Identification of genetic determinants of breast cancer metastasis to the bone

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ABSTRACT

The aim of this thesis was to identify genetic determinats of breast cancer (BC) metastasis to the bone. For that purpose we used a comprehensive approach that relies on human breast cancer cells, experimental mouse models and clinical data. First, we focused on genes specifically acquired for bone metastasis, highly expressed in bone metastatic lesions, but not enriched in the primary breast tumors that relapse to bone. Among these genes, the secreted factor NOG stand out as highly expressed in breast cancer cells, mainly ER-negative, with high capacity to develop bone metastasis in mouse models. NOG expression increases osteoclast differentiation and self-renewal capacity of breast tumors cells, thus fostering bone metastatic growth. Besides, we also centered on genomic alterations that were significantly associate with bone recurrence in primary breast tumors. Namely, by *in vivo* selection of estrogen receptor positive MCF7 human BC cell line we isolated highly specific bone metastatic derivative named BoM2. The BoM2 bone metastatic phenotype was associated with a 16q22-24 genomic gain. Interestingly, 16q22-24 genomic gain was able to specifically predict bone metastasis in primary breast tumors, including both ER positive and ER negative tumors. Next, within this genomic region, *MAF* gene emerged as a specific mediator of breast cancer metastasis to the bone. *MAF* overexpression in BC cells supported the bone metastatic colonization in immunodeffcient mice, while this process was impeded in its absence. Furthermore, we also confirmed that *MAF* is significantly overexpressed in breast tumors that relapse to the bone. *MAF* bone metastatic function is mediated through transcriptional regulation of PTHLH, which drives osteoclast differentiation and contributes to high osteolytic phenotype of *MAF* driven bone metastasis. Moreover, we showed that *MAF* controls other pro-metastatic functions that may collectively support the process of bone metastasis.

RESUMEN

El objetivo de esta tesis era identificar determinantes genéticos de cáncer de mama con metástasis óseas. Para ello se utilizó un enfoque integral que se sustenta en células humanas de cáncer de mama, modelos experimentales de ratón y datos clínicos. En primer lugar, nos centramos en los genes específicamente adquiridos para la metástasis ósea, altamente expresados en las lesiones de metástasis óseas, pero no enriquecidos en los tumores primarios de mama que recaen en el hueso. Entre estos genes, el factor secretado NOG destaca como altamente expresado en células de cáncer de mama, principalmente ER-negativas, con alta capacidad de desarrollar metástasis ósea en modelos de ratón. La expresión de NOG aumenta la diferenciación de los osteoclastos y la capacidad de auto-renovación de las células tumorales de mama, estimulando el crecimiento de metástasis óseas. Por otra parte, también nos centramos en las alteraciones genómicas que se asocian significativamente con la recurrencia ósea en tumores primarios de mama. Es decir, por selección in vivo de la línea celular humana de cáncer de mama MCF7, receptor de estrógeno positiva, aislamos una línea derivada altamente específica de metástasis ósea denominada BoM2. El fenotipo de metástasis ósea BoM2 se asoció con una ganancia genómica 16q22-24. Curiosamente, la ganancia genómica 16q22-24 fue capaz de predecir específicamente metástasis ósea en los tumores primarios de mama, incluyendo ER-positivos como ER-negativos. A continuación, dentro de esta región genómica, el gen MAF surgió como un mediador específico de la metástasis del cáncer de mama en hueso. La sobreexpresión de MAF en células de cáncer de mama apoyó a la colonización de metástasis óseas en ratones immunodeficientes, mientras que este proceso no tuvo lugar en su ausencia. También se confirmó que MAF se sobreexpresa de forma significativa en los tumores de mama que sufren una recaída en el hueso. La función de metástasis ósea de MAF está mediada a través de la regulación transcripcional de PTHLH, el cual impulsa la diferenciación de los osteoclastos y contribuye impulsando el fenotipo osteolítico de las metástasis óseas de

MAF. Es más, hemos demostrado que MAF controla otras funciones pro-metástasis que colectivamente pueden apoyar el proceso de metástasis ósea.

