specimen therefore appear to occur in reverse patterns in some specimens.

Not all normal epithelium from archival radical prostatectomy specimens stained positive for PITX2 (Figure 11A) however. It is possible that this is related to a greater distance of the normal cells from malignant glands in this section, or simply due to poor fixation of the negative tissues. Sharply contrasting with this poorly stained specimen, TRUS biopsy tissue showed abundant PITX2 staining, with the majority of nuclei showing high concentrations of this transcription factor (Figure 11B).

Based on the molecular and histological evidence that some primary tumors were positive for PITX2 protein, along with the array results indicating strong expression of the gene in one clinical bone metastasis specimen (see "Prostate Cancer Clinical Sample Array Analysis"), histological evidence for PITX2 protein involvement in bone metastasis was sought using sections of an archival clinical bone metastasis specimen (Figure 12A). PSA protein expression was patchy and present at a relatively
low level in the cancer cells (panel B) compared to the very high levels of nuclear and cytoplasmic PITX2 staining (panel C).

[0321] As is the case with most prostate cancer bone metastases, although the specimen was predominantly osteoblastic (Figure 13 panel A), there were also areas of osteolytic activity with cortical bone destruction and no evidence of bone formation (panel B). The intensity of PITX2 staining appeared to differ in a pattern related to bone response, as staining was intense in an osteolytic region (panel C) but became lighter as the lesion pathology became more osteoblastic (direction of the arrow in panel D), fading to virtual absence of PITX2 signal in the highly osteoblastic area (Panel E).

[0322] Just as not all normal prostate samples or primary cancers appeared to stain for PITX2, some prostate cancer bone metastases also appeared to be negative for PITX2 expression. Interestingly, of the samples examined to date, PSA staining was stronger in the PITX2-negative metastases and vice versa (Figure 14).

[0323] The evidence from this limited set of clinical samples therefore suggests that the quality and pattern of PITX2 staining is likely to be dependent on the degree of tissue fixation, with the larger prostatectomy specimens inadequately fixed compared to the TRUS biopsies, with their small size relative to fixative volume under typical collection protocols. Even in PITX2 positive areas of the larger prostatectomy specimens, the small number of positive nuclei may be due to leakage of nuclear PITX2 into the cytoplasm of underfixed cells, as observed for other proteins.

[0324] To summarize, the results presented here identify a previously unrecognized factor, PITX2, which appears to be over-expressed in a proportion of metastatic prostate cancer specimens. Other novel data in this study indicate that all three isoforms are over-expressed in metastatic disease with the B isoform predominant.

[0325] In some specimens of normal prostate very low levels of PITX2B and/or C isoforms are expressed, whereas other specimens do not express the gene. The level of PITX2 production is elevated in primary cancer (with all three isoforms present in some specimens) and reaches very high levels in metastatic disease. This stepwise increase in expression of PITX2 suggests a role for the gene in progression from normal to malignant disease and strongly implicates it in metastasis.
The interesting observation that some "normal" prostate epithelium is weakly positive for PITX2 whilst other specimens are completely negative suggests a possible role for the gene as a biomarker in the prognosis of prostate cancer. Similarly the incremental increase in PITX2 staining from primary to metastatic disease suggests a role for this protein in tumor progression and thus serves as a prognostic factor for metastatic disease.

A reason for the lack of consistent expression across metastasis samples may relate to the small amount of material collected by the laser capture technique. PITX2 expression is non-uniform across individual samples (as seen in the IHC data presented herein), the micro-dissection of small groups of cells for RNA preparation may have failed to collect the PITX2 expressing cells present in the metastatic specimens. Similarly, the region selected for micro-dissection may have led to the negative expression data, if for example a blastic region was chosen for sampling instead of a more lytic region.

PITX2 may therefore act as a molecular switch to convert an initial osteolytic phenotype, which would enable the establishment of the deposit, to an osteoblastic phenotype typically associated with prostate cancer metastases.

A role for PITX2 in the development of castrate resistance may be implied from the preceding results on a number of fronts. Firstly all bone metastasis samples positive for PITX2 expression at the protein or mRNA level were in the castrate resistance phase of disease. The inverse correlation of PSA staining (the quintessential androgen dependent gene) and PITX2 staining in clinical samples may also implicate this gene in such a role.

Materials and Methods

Clinical Samples

Fresh frozen clinical primary prostate cancer, normal prostate tissue, benign hypertrophy (BPH) tissue and prostate bone metastasis tissues were obtained for quantitative PCR analysis, as well as random but comparable archived paraffin embedded samples for immunohistochemical analysis under the appropriate Hospital and University Human Ethics approvals.
RNA Preparation and qRT PCR Analysis

RNA was prepared from the fresh frozen samples as per Example 1. Following assessment of RNA and cDNA quality (as per Example 1), real-time PCR analysis was carried out using the SA Bioscience RT² Profiler PCR Wnt pathway Array system (as per Example 1). One bone marrow prostate cancer metastasis sample, 2 normal prostate tissue samples, 3 BPH tissue samples and 9 primary prostate cancer tissue samples were analyzed. The bone marrow sample was in the form of reamings from insertion of a medullary nail as a prophylactic intervention for a pending pathologic fracture.

Confirmatory qRT-PCR analyses of PITX2 expression was performed on cDNA produced from tumor total RNA, using Universal and A, B and C isoform specific primers designed with the aid of Primer 3 (Rozen and Skaletsky Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S MSeBMaPM. Primer3 on the WWW for general users and for biologist programmers 2000. p. pp 365-86), Blasted for specificity (NCBI Blast) and then repeated using a second set of primers developed by Dr J Martens (see Table 4). Alignment of all primer sets to PITX2 mRNA sequence is illustrated in Figure 15.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence Forward</th>
<th>Primer Sequence Reverse</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PITX2 (Universal)</td>
<td>5’-CAGCCTGAGACTGAAAGCA-3’ [SEQ ID NO 9]</td>
<td>5’-GCCGACGCTTCTAGCAT-3’ [SEQ ID NO 10]</td>
<td>199</td>
</tr>
<tr>
<td>PITX2 A</td>
<td>5’-GCGTGTGTCATAATTAGAAAG-3’ [SEQ ID NO 11]</td>
<td>5’-CGAGGCCATTCTTGCATAG-3’ [SEQ ID NO 12]</td>
<td>302</td>
</tr>
<tr>
<td>PITX2 B</td>
<td>5’-GCCGTTGAATGGTCTTCTCTC-3’ [SEQ ID NO 13]</td>
<td>5’-CCTTTGCGTCTTCTCTTAG-3’ [SEQ ID NO 14]</td>
<td>198</td>
</tr>
<tr>
<td>PITX2 C</td>
<td>5’-ACTCTCCGTCTCCGACTT-3’ [SEQ ID NO 15]</td>
<td>5’-CGGAGCAGTCTACTAGTGCCTC-3’ [SEQ ID NO 16]</td>
<td>197</td>
</tr>
<tr>
<td>PITX2 (Universal 2nd set)</td>
<td>5’-GAGCTGGAGCCACTTTAC-3’ [SEQ ID NO 17]</td>
<td>5’-CGGAGCCATTCTTGCATAG-3’ [SEQ ID NO 18]</td>
<td>176</td>
</tr>
<tr>
<td>PITX2 A (2nd set)</td>
<td>5’-GCGTGTGTCATAATTAGAAAG-3’ [SEQ ID NO 19]</td>
<td>5’-CGGAGCCATTCTTGCAATG-3’ [SEQ ID NO 20]</td>
<td>302</td>
</tr>
<tr>
<td>PITX2 B (2nd set)</td>
<td>5’-GCCGTTGAATGGTCTTCTCTC-3’ [SEQ ID NO 21]</td>
<td>5’-CCTTTGCGTCTTCTCTTAG-3’ [SEQ ID NO 22]</td>
<td>198</td>
</tr>
</tbody>
</table>
TABLE 4: Specific qRT-PCR primers