PREFACE

Cancer is a hazardous disease that affects millions of men and women worldwide. Current clinical practice favors better outcome in patients diagnosed with cancer due to early detection and improved therapeutic treatment. However, the metastatic disease still remains a major challenge in cancer treatment. Metastasis is the most life-threatening hallmark of cancer disease and the cause of death of most cancer patients. The research done in past decades led to building a framework of tumor evolution and metastasis, with many tumor-intrinsic and tumor-extrinsic mechanisms that govern metastatic behavior been described. Despite the accumulated knowledge about genes and mechanisms underlining metastatic progression, the translation of research into clinical practice and patient treatment persists limited. In breast cancer, substantial advance in clinical oncology was accomplished with targeted therapies based on the use of estrogen receptor (ER) and HER2/neu gene amplification in standard diagnosis. These biomarkers distinguish patients that will benefit from targeted therapy, rather than being exposed to cytotoxic chemotherapy. Moreover, molecular profiling of breast cancers help systematizing its remarkable heterogeneity; revealed basic molecular subtypes, characterized by certain molecular signatures that exist within breast tumors and have meaningful prognostic value for breast cancer patients. However, even with treatment, metastatic relapse occurs in 20%-30% of breast cancer patients, often after a long latency period after primary tumor diagnosis. Consistently, rising evidence indicates that metastatic genes represent a separate group of genes to those that are altered in the early steps of tumorigenesis. Many of metastasis genes are supporting the infiltration, survival and colonization of distant organ, and in breast cancer they specifically enable outgrowth from dormancy. These steps in metastatic progression are often related with activation of local and in particular distant, metastatic, stroma. Our research brings novel insights into genes and genomic alterations that are important for metastatic colonization of bone, the most common site of relapse in breast cancer. We identify *NOG* as metastatic virulence gene highly

expressed in bone metastasis but not in primary breast tumors that relapse to bone. *NOG* fosters outgrowth of breast tumor cells in bone by increasing self renoval capacity of tumors cell as well as supporting tumor stroma interaction in bone environment. Moreover, we describe 16q22-24 genomic gain and overexpression of transcription factor *MAF* as novel markers of bone metastasis in breast cancer. 16q22-24 genomic gain, and consequent *MAF* overexpression, is detected in breast cancer primary tumors that are at high risk of relapse to the bone. Furthermore, *MAF* emerges as specific mediator of breast cancer metastasis to bone. It contributes to bone metastatic growth through its target gene *PTHrP* that enhance osteoclast differentiation, thus promoting progression of osteolytic lesions. 16q22-24 genomic gain and *MAF* overexpression are biomarkers that could be directly translated into clinical practice, since their expression is detected by means of clinically standardized methods, FISH and IHC, respectively, and they significantly predict relapse to bone. Moreover, such markers could improve the treatment of breast cancer patients by stratifying patients that can directly benefit from bone modifying agents such as bisphosphonates and RANKL neutralizing antibody in systemic adjuvant setting. Moreover, *MAF* gene present potential molecular targets for anti-metastatic therapy. We believe that our work conceptually contributes to the reshaping breast cancer metastasis framework and possibly has a direct impact on the standard of care of breast cancer patients.

INDEX

LIST OF ABREVIATIONS

ACE	Analysis of CAN by expression data
ADH	Atypical ductal hyperplasias
AI	Aromatase inhibitors
AIC	Akaike information criterion
AITL	Angioimmunoblastic T-cell lymphoma
AJCC	American committee on cancer
ALDH	Aldehyde dehydrogenase
ALH	Atypical lobular hyperplasia
AMPK	AMP-activated protein kinase
AUC	Area under curve
BLI	Bioluminescence imaging
BC	Breast cancer
BMP	Bone morphogenetic proteins
BoM	Bone metastatic derivative
BP	Bisphosphonates
bZIP	Basic leucine zipper
C/EBPs	CCAAT/enhancer binding proteins
CGH	Comparative genomic hybridization
ChIP	Chromatin immunoprecipitation
CI	Confidence interval
CIN	Chromosomal instability
CNA	Copy number alterations
CRE	cAMP responsive element
CT	Chemotherapy
CTIBL	Cancer treatment induced bone loss
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DTC	Disseminated tumor cell
E2	Estrogen
EBCTTG	Early breast cancer Trislists' collaborative group
ECM	Extracellular matrix
EGF-R	Epidermal growth factor receptor
EHR	Extended homology region
EMC	Erasmus medical center
EMT	Enothelial-mesenchymal transition