<table>
<thead>
<tr>
<th>( \text{PITX2 C (2nd set)} )</th>
<th>5'-CTCATCTTCTCTGTCCATCCATC-3' [SEQ ID NO: 23]</th>
<th>5'-CCTTTGGCCCTTCTTCTTAG-3' [SEQ ID NO: 24]</th>
<th>211</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplphilin</td>
<td>5'-GCCGATAGGAGGCTTGGGCTG-3' [SEQ ID NO: 25]</td>
<td>5'-ACCAGTTGATTGTTGTTCG-3' [SEQ ID NO: 26]</td>
<td>187</td>
</tr>
<tr>
<td>18S</td>
<td>5'-TTCCGAACCTGAGCCATGAT-3' [SEQ ID NO: 27]</td>
<td>5'-CGAACCTCCGACTTTGTTCC-3' [SEQ ID NO: 28]</td>
<td>150</td>
</tr>
</tbody>
</table>

**Immunohistochemical Assessment of Paraffin Embedded Clinical Samples**

[0333] Archived paraffin embedded samples of primary prostate cancer, normal prostate and prostate cancer bone metastases were obtained as per the appropriate Princess Alexandra Hospital human ethics approval. Samples were either obtained at the time of radical prostatectomy, TRUS biopsy of the prostate or at Transurethral Resection of the prostate (TURP) with prior patient consent. Fresh samples were snap frozen in liquid nitrogen and then stored at -80°C. Sections were stained using H&E chemistry, or using Immunohistochemistry using the PSA antibody at 1:10000 (Dako) without antigen retrieval or the PITX2 P2R10/P2Y4 antibody at 1:100 or 1:200 (Capra Bioscience) after antigen retrieval at pH 6 (Dako). PITX2 positive and negative control cell blocks were created using cultured cells with and without PITX2 expression, respectively.

**Immunoblot Verification of PITX2 Antibody Specificity**

[0334] Western immunoblot analysis to test specificity of binding of the P2R10/P2Y4 antibody (1:5000) was performed on whole cell lysates of positive cells (PC3 cells transiently over-expressing PITX2) and negative control (LNCaP) cell lines as detailed below with signal detection using the LI-COR Odyssey Infrared Imaging System.

[0335] Immunoblot analysis was carried out as follows:

[0336] Lysates were boiled in loading dye at 100°C for 10 min and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-FL (for Odyssey detection) or Immobilon-P (for ECL detection) PVDF membrane (Millipore, Bedford, Massachusetts, USA). Immobilon-FL membranes were blocked with Odyssey blocking solution [a 1:1 solution of PBS and Odyssey blocking buffer
(LI-COR Biosciences)] for 1 h at room temperature, and incubated with primary antibody (1:1000) diluted in fresh PBS Odyssey blocking solution at 4°C overnight. Membranes were washed with four changes of 0.1% Tween-20/PBS (PBS-T) for a total of 20 min, and incubated in fluorescent conjugated secondary antibodies (1:10000) diluted in fresh PBS Odyssey blocking solution at 4°C overnight.

Membranes were washed with four changes of 0.1% Tween-20/PBS (PBS-T) for a total of 20 min, and incubated in fluorescent conjugated secondary antibodies (1:10000) diluted in fresh PBS Odyssey blocking buffer for 1 h. Membranes were washed in PBS-T as above and scanned and analysed using the LI-COR Odyssey Infrared Imaging System and software.

[0337] Immobilon-P membranes were blocked in 5% skim milk/0.05% Tween-20/PBS for 1 h at room temperature, and incubated in primary antibody diluted (1:5000) in 2% skim milk/0.05% Tween-20/PBS at 4°C overnight. Membranes were washed in PBS-T four times and incubated in horse-radish peroxidise conjugated secondary antibody (1:20000) in 2% skim milk/0.05% Tween-20/PBS for 1 h at room temperature and washed four times. Blots were incubated with the Supersignal West Pico, West Dura Extended Duration, or West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer's instruction. Blots were exposed to film (Fujifilm) or detected using the CHEMI 5000 chemiluminescence documentation system (Viber Lourmat, Marne-la-Vallee Cedex, France), and signal quantitated using ImageQuant version 5.2 software.

**Statistics**

[0338] Sample data from arrays were normalised to combined housekeeping gene data using the ΔΔCt method. Differences in expression levels were then assessed statistically using one-way analysis of variance followed by Student's T-test (P<0.05 considered significant). Data from other experiments were analysed using ANOVA and the Student's t test to evaluate the significance of the difference in mean values between cell lines, treatments and specimens. P values <0.05 were considered to indicate statistically significant differences. Data are represented as mean ± standard error (SE) unless otherwise stated.
EXAMPLE 3
IN VITRO INVESTIGATION OF PITX2 FUNCTIONAL ROLES IN PROSTATE CANCER PROGRESSION AND METASTASIS

Effect of co-culture with Saos-2 on expression of PITX2 in PC3 cells

[0339] As high levels of PITX2 expression had been detected in some metastatic prostate clinical samples (see Example 2), changes in PITX2 gene expression by two different prostate cancer cells lines in response to co-culture with the Saos-2 osteoblastic osteosarcoma cell line were investigated. A transwell co-culture system was employed to test for paracrine interactions via secreted factors. Co-culture with the Saos-2 cells increased PITX2 expression in the osteolytic PC3 cell line compared to control PC3 cultures grown on the inserts with no cells in the lower chamber, suggesting the production of a diffusible factor(s) by the osteoblastic bone cell that increased expression by the cancer cell (Figure 16). In contrast, no PITX2 transcripts were detected in the lymph-node derived LNCaP cells, whether cultured in the presence or absence of Saos-2 bone cells.

Overexpression of PITX2 isoforms in PC3 and LNCaP cells

[0340] Overexpression of genes of interest is a way to investigate protein function, and this approach was chosen to study the role of PITX2 in prostate cancer. Although the functions of different PITX2 protein isoforms are not well understood, their distinct N-terminal structures may lead to functional diversity in other systems (Lamba et al, 2008, BMC Molecular Biology 9(1):31). Therefore it was decided to test the functional significance of the three major isoforms of the PITX2 protein individually.

[0341] LNCaP cells were initially chosen for individual over-expression of PITX2 isoform A, B or C. Stably transfected lines were generated using antibiotic selection, and assessed for PITX2 isoform expression by qRT-PCR. Although PITX2 transcripts were readily detected using universal and B isoform-specific primers in the PITX2 5-transfected line (Figure 17A), levels of PITX2 A and C transcripts in the stable cell lines were very low with any primer pair tested (panel B).

[0342] LNCaP native cells had been chose for this experiment because they do not express measurable levels of any of the PITX2 gene isoforms. However, the
above results suggested that expression of PITX2 A or C may be incompatible with LNCaP cell viability, an impression that was upheld when identical results were obtained from a repeat of the experiment (not shown).

[0343] Investigation of the roles of the PITX2 isoforms in prostate cancer cell function was therefore pursued using a transient transfection approach in the PC3 cell line, which had previously been found to express endogenous transcripts encoding all three isoforms of PITX2. Very high levels of appropriate isoform-specific transcripts were detectable in the individual transiently transfected cultures (Figure 18).

[0344] PITX2 protein levels in transiently transfected cultures were then assessed by Western immunoblots. For this experiment, which was to serve as the basis for subsequent knock-down studies, the amount of input plasmid DNA was varied to define the minimum amount necessary to produce a measureable PITX2 protein signal (Figure 19). All three protein species were detected using the P2R10 anti-PITX2 antibody and M2 anti-FLAG antibody, with the signal intensity for the PITX2A protein lower than for the B and C isoforms. In addition, the signal for the A protein isoform dropped off more rapidly, becoming undetectable at the lowest input DNA value of 0.25 µg). By contrast the signals for B and C isoforms were robust at all DNA inputs tested. Upon quantification of signal intensities, these patterns were clearly visible (Figure 20).

[0345] Based on the minimum input DNA concentration for the PITX2 A plasmid, all subsequent over-expression and knockdown experiments were conducted using 0.5 µg of expression plasmid per well of a 6 well plate, or with input DNA and collection volumes scaled from this level for other culture configurations as appropriate. This experimental design allowed total DNA input for co-transfection experiments to remain in a non-toxic range.

Knockdown of PITX2 with shRNA

[0346] Knockdown of PITX2 in prostate cancer cell lines was undertaken using the purchased shRNA constructs, including four different anti-PITX2 shRNA sequences (each designed to target all three major isoforms) and a scrambled negative control sequence (NC). The capacity of each shRNA species to knockdown exogenous PITX2 transcripts as tested by transient co-transfection in PC3 cells with the isoform-specific PITX2 expression constructs, followed by transcript and protein analyses. Reasoning that endogenous PITX2 expression is substantially lower than that produced
by transient transfection of the expression constructs, a pilot study to determine the minimum amount of shRNA plasmid capable of knocking down endogenous PITX2 levels was performed. Each isoform-specific expression plasmid (0.5 µg) was co-transfected with increasing amounts of each shRNA (ranging from 0.25 µg to 5 µg).

Results shown here used 0.5 µg input shRNA plasmid, the minimum effective shRNA input selected for all subsequent knockdown experiments.

[0347] Successful knockdown over PITX2 over-expression by 30% or greater was achieved in all isoforms at the protein level by co-transfection with at least 2 independent shRNA constructs compared to co-transfection with NC (Figures 21 and 22). In the PITX2A co-transfected cultures the two most potent shRNA constructs were sh3 and sh4 with knockdown of 50% and 70% respectively observed. The shl and sh2 constructs in these cultures had essentially little to no effect. In the PITX2B co-transfected cultures shl again had no effect on 2B protein levels, however sh2, sh3 and sh4 all successfully produced knockdown of the 2B protein isoform by approximately 42%, 32% and 35% respectively. In contrast, in the PITX2C co-transfected cultures, shl produced the most significant knockdown of the 2C protein isoform compared to the other three shRNA constructs with 60% knockdown achieved compared to 25%, 45% and 25% respectively for the sh2, sh3 and sh4 shRNA constructs.

[0348] Due to the unknown effects of individual PITX2 isoforms in prostate cancer, the two shRNA constructs with the highest successful knockdown of all three isoforms were chosen for use in all remaining experiments including co-transfection with isoform specific expression constructs and knockdown of endogenous PITX2 in the PC3 parental cells. The two constructs therefore with the most consistent effects across all three isoforms were sh3 and sh4 as they produced successful knockdown of each isoform at the protein level to a minimum of 25%.

Effects of altered PITX2 levels on PC3 cell proliferation and number

Effects of PITX2 over-expression

[0349] PC3 cells were transfected with individual PITX2 isoform expression vectors. By four days post transfection, cultures transfected with A or C isform vector appeared to be less dense than native PC3 cells or cultures transfected with the B isoform or empty vector construct (Figure 23) PC3 native cells after mock transfection
and PC3 *PITX2B* overexpressing cells showed 100% confluence whilst PC3 *PITX2A*
and *PITX2C* overexpressing cells showed decreased confluence.

[0350] As cell densities of the isoform-overexpressing cultures appeared to differ from the parental cultures and each other, cell number and proliferation were assessed. By 24 hr after transfection, cell number, assessed by fluorescence on binding of the cyanine cell permeable Syto 60 dye to nucleic acids, was significantly reduced in all cultures that had received plasmid compared to mock transfected PC3 parentals (mean mock transfected value indicated by dashed lines on graph), including the vector control (p=0.007), with those transfected with *P/7X2-S*-expression vector further reduced compared to the vector control cultures (p=0.004) (Figure 24, panel A). Cell proliferation assay indicates a decrease in cellular proliferation at 24 hrs due to transfection of plasmid compared to mock transfection (mean mock transfected value indicated by dashed line on graph panel B) in all groups including vector control (PC3 vs Vector p=1.4 E-07) and the reverse result to cell number for transfection with the B isoform with increased proliferation (B vs Vector p=0.02) and a non-significant trend of increased proliferation rate with the A and C isoforms.

[0351] The results in Figure 24 were somewhat unexpected give the previously observed effects of overexpression of *PITX2* A and C. The experiment was therefore repeated at 48hrs post transfection in order to allow for temporal effects of overexpression on the cells (Figure 25). Syto 60 assays at 48hrs post transfection indicated that there were significant differences due to transfection in all transfected groups (mean mock transfected value indicated by dashed line on graph) including vector control (PC3 mock transfected vs. Vector control p=6.8E-08). At 48hrs in comparison to the 24hr data however, there was no significant difference in cells transfected transiently with *P/7X2-S*-expression vector compared with vector control cultures (p=0.27), but a significant increase in cell number in *PITX2A*-expression vector transfected cultures (p=2.8E-07) and a decrease in cell number in the *PITX2C*-expression vector transfected cultures compared to vector control cultures (p=0.004) was observed. 48hr BrdU proliferation results maintained a significant transfection effect with a decrease in proliferation all transfected groups (mean mock transfected value indicated on graph by dashed line) including the vector control cultures (PC3 vs. Vector p=0.0009). The trend of increased proliferation with *P/7X2-A*-expression vector and *P/7X2C*-expression vector transfection compared to empty vector became
statistically significant (Vector vs. PITX2A p=0.024 and Vector vs PITX2C p=0.014) and the significantly increased proliferation rate observed with P/7X25-expression vector transfection was maintained (Vector vs. PITX2B p= 0.009).

0352 The results from Figures 24 and 25 therefore indicate a transfection effect on proliferation and cell number in PC3 cells that is seen in the vector control line compared to mock transfected cells. There is, however, an additional effect of PITX2 isoform overexpression seen in both cell number and proliferation rate with uniform significant increases in proliferation rate with all isoforms seen. PITX2 therefore appears to increase cellular proliferation. The effect on cell number appears isoform specific with increased cell numbers seen in PITX2A transfected cells, no effect in PITX2B transfected cells and a decrease in cell numbers in PITX2C transfected cells. The cause of the alteration in cell numbers may be due to downstream pathways activated by PITX2 overexpression having deleterious or beneficial effects on cell viability post transfection.

Effects of PITX2 Knockdown

0353 Syto 60 and BrdU assays were repeated 24 hr and 48 hrs post transient transfection of PC3 cells with sh3, sh4 and NC (Figures 26A and B and 27A and B) to investigate the effects of knockdown of endogenous PITX2 expression.

0354 Syto 60 assay at 24hrs post transfection indicated that there were significant differences due to transfection in all groups (mean mock transfected value indicated by dashed line on graph) including NC (PC3 vs. NC p=5.2E-05) and an increase in cell number with transfection of shRNA 3 and 4 (sh3 vs. NC p= 0.004, sh4 vs NC p=0.028). Proliferation assay indicated a decrease in cellular proliferation at 24hrs due to transfection (mean mock transfected value indicated by dashed line on graph) in all groups including NC (PC3 vs. NC p= 0.009) and a significant further decrease when endogenous PITX2 expression is knocked down by transfection by either shRNA (NC vs. sh3 p=0.01, and NC vs. sh4 p=0.001).

0355 The experiment was repeated at 48 hrs post transfection in order to allow for temporal effects of shRNA transfection on the cells. Syto 60 assays at 48 hrs post transfection indicated that there were significant differences due to transfection in all groups (mean mock transfected value indicated by dashed line on graph) including NC (PC3 vs. NC p=2.6E-06). At 48 hrs in comparison to the 24 hr data, however, there
was no significant difference in the cell numbers transfected with either shRNA (NC vs. sh3 p=0.11 and NC vs. sh4 p=0.34). 48 hr proliferation assay results maintain the pattern of transfection effect however (mean mock transfected value indicated by dashed line on graph), this is no longer significant for the vector control (PC3 vs. NC p=0.055) but remained significant for the sh3 and sh4 cultures (PC3 vs. sh3 p=0.003; PC3 vs. sh4 p=0.007) as was the case with the decreased proliferation rate seen in shRNA transfected, PITX2 knockdown cultures (NC vs. sh3 p=0.11, NC vs. sh4 p=0.23).

The effect of knockdown of PITX2 in PC3 cells is essentially the opposite of that seen with overexpression of PITX2 isoforms, that is decreased proliferation rates and increased cell numbers at 24 hrs and the trend was maintained at 48 hrs.

Effects of altered PITX2 expression on Motility

Effects of PIIXT2 overexpression

The effect of overexpression of individual PITX2 isoforms in PC3 cells was assessed by "scratch" motility assays (also known as wound healing assays). Cells were pre-treated with mitomycin C a cytostatic drug to inhibit cellular proliferation and therefore remove any confounding issues with proliferation rates on scratch closure and assess purely motility. Photos of scratches were taken at zero, four, eight, twelve and twenty-four hours post scratch formation and motility assessed as percent closure of the original scratch over time (Figure 28).