ER	Estrogen receptor
ERBB2	V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2
ERE	Estrogen response elements
ESA	Epithelial specific antigen
ET	Endocrine therapy
FBS	Fetal bovine serum
FDA	Food and drug administration
FFPA	Formalin-fixed paraffinembedded
FGF	Fibroblast growth factor
FISH	Fluorescent *in situ* hybridization
G	Glutamine
GSEA	Gen set enrichment analysis
GEO	Gene expression omnibus
HE	Hematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HSC	Hematopoietic stem cell
HP1	Heterochromatin protein 1
HR	Hazard ratio
HR	Hormone receptor
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IQR	Interquartile range
LCIS	Lobular carcinoma *in situ*
LN	Lymph node
LOH	Loss of heterozigosity
MAF	Musculo aponeurotic fibrosarcoma
MARE	MAF-recognition element
MaSC	Mammary stem cell
MSC	Mesenchymal stem cells
MCSF	Macrophage colony stimulating factor
MET	Mesenchymal-endothelial transition
MM	Multiple myeloma
MSKC	Memorial Sloan-Kettering center
MUC	Sialomucin
NPV	Negative predictive value
OD	Optical density
ORF	Open reading frame
PCA	Principal component analysis

PDGF	Platelet-derived growth factor
PI-MEC	Pregnancy-identified mammary epithelial cells
PP	Pyrophosphate
PPV	Positive predictive value
PR	Progesterone receptor
PTHLH (PTHrP)	Parathyroid hormone related protein
pRB	Retinoblastoma protein
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative real-time PCR
RTKs	Receptor tyrosine kinases
RANK	Nuclear factor-kB (NF-kB) receptor
RANKL	Nuclear factor-kB (NF-kB) ligand
RMA	Robust multi-array average
ROC	Receiving operating characteristic
SD	Standard deviation
Se	Sensitivity
Sp	Specificity
SERM	Selective estrogen receptor modulators
SRE	Skeletal related events
T	Tumor
TCGA	The cancer genome atlas
TDLU	Terminal duct lobular units
TEB	Terminal end bud
TGFβ	Transforming growth factor β
TMA	Tissue microarray
TN	Triple negative
TRAP	Tartrate-resistant acid phosphatase
TRE	TPA-responsive element
TP53	Tumor protein p53
uAUG	Upstream AUG
UICC	International union against cancer
UL	Unfolded lobules
uORF	Upstream open reading frame
UPR	Unfolded protein

CHAPTER 1. INTRODUCTION

INTRODUCTION

1.1 Human cancer disease

Cancer is a collection of diseases that are characterized by misregulation of genes and pathways which results in disruption of cellular and tissue homeostasis. Cancer is defined as a malignant neoplasm. A neoplasm, 'new growth', represents abnormal growth of tissue. In contrast to benign neoplasms that remain confined to their original localization, malignant neoplasms are both capable of invading adjacent tissue and of spreading to other tissue or organs (metastasize). The term tumor is used synonymously for neoplasm. Hence, cancer is a vastly complex and chronic disease exhibiting a plethora of changes in multiple genes (Cao et al., 2011). Cancer diseases have diverse risk factors and epidemiology and originate from most of the cell types and organs of the human body.

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. About 12.7 million of new cancer cases and 7.6 million cancer deaths occur per year worldwide. Breast cancer in females and lung cancer in males are the most frequently diagnosed cancers in both economically developed and developing countries. These cancers are followed by colorectal and lung cancer in females and prostate cancer in males in economically developed world; or cervix and lung cancer in females and stomach and liver cancers in males in economically developing countries (Jemal et al., 2011).

Epidemiological analysis of cancer showed that environmental factors can influence cancer development. Such notion is supported by the fact that cancer incidence varies between geographic regions. Moreover, cancer incidence among migrants more closely reflects the rates in the adoptive country supporting the observation that the environmental exposure and medical practices are determinants of cancer risk (Colditz et al., 2006; Rastogi et al., 2004). Among non-genetical factors, tobacco is up to 10 times increasing the risk for lung cancer. Moreover, exposure to radiation, as well as obesity and lack of physical activity increase the rates of oesophageal, colon, uterine, kidney and post-menopausal breast cancer (Colditz et al., 2006; Fry and Fry, 1990; Polednak, 2003). Additionally, viral infections can cause certain type of cancer with examples of cervical cancer caused by human papilloma virus, hepatocellular

carcinoma by hepatitis B and C viruses, T cell leukemia by human lymphotropic virus type I (Colditz et al., 2006; Franco et al., 1999; Schiffman et al., 1993). All these factors can cause a genetic damage to normal cells which eventually leads to malignant transformation.

Cancer is a genetic disease arising from an accumulation of mutations and genetic rearrangements in cancer-susceptibility genes that promote clonal selection of cells with increasingly aggressive behaviors (Ding et al., 2010; Fearon and Vogelstein, 1990; Michor et al., 2004; Shah et al., 2009; Shah et al., 2012). The vast majority of mutations in cancer are somatic, arising in non-germline cells, and are found only in individual cancer cells. However, about 1% of all cancers arise in individuals with hereditary cancer syndrome that carry a particular germline mutation (Antoniou and Easton, 2006; Fearon and Vogelstein, 1990). Cancer-susceptibility genes comprise three classes of genes: gatekeepers (oncogenes and tumour-suppressor genes), caretakers (function in maintaining the genomic integrity of the cell) and landscaper genes that generate an abnormal stromal environment that contributes to the neoplastic transformation of cells. (Bissell and Radisky, 2001; Sharan et al., 1997; Sieber et al., 2003). Genetic instability is defining characteristics of most human cancers. The two key types of genetic instabilities include a defect in mismatch repair and chromosomal instability (CIN). The mismatch repair mutations result in an increased rate of point mutations and the consequent widespread microsatellite instability, which is found in some colorectal cancers (Boland and Goel, 2010). Most cancers, however, display chromosomal instability which refers to an increased rate of loss or gain of whole chromosomes or large parts of chromosome during cell division. The consequence of chromosomal instability is an imbalance in chromosome number. Moreover, as a result of chromosomal instability, one parental copy of a gene region can be lost, which is referred as loss of heterozigosity. Loss of heterozigosity is a mechanism commonly involved in inactivation of tumor suppressors (Michor et al., 2004).

Although key mutational changes are necessary for the initiation of neoplastic growth and are likely to be required for escape from a cellular niche, epigenetic modifications also have a crucial role in tumorigenesis. These modifications allow rapid cellular selection in a changing environment, thus leading to growth advantage for tumor cells at the expense of the host (Timp and Feinberg, 2013). The dynamic interplay between epigenetic signatures and post-translational modifications including phosphorylation, acetylation and ubiquitylation, attributes to the complicated regulation of tumorigenesis.

In addition, non-coding RNAs, such as miRNAs, are also involved in the signaling networks that are associated with tumorigenesis (Cao et al., 2011).

Several lines of evidence indicate that tumorigenesis in humans is a multistep process. These steps reflect genetic alterations that drive the progressive transformation of normal human cells into premalignant lesions. Eventually premalignant lesions evolve into malignant lesions that produce primary tumors, and finally into highly malignant derivatives which are invasive and metastatic (Hanahan and Weinberg, 2000; Yokota, 2000). However, only a restricted fraction of the cells in a primary tumor are considered to be highly metastatic. Namely, cells in a primary tumor are phenotypically and biologically heterogeneous and such heterogeneity is caused by the difference in the genes altered in each cancer cell. Therefore, highly malignant cells often acquire alterations in more genes than non-metastatic cells and various genes are differentially expressed between metastatic and non-metastatic cells (FIG 1).