Overexpression of all three isoforms increased cellular motility significantly compared to empty vector control cultures (Vector vs. PITX2A p= 4h 0.053, 8h 0.02, 12h 0.04, 24h 0.25; Vector vs. PITX2B p= 4h 0.005, 8h 0.00012, 12h 0.00019, 24h 0.00022; Vector vs. PITX2C p= 4h 0.1, 8h 0.037, 12h 0.04, 24h 0.07) with the B isoform having the greatest effect on increasing motility.

In order to ensure that the effect of increased motility was due to overexpression of each PITX2 isoform, co-transfection of each isoform with sh3 and 4 shRNA was performed in order to confirm reversal of the phenotype seen in Figure 28 (Figures 29-31).
Significant decreases in cellular motility occurred with knockdown of PITX2A by sh3 and sh4, reversing the phenotype of increased motility seen with overexpression of PITX2A (Figure 29).

Additionally, significantly decreased cellular motility occurred with knockdown of PITX2B by co-transfection with sh3 and sh4, reversing the phenotype of increased motility seen with overexpression of PITX2B (Figure 30).

Moreover, significant decreases in cellular motility occurred with knockdown of PITX2C by sh4, reversing the phenotype of increased motility seen with overexpression of PITX2C (Figure 31). The trend was also seen with sh3 co-transfection but was not statistically significant.

Accordingly, the results presented in Figures 29-31 indicate that the increased motility seen in PC3 cells with PITX2 isoform transfection is a specific effect caused by PITX2 as this phenotype can be reversed with knockdown of the overexpression by shRNA co-transfection.

**Effects of Knockdown of Endogenous PITX2 on Motility**

**Scratch Motility Assay**

In order to assess the effect of endogenous PITX2 expression on motility and to correlate the effects seen with overexpression of PITX2 isoforms in PC3 cells, scratch assays were performed in PC3 cells transiently transfected with shRNA 3 and 4 (Figure 32). Knockdown of endogenous PITX2 isoforms in PC3 cells significantly decreases cellular motility compared to negative control (NC vs. sh3 p= 4h 0.06, 8h 0.03, 12h 0.06, 24h 0.13; NC v sh4 p= 4h 0.16, 8h 0.05, 12h 0.006, 24h 0.01).

Figure 32 indicates that the effect of knockdown of endogenous PITX2 in PC3 cells is the reverse of that seen with overexpression and strongly supports the role of PITX2 in promoting cellular motility in PC3 prostate cancer cells.

**Transwell Motility Assay**

The results from the co-culture of PC3 cells and Saos-2 osteosarcoma cells presented above indicate a role for PITX2 in the interaction between the metastatic prostate cancer cells and the host bone cells at the metastatic site. To investigate if PITX2 is involved in the "homing" of the cancer cells to the bone (a process which has
been proposed to be integral in explaining the tropism for bone as a preferred metastatic site by prostate cancer), the use of a transwell system and chemoattractants were utilized.

[0367] Prostate cancer cells transiently transfected with either NC, sh3 or sh4 plasmids or mock transfection, were seeded in serum free media in a transwell insert containing 8 μM pores, which unlike the inserts used in the co-culture experiments allow the cells to mobilize through the membrane along a chemoattractant gradient. Motility after 24 hrs incubation was then assessed (Figure 33).

[0368] Approximately a 10% increase in motility towards 10% FBS occurs in all groups which was not affected by transfection or knockdown of PITX2. Very significant differences in motility towards Saos conditioned media was seen within groups and between groups. Comparisons within groups showed massively increased motility in PC3 cells towards Saos CM compared to FBS (p=1.3E-06). A transfection effect was noted with a smaller but significant increase in motility towards Saos CM seen in the NC control (p=0.018). Comparison between PC3 and NC motility toward Saos CM also indicated significant transfection effect with decreased motility in the NC transfected cells (NC Saos CM motility vs. PC3 Saos CM motility p= 1.8E-06).

[0369] Knockdown of PITX2 with shRNA however had the effect of decreasing motility toward Saos CM (sh3 p=0.017, sh4 p=0.15). Whilst the sh4 intragroup comparison did not reach statistical significance, intergroup comparisons of motility toward Saos CM using the NC as the control did (NC Saos CM motility vs. sh4 Saos CM motility p=0.0006; NC Saos CM motility vs. sh3 Saos CM motility p=0.003).

[0370] Taken together these results indicate that PITX2 is involved in promoting migration and homing of the prostate cancer cells specifically towards the bone metastatic site.

*Effects of altered PITX2 expression on Cellular Attachment to Extracellular Matrix (ECM) Molecules*

**Effects of PITX2 Overexpression**

[0371] As cellular attachment is integral to the EMT process, and the preceding results indicate a strong role of PITX2 in the promotion of this process, the role of PITX2 in cellular attachment was investigated through attachment assays using
the ECM molecules Collagen I (the major collagen found in bone), Collagen IV (the major collagen in basement membranes) and Fibronectin (an ECM molecule which can bind collagen as well as integrins and is also found in a soluble form in blood). In cancer and metastasis, decreased cellular attachment to these factors is often associated with the EMT process and metastatic cascade, as decreased cellular adhesiveness is necessary for movement of the cells through the ECM to gain access to the vascular "highway" and on the opposite end for egress from the vascular space into the metastatic destination.

[0372] Attachment of cells transiently transfected to overexpress PITX2 isoforms, empty vector or mock transfected to ECM molecules was assessed by absorbance of crystal violet dye eluted from bound cells and presented as percent change in absorbance to control wells containing no ECM coating [Figure 34].

[0373] A trend of decreased attachment to collagen I and IV with overexpression of PITX2 isoforms A and C was observed. A significant decrease in attachment to collagen I (p=0.04) and a trend of decreased attachment to collagen IV with PITX2B overexpression was also observed. No significant difference to attachment of fibronectin was observed with overexpression of any PITX2 isoform.

[0374] In summary, the results presented above provide solid evidence for the involvement of PITX2 in the promotion of the metastatic process in prostate cancer. Through the demonstrated effects of increasing cellular motility, decreasing cellular adhesiveness (and thereby increasing cellular motility through the ECM and egress from the primary cancer site and ingress to the metastatic site), coupled with the demonstrated homing mechanism toward bone, it would appear that PITX2 is a very significant molecular facilitator of prostate cancer metastasis.

[0375] Apart from these metastatic promoting effects, it also appears that PITX2 has roles in cellular proliferation with overexpression increasing proliferation rate but having variable effects on cell number. Knockdown of endogenous PITX2 essentially has the reverse effects - that is increased cell number and decreased proliferation rate. Given the clinical correlations seen in Example 2, these results strongly implicate this previously unidentified factor as playing a major role in at least a proportion of prostate cancers’ progression and metastasis.
These results again indicate this factor as a potentially important therapeutic target given the multiple physiologic and oncologic properties it imparts to the cancer cells. Small interfering molecules designed to inhibit all three isoforms could potentially have a significant role in metastasis prevention (given the results showing all three isoforms of PITX2 have similar effects in increasing cellular motility and the homing results seen with decreased motility towards Saos CM with knockdown of endogenous PITX2 in PC3 cells). This approach of metastasis prevention is lacking in the current arsenal of prostate cancer therapies.

Given the data showing upregulation of PITX2 expression in co-culture of PC3 cells with Saos-2 cells, these small interfering molecules may well also inhibit establishment and promotion of new metastatic deposits as well as have deleterious effects on established sites.

Materials and Methods

Cell culture

The prostate cancer cell lines PC3, LNCaP, MDA PCa 2b and the osteosarcoma line Saos-2, and stable and transiently transfected versions of the PC3 and LNCaP lines were grown and prepared according to methods described in Example 1.

PITX2 shRNA

PITX2 shRNA Constructs

Sure Silencing PITX2 shRNA plasmids were obtained from SA Bioscience designed to target all three major isoforms of PITX2 (isoforms A, B and C). Four shRNA clones and one negative control scrambled selected with a puromycin resistance marker.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Insert Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh clone 1</td>
<td>5'-CAGTCTCAACAGCCTGAATAA-3' [SEQ ID NO: 29]</td>
</tr>
<tr>
<td>sh clone 2</td>
<td>5'-CAGGCGAGCTCTGCAAGAAT-3' [SEQ ID NO: 30]</td>
</tr>
<tr>
<td>sh clone 3</td>
<td>5'-GTCTCAACAGCCTGAATAACT-3' [SEQ ID NO: 31]</td>
</tr>
<tr>
<td>sh clone 4</td>
<td>5'-GACATGTCCACACGGAAGA-3' [SEQ ID NO: 32]</td>
</tr>
<tr>
<td>Negative Control</td>
<td>5'-GGAATCTCATTGCATGCATAC-3' [SEQ ID NO: 33]</td>
</tr>
</tbody>
</table>

TABLE 5: shRNA clones and insert sequences
Transformation, Amplification and Purification of Plasmids

[0380] Transformation, amplification and purification of shRNA plasmids were required prior to transfection of cells.