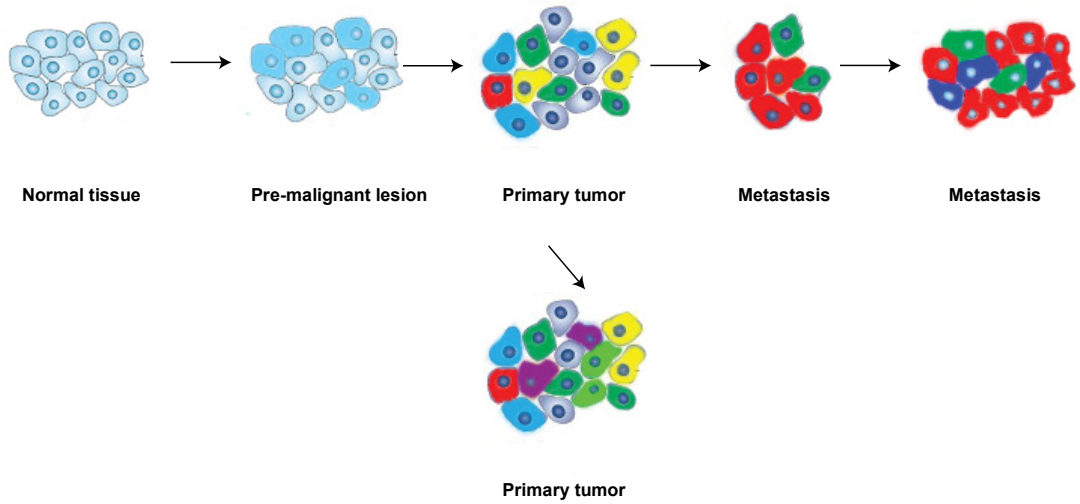


Figure 1. Stepwise progression of human cancer and clonal selection of metastasis. Normal cells evolve to highly malignant state due to accumulation of genetic alterations in the cells. Cells in primary tumors are phenotypically different harboring different mutations. Only a subpopulation of cells has the genetic prerequisites required for metastatic capacity. Furthermore, cells within both the primary tumor and the metastatic lesion(s) can continue to diversify as the lesions grow.

There are more than 100 of distinct types of cancers, which originate in different tissues and organs in human body. Moreover, the subtypes of tumors can arise within a specific organ. In order to decipher such complexity, Hanahan and Weinberg have proposed six hallmarks of cancer, which were later expanded with four new emerging

hallmark capabilities. These hallmarks constitute organizing principles for understanding the remarkable diversity of neoplastic diseases (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Hallmarks of cancer include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulation of cellular energetics, avoiding immune destruction, tumor-promoting inflammation and genomic instability and mutations. These principals reflect defects in regulatory circuits in cancer cells that lead to disturbance of regulated cell proliferation and homeostasis. Therefore, as normal cells evolve progressively to a neoplastic state, in a multistep process of human tumor pathogenesis, they need to acquire the hallmark traits that enable them to become tumorigenic and ultimately malignant. However, tumors are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another.

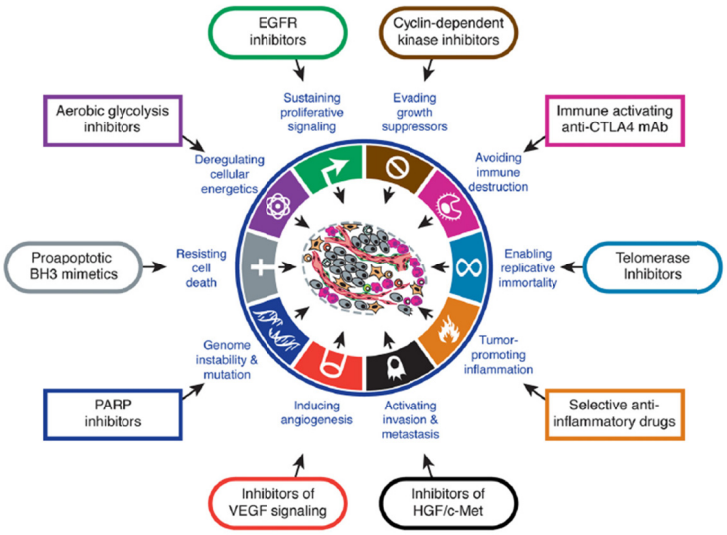


Figure 2. The hallmarks of cancer and their therapeutical targeting. The functional capabilities are acquired by almost all cancers and are necessary for tumor growth and progression. Drugs that interfere with mentioned tumor capabilities have been developed and are in clinical trials or in some cases approved for clinical use in treating certain forms of human cancers. The drugs listed are illustrative example; there is a deep pipeline of candidate drugs with different molecular targets and modes of action in development for most of these hallmarks. Adopted from (Hanahan and Weinberg, 2011).

The recruited normal cells which form the tumor-associated stroma, act as active participants in tumorigenesis rather than as passive bystanders. As such, these stromal

cells contribute to the development and expression of certain hallmark capabilities. Hence, simple enumeration of the traits of the cancer cells is not sufficient to describe tumor complexity. Rather, hallmarks of cancer must encompass contributions of tumor microenvironment to tumorigenesis. Deeper understanding of features of cancer cells and the tumor associated stroma will foster the development of putative therapeutic treatments and implementation of cancer research into clinical practice (FIG 2).

At present, tumors are seen as complex organs composed of specialized cell types and tumor microenvironment that recruit during multiple steps of tumorigenesis (FIG 3).

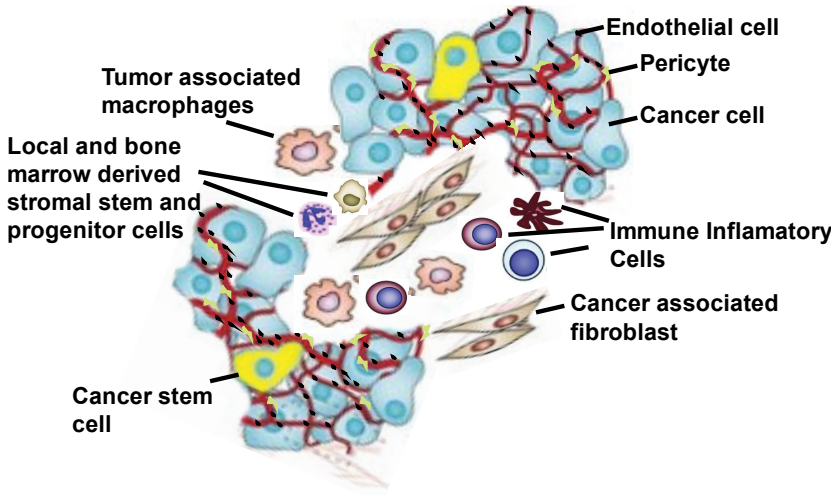


Figure 3. The tumor microenvironment. An assembly of distinct cell types constitutes most solid tumors. Both the parenchyma and stroma of tumors contain distinct cell types and subtypes that collectively enable tumor growth and progression. The multiple stromal cell types create a succession of tumor microenvironments that change as tumors invade normal tissue and thereafter seed and colonize distant tissues. Modified from (Hanahan and Weinberg, 2011; Quail and Joyce, 2013).

1.2 Breast cancer

Breast cancer (BC) is a malignant neoplasm originated in the mammary gland, mainly affecting women but also men. It is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths in 2008 worldwide. Its incidence rate varies around the world and increases with economic development. Yearly, 370.000 new cases are diagnosed in Europe and 230.000 in the United States (Jemal et al. 2011). Therefore, breast cancer constitutes a major health problem. However, death rates of breast cancer have been decreasing in European countries over the past 25 years, as a result of early diagnosis and improved treatment. Sporadic tumors represent the majority of breast cancer patients with somatic mutations and genomic rearrangements in oncogenes and tumor suppressors acquired during tumor progression. However, up to 5-10 % of breast cancer are hereditary, with large proportions characterized by mutation in *BRCA1* and/or *BRCA2* genes. The lifetime risk of breast cancer in women with a *BRCA1* or *BRCA2* mutation is approximately 75% (Narod and Salmena, 2011).