[0381] Competent JM1 09 E coli cells (Promega) were incubated with ligation reaction on ice for 20 min, at 42°C for 1 min, then on ice for 2 min. Luria Broth (LB) (Sigma) was added to cells prior to incubation at 37°C for 1 h. Cells were then plated on antibiotic selective LB agar plates (ampicillin 50 µg/ml) and incubated at 37°C overnight. Single colonies were picked and grown in selective LB at 37°C overnight. Cells were harvested by centrifugation at 2000 rpm for 5 min and resuspended in 30% glycerol in nonselective LB media for storage at -80°C.

[0382] The remaining cultures were purified using a QIAprep Spin Miniprep Kit (Qiagen) as per manufacturers’ instructions. DNA was eluted in 50µL of elution buffer and quantified by Nanodrop spectrophotometry.

[0383] Quality control of product was performed by PstI restriction enzyme digestion (Cell Signalling Technology) as per manufacturer's instructions. Undigested and digested plasmid was then run on a 2% agarose gel with a lkb DNA ladder. PstI digested fragments of plasmids showed bands at 3209bp and 1402bp.

[0384] Remaining plasmid stocks were stored at 4°C for later use.

shRNA Transfection of PC3

[0385] Cells were grown to 90-95% confluence in 6 well plates. Previously amplified and purified shRNA plasmids were transfected into cells at 0.5 µg/well using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions upscaling for a 6 well plate. For transient transfection experiments cells were utilized 24hrs post transfection. For stable selection, 24 hrs post transfection cells were confluent and therefore passaged 1:10 into a T25 flask. 24hrs post passage 4µg/mL Puromycin (Invitrogen) was added to culture media for selection of stably transfected PC3 cells. The selection dose of puromycin was previously determined by growing untransfected PC3 cells in varying concentrations of puromycin to find the minimum effective dose at killing all cells. Cultures were maintained under puromycin selection pressure at 2µg/ml long term.
**PITX2 Isoform Constructs**

[0386] Origene TrueORF human cDNA clones for transcript variant 1, 2 and 3 were obtained (Origene Technologies) in a transfection ready pCMV6 entry vector (myc and flag tagged) with kanamycin and G418 resistance. Insert sequences for each transcript variant were reported as having 100% homology to the following references: variant 1 NM_153427.1; variant 2 NM_153426.1; variant 3 NM_000325.5. Each plasmid was reconstituted in sterile H₂O to produce a concentration of 100 ng/µL.

**Transformation, Amplification and Purification of Plasmids**

[0387] Transformation and amplification and purification of origene TrueORF clones was performed as above except Kanamycin (Invitrogen) at 30 µg/mL concentration was used instead of Ampicillin in LB agar and broth and no restriction digestion quality control step was performed.

**Sequencing of Plasmids**

[0388] Plasmid was prepared from glycerol stock using QIAprep Spin Miniprep kit or QIAfilter Plasmid Maxi kit (Qiagen; Doncaster, VIC, Australia) according to the manufacturer's protocols. Sequences of plasmid inserts was confirmed by automated DNA sequencing using the ABI 377 DNA Sequencer instrument, or confirmed using the Xpress Sequencing Service (AgGenomics; Fitzroy, VIC, Australia).

**TrueORF Clone Transfection of LNCaP and PC3 Cell Lines**

[0389] Transfection of TrueORF clones was performed as above except both stable and transient transfections were performed. For transient transfections 0.5 µg of DNA was used per well of a six well plate and 1 µg of DNA was used per 6 cm diameter plate for stable transfections. Reaction sizes were appropriately scaled as per Lipofectamine 2000 protocol. Transient transfections were then collected at 24-48 hours post transfection for RNA and protein as described above and for use in functional assay experiments. For stable selection, 24 hours post transfection, cells were passaged into a T75 flask and media changed to selection media containing 0.6mg/ml G418. Cultures were maintained under G418 selection pressure at 0.3mg/ml long term.
**Co-transfection of TrueORF PITX2 Clones and shRNA Plasmids**

[0390] In order to verify the function of the shRNA plasmids in knockingdown PITX2 function, transient co-transfection of each PITX2 isoform clone (0.5µg) together with each shRNA clone (0.5µg) was performed in 6 well plates as per protocol. RNA and protein were then collected as per protocol and utilized for qRT-PCR and immunoblot analysis as per protocol.

**Transient transfection of PITX2 shRNA and expression plasmids**

[0391] Transient and stable transfection and transient co-transfection of PITX2 shRNA plasmids and PITX2 isoform overexpression plasmids (as described above) into PC3 and LNCaP cells were performed. Correct DNA sequences of PITX2 open reading frame (ORF) plasmids (as described above) were confirmed as per section protocol described above (data not shown). Sequence alignment of shRNA targets to human PITX2 locus indicated that all four shRNA constructs targeted exon 6, which is common to the transcripts encoding all three PITX2 protein isoforms (Figure 36).

**RNA preparation and quantitative RT PCR analysis**

[0392] RNA and cDNA were prepared from individual wells of cell cultures and quality of preparations was assessed. Quantitative real-time PCR analysis was performed on the Rotor-gene RG-3000 or Rotor-gene RG-6000 thermocyclers using specific primers as per Table 4. Data were assessed by normalised to a housekeeping gene (cyclophilin), assessed utilising the ΔC<sub>t</sub> method and analysed statistically by One way analysis of variance followed by Student's T-test. Experiments consisted of biologic replicates or triplicates with analysis performed as a combined average of two technical replicates of the biologic replicates.

**Protein lysate preparations and immunoblot analysis**

[0393] Whole cell lysate were prepared from cell cultures according to Example 1, and protein concentration assessed by BCA assay according to Example 1. Immunoblot analyses using P2R10 anti-PITX2, M2 anti-Flag and anti-β-tubulin antibodies were performed according to Example 1 with visualization by the LI-COR Odyssey Infrared Imaging System and/or enhanced chemiluminescence.
Cell Co-culture Experiments

[0394] Cell lines prepared and grown as described above were used in cell co-culture experiments. Cell lines for co-culture experiments were grown in media designated "Experimental Media" (ExpM) which consisted of a 50:50 mixture of DMEM and RPMI + 1% FBS + 1% P/S for all lines except MDA PCa 2b which was grown in a 50:50 mixture of DMEM and BRFF-HPC1 +1% FBS +1% P/S. Initial trial experiments were performed in serum free ExpM, but due to extremely poor cell growth and survival, serum was added for all following experiments as documented.

[0395] 0.4 µM pore transwell inserts (BD Biosciences) in 6 well plates were used for co-culture experiments. These inserts allowed interaction of secreted factors in ExpM from each cell line without direct contact. Cells were seeded at 1 x 10⁵ cells for each insert/well. Saos cells were seeded in the bottom well and prostate cancer cell lines were seeded in the insert. Inserts were left for 48 hours in standard media containing 10% FBS alone before bringing inserts and bottom well cells together in ExpM containing 1% FBS for 48hrs before collection of protein or RNA preparations as described in Example 2. Experiments were completed in triplicate on two separate occasions for RNA and in triplicate for protein.

Transwell co-culture prostate cancer cells and Saos-2 osteosarcoma cells

[0396] PC3 or-LNCaP cells were co-culture in Transwell inserts (0.4 µm pore size) in 6 well plates at 1 x 10⁵ seeding density, with Saos-2 osteosarcoma cells plated at 1 x 10⁵ density in the lower chamber using a modified Boyden protocol (as described in Example 2). RNA and cDNA were prepared from individual wells, with insert and lower cultures separated (as described above). Real time PCR analysis was performed on cDNA preparations using PITX2 universal primers as per Table 4. Experiments were performed in triplicate on two occasions. Real time PCR analysis consisted of combined average results of technical duplicates of each biologic replicate.

Syto 60 cell quantitation and BrdU proliferation assays

[0397] Syto 60 and BrdU assays were performed using transiently transfected and mock transfected PC3 cells (as described in Example 2 at 24 hrs and 48 hrs post transfection. Syto 60 assays were performed in 6 well plates (one plate per cell line
seeded at 5 x 10^5 cell per well and grown to ~50% confluence) and BrdU assays were performed on cells grown in 96 well plates (seeded 4 x 10^3). Experiments were performed x6 replicates for each time point twice.

Transwell migration assays

5 [0398] Transiently transfected PC3 cells were plated in Transwell (8 µm pore size) inserts in 12-well plates at 2 x 10^5 cells per well and migration through the pores was assayed as described below.

[0399] Migration assays were also performed in 12-well cell culture inserts with 8 µm pores (BD Biosciences). Ten percent FBS or ten percent Saos conditioned media was placed in the lower chamber to act as a chemoattractant. 2 x 10^5 cells from each cell line were seeded in 200 µL of serum free media containing 0.1% BSA (w/v) into the top chamber of the well and incubated for 12 hours. Wells containing serum free RPMI in the lower chamber were used as controls for random migration of cells from the top chamber to the bottom. After incubation, cells remaining on the upper surface of the insert were removed using a cotton bud and rinsed in PBS x2. To quantify the number of cells that migrated, cells on the bottom of the insert were fixed in 100% - 20° C methanol for five minutes and stained in 0.5% (w/v) crystal violet. Plates and inserts were rinsed in PBS x2 and the stain eluted with 10% (v/v) acetic acid. The eluted acetic acid/stain was quantified on the Multiskan plate reader (Labsystems) at 595 nm and normalized back to the serum free controls. Experiments were performed on three occasions with five replicates per cell line, per chemoattractant agent.

Attachment assays to extracellular matrix proteins (ECM)

[0400] Attachment of knockdown, over-expressing, parental and empty vector control cell lines to extracellular matrix (ECM) components was performed in 96-well plates. 96-well plates were coated with 50 µL per well of human collagen I, human collagen IV or human fibronectin (BD Biosciences) diluted to 10 µg/mL. Following overnight incubation of plates at 4° C, plates were washed with sterile PBS and nonspecific binding sites blocked with 1% BSA (Sigma) for 2 hours at room temperature before washing in PBS.

[0401] Cells were seeded at 2 x 10^4 cells per well in 100µL of serum free RPMI with 0.1% (w/v) BSA. Wells blocked with 1% (w/v) BSA only were used to quantify background adherence. Plates were incubated at 37° C for 1 hour. Following
incubation, plates were fixed in 100% -20°C methanol for five minutes and stained with 0.5% (w/v) crystal violet/20% (v/v) methanol (Sigma) for one minute and were destained and quantified as per the transwell migration assay described above. Results were normalized to BSA background attachment. The experiments were done twice with four replicates per ECM treatment per cell line. Experiments were performed on two occasions with 4 replicates per transfected cell population, per ECM protein.

Statistics

[0402] Data were analysed using ANOVA and the Student's t test to evaluate the significance of the difference in mean values between cell lines, treatments and specimens. P values <0.05 were considered to indicate statistically significant differences. Data are represented as mean ± standard error (SE) unless otherwise stated.

EXAMPLE 4

PROGNOSIS AND THERAPY OF PROSTATE CANCER

[0403] Currently the usual screening examination for prostate cancer includes (1) a PSA blood test and (2) a DRE (digital rectal examination). If either of these (or both) is abnormal then patients are routinely referred to a urologist for further investigation.

[0404] Depending on age, co-morbidities etc most patients would then undergo TRUS biopsy (Transrectal Ultrasound guided biopsy) of the prostate for a tissue diagnosis. This targets the peripheral zone of the prostate in which ~70% or higher of prostate cancer develops. BPH is derived from the transition zone. If this indicated prostate cancer, a bone scan and CT abdomen would often be performed to investigate for metastatic or locally advanced disease. If there is no indication of bone metastases, lymph node metastases or locally advanced disease, a curative therapy would be offered (again taking into consideration age, co-morbidities, life expectancy etc). This would either be in the form of surgery (radical prostatectomy) or radiation therapy (external beam radiotherapy or brachytherapy) with or without adjuvant hormonal therapy.

[0405] Patients with metastatic disease at diagnosis are considered currently incurable and would be placed on androgen deprivation therapy, with no possibility of cure. This is effective for a variable time course, but inevitably leads to castrate
resistance in the tumor with the therapy becoming ineffective. Current chemotherapy
treatments have been universally disappointing with prolongation of life by months the
most success claimed by any therapy.

[0406] There are a number of deficiencies associated with the current
5 techniques in diagnosis, staging and treatment of prostate cancer. PSA is not terribly
specific in that it can be elevated by other conditions in the prostate including Benign
Prostatic Hypertrophy (BPH) which is extremely common in the same population of
patients and infection (prostatitis). Some high grade cancers can also be non-secreters
i.e. excrete normal or low levels of PSA, thereby proving difficult to diagnose with this
test. The level of PSA also does not generally provide an indication of the behaviour of
the cancer i.e. will it behave in an indolent fashion with slow growth and little chance of
metastasis or will it be an aggressive, early metastasizing cancer which if not treated
will bring risk to the patient's life?

[0407] The TRUS bx histopathologic assessment, whilst gives some
15 indication of likely tumor behaviour through Gleason grade and rough volume of
disease cannot again give any definite assessment of likelihood of metastasis at the time
of diagnosis by itself. The staging imaging modalities currently used are also
insufficiently sensitive and specific to pick up all deposits of metastatic disease as up to
20% of patients offered therapy of curative intent (by surgery or radiation) will
20 ultimately develop metastatic disease. This is often not a failure of the therapy but more
an indication that these patients already had micro-metastatic disease at the time of the
initial therapy which was unable to be diagnosed with the current modalities. The TRUS
biopsy also has an associated false negative rate, which is either due to sampling of non-
cancerous tissue and missing areas of cancer (i.e. a very fine needle biopsying a very
large prostate gland) or a gland that whilst not definitely cancerous may progress to
cancer at a later stage through malignant transformation.

[0408] In accordance with the present invention PITX2 is proposed to address
several of these current clinical deficiencies pursuant to the following illustrative
procedure.

[0409] On suspicion of prostate cancer
- PITX2 qPCR of cells in ejaculate or urine is used as a screening test to increase the sensitivity and specificity of routine PSA screening as normal prostate expresses very low levels of PITX2 and primary cancer significantly higher levels.

PITX2 staining of TRUS bx specimens is used to indicate:

(a) Metastatic potential of the primary cancer, tumor biology and likelihood of micrometastatic disease (as mets show high PITX2 expression) if a ratio of PITX2 expression in localized and metastatic cancers could be determined or indolent versus aggressive primary cancers. Patients with clear bone scans or CTs but high levels of PITX2 in their primary cancers could therefore be offered either more aggressive therapies with early adjuvant or neoadjuvant hormonal or chemotherapy along with their primary therapies, or alternatively less aggressive primary therapy as their likelihood of incurable disease would be higher thereby limiting unnecessary interventions with significant side effects and risks. Patients with low PITX2 expression in their primary cancer could be offered watchful waiting as a safe alternative to aggressive therapies knowing that the risk of metastasis and progression in these tumors was less than high PITX2 expressing primary tumors.

(b) TRUS bx which are negative for cancer and negative for PITX2 expression may be patients who can be safely discharged from further investigation or biopsy as their potential for malignant transformation is very low, whereas negative biopsies for cancer, but positive for PITX2 expression may indicate higher likelihood for later cancer formation or potential false negative results which require either repeat "saturation" biopsies (i.e. multiple biopsies compared to the usual paired sextant biopsies performed) or more intensive surveillance as they have a higher risk of cancer formation.

In diagnosed primary cancer:

(1) In known cancer patients during the staging phase, circulating tumor cells (CTCs) from the patient's serum could be used for PITX2 qPCR, and those with high PITX2 expression could indicate likelihood of micrometastatic disease and therefore alter treatment accordingly as previously described.
As PITX2 has been shown to promote cellular motility and metastasis in prostate cancer cell lines, small molecule inhibitors could then be used as adjuvant therapy in primary treatment in the prevention of metastatic disease.

PITX2 is proposed to be involved in the promotion of the establishment and progression of bone metastases and is therefore considered a novel target for treatment of established metastatic disease.