The research in past 30 years led to greater understanding of breast cancer disease elucidating its complexity and describing molecular pathways involved in breast cancer progression. The current view is that the breast cancer represents a collection of diseases which display high heterogeneity at both molecular and clinical level. The discoveries in breast cancer field resulted in significant advances in diagnoses and treatment of breast cancer. Nevertheless, several major unresolved clinical and scientific issues remain. These are related to (a) prevention (who needs it and when), (b) diagnosis (more specific and sensitive methods are warrant), (c) tumor progression and recurrence (what causes it and how to predict it), (d) treatment (who should be treated and how), and (e) therapeutic resistance (how to predict, prevent, and overcome it).

1.2.1 The mammary gland

The mammary gland is a unique organ that undergoes extensive remodeling and differentiation during the life of humans, being its main responsibility to provide nutrition to the offspring (FIG 4). The mammary gland is derived from the ectoderm. In human embryo the breast bud arises as a result of proliferation of basal cells of the epidermis. This is induced by factors secreted by mesenchymal cells present in the breast bud (Robinson, 2007). Mammary development occurs in three distinct and differentially regulated stages: embryonic, pubertal and adult. In humans, males and females have a similar rudimentary mammary gland at birth. Subsequent mammary development is initiated with the onset of female puberty and is dependent on the high levels of ovarian hormones oestrogen and progesterone. After puberty, the mammary gland undergoes cycles of growth and involution, which are regulated with the menstrual cycle, and with cycles of pregnancy and lactation (Ali and Coombes, 2002).

At histological level, the mammary gland comprises a rudimentary branching duct system lying in a fat pad (FIG 4). During puberty rudimentary ducts proliferate and swell into distinct multilayered epithelial structures known as terminal end buds (TEBs). These ductal structures then undergo successive rounds of elongation, bifurcation and lateral branching until reaching the limit of the fat pad and a full epithelial tree is being formed. Post-pubertal development results in cyclical increases in ductal branching. The end-buds of mammary ductal system are called alveoli and multiple alveoli are organized into lobes.

Each mammary gland contains 15-20 lobes that drain to the nipple. During pregnancy, further branching and end-bud (alveoli) development are occurring. After weaning, mammary gland regresses to a near prepregnancy state through massive programmed cell death (apoptosis). Branching network of ducts is lined by an inner layer of secretory luminal epithelial cells. Luminal cells are responsible for milk production during lactation and are surrounded by contractile myoepithelial cells that also produce the basement membrane (BM) that forms a physical barrier separating the epithelial and stromal compartments. Ductal myoepithelial cells are arranged in a more or less continuous monolayer, whereas alveolar myoepithelial cells are basket-shaped and do not form a continuous layer between the secretory epithelium and the surrounding basement membrane (Warburton et al., 1982).