As PITX2 is considered to play a role in development of castrate resistance it could be targeted as a cotherapy with androgen ablation for hormone sensitive disease to prevent development of androgen independence or as monotherapy once castrate resistance develops.

EXAMPLE 5

PRELIMINARY STUDY OF PITX2 EXPRESSION IN BREAST CANCER

The expression of PITX2 in a series of breast cancer cell lines was investigated using immunohistochemistry on tissue microarray sections. Nine breast cancer cell lines (MCF7, T47D, BT474, SKBR3, SUM159, MDA231, 8701BC, KPL1, MCFIOA, HS578T and HCC1937) were analysed and all showed positivity for PITX2. In one of these cell lines, PITX2 localisation was restricted to the nucleus, whilst in others PITX2 was localised in the cytoplasmic compartment, and still in others PITX2 was localised in both the cytoplasm and nucleus. Of interest, nucleoli were negative for PITX2 in all cases. Figure 37 shows a representative cell line (SKBR3) in which localization of PITX2 was largely restricted to the cytoplasm.

PITX2 expression was also analysed in tumour samples (i.e., about 100 invasive lobular carcinomas, 50 invasive ductal carcinomas and 50 brain metastases arising from breast cancer) and preliminary results indicate that PITX2 is expressed in a majority of the tumours. Figure 38 shows a representative tissue microarray section of human breast cancer ductal tissue in which a majority of the cancer cells is shown to express PITX2.

Studies are being currently undertaken to compare PITX2 expression between primary breast tumours, breast cancer metastases and normal breast tissue.
[0422] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[0423] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

[0424] Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Those of skill in the art will therefore appreciate that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention. All such modifications and changes are intended to be included within the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A method for diagnosing the presence or risk of development of a skeletal metastasis-associated, hormone-related cancer in a subject, the method comprising detecting in the subject overexpression of PITX2, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

2. A method according to claim 1, wherein the skeletal metastasis-associated, hormone-related cancer is selected from prostate cancer, breast cancer, endometrial cancer and ovarian cancer.

3. A method according to claim 1, wherein the skeletal metastasis-associated, hormone-related cancer is selected from prostate cancer and breast cancer.

4. A method according to claim 1, comprising detecting overexpression of a PITX2 polynucleotide selected from the group consisting of PITX2A, PITX2B and PITX2C.

5. A method according to claim 4, wherein the P/7X2polynucleotide is selected from the group consisting of: (a) a polynucleotide comprising a nucleotide sequence that shares at least 80% sequence identity with the sequence set forth in any one of SEQ ID NO: 1, 3, 5 or 7, or a complement thereof; (b) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence set forth in any one of SEQ ID NO: 2, 4, 6 or 8; (c) a polynucleotide comprising a nucleotide sequence that encodes a polypeptide that shares at least 80% sequence identity or similarity with at least a portion of the sequence set forth in SEQ ID NO: 2, 4, 6 or 8, wherein the portion comprises 15 or more contiguous amino acid residues of that sequence; and (d) a polynucleotide comprising a nucleotide sequence that hybridizes to the sequence of (a), (b), (c) or a complement thereof, under at least medium or high stringency conditions.

6. A method according to claim 4, comprising detecting overexpression of a PITX2A polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

7. A method according to claim 4, comprising detecting overexpression of a PITX2B polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.
8. A method according to claim 4, comprising detecting overexpression of a PITX2C polynucleotide, which indicates the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer.

9. A method according to claim 1, wherein overexpression is detected by: (1) providing a biological sample from the subject; (2) measuring in the biological sample the level or functional activity of at least one PITX2 expression product; and (3) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample obtained from one or more normal subjects or from one or more subjects lacking a skeletal metastasis-associated, hormone-related cancer, wherein a higher level or functional activity of the or each expression product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the presence or risk of development of the skeletal metastasis-associated, hormone-related cancer in the subject.

10. A method according to claim 9, further comprising diagnosing the presence, stage or degree of the skeletal metastasis-associated, hormone-related cancer in the subject when the measured level or functional activity of the or each expression product is higher than the measured level or functional activity of the corresponding expression product.

11. A method according to claim 9, wherein the skeletal metastasis-associated, hormone-related cancer is prostate cancer, and the biological sample largely comprises prostate cells from the peripheral zone of the prostate.

12. A method according to claim 11, wherein the biological sample largely does not comprise prostate cells from the transition zone of the prostate.

13. A method according to claim 9, the methods further comprise diagnosing the absence of the skeletal metastasis-associated, hormone-related cancer when the measured level or functional activity of the or each expression product is the same as or similar to the measured level or functional activity of the corresponding expression product.

14. A method according to claim 9, wherein the biological sample comprises cells or cell lines, histological slides, biopsies, paraffin-embedded tissue, bodily fluids, ejaculate, urine, blood, sputum, bone, stool, tissue, prostate tissue, ovarian tissue,
endometrial tissue, colon tissue, lung tissue, bladder tissue, testicular tissue and thyroid tissue.

15. A method according to claim 9, wherein the biological sample comprises mammary cells, ovarian cells, endometrial cells or prostate cells.

16. A method according to claim 15, wherein the biological sample is selected from mastectomy specimens, isolated breast cells, breast milk, menstrual fluid, ovariectomy specimens, isolated ovary cells, endometrectomy specimen, isolated endometrium cells, core needle biopsies, sentinel lymph node biopsies, bone marrow biopsies, bone marrow aspirations, prostatic excretions, semen, isolated prostate cells, prostatectomy specimens and TRUS biopsies.

17. A method according to claim 9, wherein the expression product or corresponding expression product is selected from a target RNA or a DNA copy of the target RNA whose level is measured using at least one nucleic acid probe that hybridizes under at least medium or high stringency conditions to the target RNA or to the DNA copy, wherein the nucleic acid probe comprises at least 15 contiguous nucleotides of a PITX2 polynucleotide.

18. A method according to claim 9, wherein the expression product or corresponding expression product is a PITX2 polypeptide whose level is measured using at least one antigen-binding molecule that is immuno-interactive with the PITX2 polypeptide.

19. A method for providing a prognosis to a subject with a skeletal metastasis-associated, hormone-related cancer, the method comprising detecting overexpression of PITX2 in the subject, wherein the overexpression is characterized by higher expression of PITX2 in the subject than the expression of PITX2 in a primary skeletal metastasis-associated, hormone-related cancer or a benign prostatic hypertrophy, and wherein the overexpression is indicative of a negative prognosis.

20. A method according to claim 19, wherein overexpression is detected by: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a primary skeletal metastasis-associated, hormone-related cancer or with a benign organ hypertrophy, wherein a higher level or functional activity of the or each expression product
product in the biological sample as compared to the level or functional activity of the corresponding expression product in the reference sample is indicative of the negative prognosis.

21. A method according to claim 19, wherein overexpression is detected by: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a secondary skeletal metastasis-associated, hormone-related cancer, wherein negative prognosis is determined when the level or functional activity of the or each expression product in the biological sample is the same as or similar to the level or functional activity of the corresponding expression product in the reference sample.

22. A method according to claim 19, wherein the biological sample is from a subject following surgical removal of a primary skeletal metastasis-associated, hormone-related cancer-affected organ or tissue and PITX2 overexpression identifies recurrence of the skeletal metastasis-associated, hormone-related cancer in the subject following the surgical removal.

23. A method for diagnosing the presence of a primary skeletal metastasis-associated, hormone-related cancer, the method comprising: (1) measuring in a biological sample from the subject the level or functional activity of at least one PITX2 expression product; and (2) comparing the measured level or functional activity of the or each expression product to the level or functional activity of a corresponding expression product in a reference sample from one or more subjects with a primary skeletal metastasis-associated, hormone-related cancer, wherein a positive diagnosis of the primary skeletal metastasis-associated, hormone-related cancer is determined when the level or functional activity of the or each expression product in the biological sample is the same as or similar to the level or functional activity of the corresponding expression product in the reference sample.

24. A method according to claim 23, wherein the skeletal metastasis-associated, hormone-related cancer is prostate cancer, and the biological sample largely comprises prostate cells from the peripheral zone of the prostate.