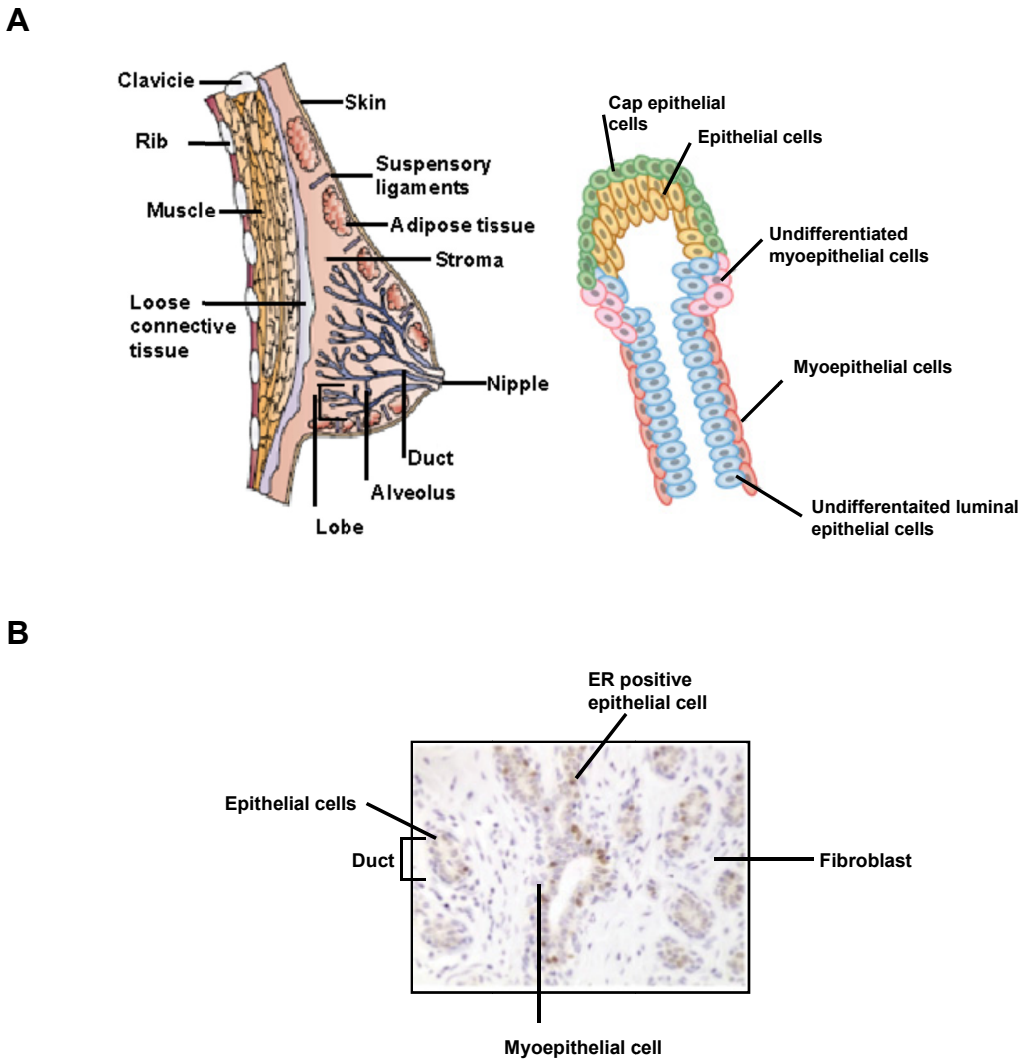


Figure 4. Structure of the mammary gland. (A) Anatomy of the human mammary gland. Each mammary gland contains 15–20 lobes, each lobe containing a series of branched ducts that drain into the nipple. The scheme of terminal end buds. A layer of epithelial cells, responsible for milk production are surrounded by an outer layer of myoepithelial cells with contractile properties. **(B)** The glandular ducts, embedded in fibroblast stroma to the estrogen receptor (ER; brown stained nuclei), showing that only a small proportion of epithelial cells are ER positive in the normal breast. Modified from (Capuco and Ellis, 2013)(Ali and Coombes, 2002).

Luminal epithelial cells display apical-basal polarity as shown by the localization of sialomucin (MUC1), epithelial specific antigen (ESA), and occludin on the apical membrane and integrin b4 on the basolateral membrane. Myoepithelial cells influence the differentiation, polarity, proliferation, and invasion/migration of adjacent luminal epithelial cells.

The mammary stroma is comprised of extracellular matrix and various cell types that

not only provide a scaffold to the organ, but also regulate mammary epithelial cell function via paracrine interactions and that include fibroblasts, adipocytes, blood vessels, nerves and various immune cells, all of which are important for normal mammary development and function. The epithelium is subjected to a highly dynamic pressure, undergoing dramatic morphogenetic changes during puberty, pregnancy, lactation and regression. Under these conditions the mammary epithelium is more susceptible to acquire mutations allowing cells to undergo transformation.

1.2.2 Mammary stem cells and