25. A method according to claim 24, wherein the biological sample largely does not comprise prostate cells from the transition zone of the prostate.
26. A method for providing a prognosis of prostate cancer in a subject comprising: (1) detecting overexpression of PIIX2 in the subject; (2) detecting or determining at least one factor selected from the group consisting of: the subject's pre-treatment PSA; the subject's post-treatment PSA; primary Gleason grade in a biopsy specimen obtained from the subject; secondary Gleason grade in a biopsy specimen obtained from the subject; Gleason sum in a biopsy specimen obtained from the subject; pre-radical primary therapy of the subject; total length of cancer in biopsy cores obtained from the subject; number of positive biopsy cores obtained from the subject; percent of tumor biopsy in a multiple core biopsy set obtained from the subject; primary Gleason grade in a pathological specimen obtained from the subject; secondary Gleason grade in a pathological specimen obtained from the subject; Gleason sum in a pathological specimen obtained from the subject; the subject's pre-operative TGF-ssl level; the subject's prostatic capsular invasion level (PCI); the subject's surgical margin status; the subject's seminal vesicle involvement; the subject's lymph node status; the subject's pre-operative IL6sR level; the sensitivity of the subject's cancer to hormone therapy; the resistance of the subject's cancer to hormone therapy; the subject's prior therapy and/or clinical stage; and (3) correlating (1) and (2) with disease outcome.

27. A method according to claim 26, wherein the factor is selected from the group consisting of primary Gleason grade; secondary Gleason grade; Gleason sum. Suitably, the subject's clinical stage is selected from T3a, T3, T2c, T2b, T2a, T2, Tie, T1b, T1a or T1. In some embodiments, the subject's prior therapy is a primary therapy (e.g., surgical treatment, chemotherapy, cryotherapy, radiation therapy, brachytherapy and hormonal therapy).

28. A method according to any preceding claim, further comprising detecting overexpression of at least one other skeletal metastasis-associated, hormone-related cancer marker gene.

29. A method according to claim 28, wherein the at least one other skeletal metastasis-associated, hormone-related cancer maker gene is selected from PCAS, Claudin 4, Hepsin, PSMA, SPINKI, GOLPH2, TMPRSS2.ERG, GalNAc-T3, HER2/new/ERbB2, Cathepsin D, BRCAl, BRCA2, ER, PR, AR, MUC1, EGFR, mutant p53, cyclin D, PCNA, Ki67, uPA and PAI.

30. A method according to claim 28, wherein the at least one other skeletal metastasis-associated, hormone-related cancer maker gene is overexpressed.
31. A method according to claim 29 or claim 30, wherein detecting the overexpression and/or presence identifies the skeletal metastasis-associated, hormone-related cancer in the subject.

32. A method for treating, preventing or inhibiting the development or progression of a skeletal metastasis-associated, hormone-related cancer in a subject, the method comprising detecting overexpression of PITX2 in the subject, and administering to the subject at least one therapy that treats or ameliorates the symptoms or reverses or inhibits the development or progression of the skeletal metastasis-associated, hormone-related cancer in the subject.

33. A method according to claim 32, wherein the therapy is selected from surgery, radiation therapy, chemotherapy, stem cell transplant; hormone therapy, and antibody therapy.

34. A method for treating or preventing a skeletal metastasis-associated, hormone-related cancer in a subject, the method comprising administering to the subject a PITX2 modulator in an amount that is effective to treat or prevent, or ameliorate the symptoms or reverse or inhibit the development or progression of the skeletal metastasis-associated, hormone-related cancer.

35. A method according to claim 34, wherein the agent reduces the expression of PITX2 or the level or functional activity of a PITX2 expression product.

36. A method according to claim 35, wherein the agent reduces PITX2 expression or the level or functional activity of a PITX2 expression product to less than 9/10 of the expression, level or functional activity of a corresponding PITX2 expression product in the absence of the agent.

37. A method for identifying agents that modulate the development or progression of a skeletal metastasis-associated, hormone-related cancer, the method comprising contacting a preparation with a test agent, wherein the preparation comprises (i) a polypeptide comprising an amino acid sequence corresponding to at least a biologically active fragment of a PITX2 polypeptide, or to a variant or derivative thereof; or (ii) a polynucleotide comprising at least a portion of a genetic sequence that regulates the expression of PITX2, which is operably linked to a reporter gene, wherein a detected reduction in the level and/or functional activity of the polypeptide, or an expression product of the reporter gene, relative to a normal or reference level and/or functional activity in the absence of the test agent, indicates that the agent modulates the...
development or progression of the skeletal metastasis-associated, hormone-related cancer.
FIGURE 1

Percent Tumour Formation by Cell Line

A

Tumour Volume Vs Cell Line

B

FIGURE 2
FIGURE 3

Mitotic Index Vs Cell Line

FIGURE 4

PITX2 Expression in Cell Line Tumors
Confirmation of PITX2 Expression in Cell Line Tumors

FIGURE 5

Normalised PITX2 Expression
Vs Sample

A

Normalised PITX2 Expression minus HuMet

B

FIGURE 6
FIGURE 7

Comparison of Gleason Grade Vs PITX2 Expression

FIGURE 8
FIGURE 15

Average Normalised PITX2 Expression in Co-Culture System

FIGURE 16
FIGURE 17

FIGURE 18
FIGURE 19

Flag Signal Normalised to βTubulin v Input Plasmid DNA Concentration

FIGURE 20
FIGURE 21

PITX2A and shRNA

PITX2B and shRNA

PITX2C and shRNA

FIGURE 22
FIGURE 24

FIGURE 25
FIGURE 26

Syto 60 Average Normalised Absorbance at 24hrs Post Transient Transfection

A  NC  sh3  sh4

BrdU Average Normalised Absorbance at 24hrs Post Transient Transfection

A  NC  sh3  sh4

FIGURE 27

Syto 60 Average Normalised Absorbance at 48hrs Post Transient Transfection

A  NC  sh3  sh4

BrdU Average Normalised Absorbance at 48hrs Post Transient Transfection

A  NC  sh3  sh4

Average Percent Closure of Scratch With PITX2 Isoform Overexpression

FIGURE 28

Co-Transfection of PITX2A and shRNAs

FIGURE 29
Co-Transfection of PITX2B and shRNAs

FIGURE 30

Co-Transfection of PITX2C and shRNAs

FIGURE 31
Average Percent Closure with Endogenous PITX2 Knockdown

FIGURE 32

Average % Change in Motility Toward Different Chemoattractant Agents

FIGURE 33
Average Attachment to Collagen I

Average Attachment to Collagen IV

Average Attachment to Fibronectin

FIGURE 34
**FIGURE 35**

**Average Attachment to Collagen I**

A

**Average Attachment to Collagen IV**

B

**Average Attachment to Fibronectin**

C

Cell Line

PC3  sh3  sh4  NC
FIGURE 36
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl
C12Q 1/68 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, MEDLINE, BIOSIS, CAPLUS (PITX2, pituitary homeobox 2, RIEGL, ARPI IDG2, proliferative disorder, cancer, neoplasm, tumor, tumour, carcinoma)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>WO 2007/039290 A2 (EPIGENOMICS AG) 12 April 2007 (see page 1 line 13, page 6 line 1 to page 7 line 11, page 11 lines 1-9, page 12 line 3, page 17 line 25, page 20 line 34, Claims 1, 5-6)</td>
<td>1-25, 32-37</td>
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<td>(see page 1 line 13, page 6 line 1 to page 7 line 11)</td>
<td>26-31</td>
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<td>x</td>
<td>MILLS, S. E. et al., &quot;Gleason Histologic Grading of Prostatic Carcinoma&quot;, Cancer. 1986, Vol. 57, Iss. 2, pages 346-349 (see page 346)</td>
<td>26-27</td>
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x Further documents are listed in the continuation of Box C

x See patent family annex

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  "O" document referring to an oral disclosure, use, exhibition or other means

  "P" document published prior to the international filing date but later than the priority date claimed

  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

  "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

  "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search
7 April 2010

Date of mailing of the international search report
U APR 2019

Name and mailing address of the ISA/AU
AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pca@ipaustralia.gov.au
Facsimile No. +61 2 6283 7999

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<td>NIMMRICH, I. et al., &quot;DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients&quot;, Breast Cancer Research and Treatment 2008, Vol. 111, Iss. 3, pages 429-437 (see whole document)</td>
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<td>A</td>
<td>WO 2007/039291 A2 (EPIGENOMICS AG) 12 April 2007 (see whole document)</td>
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX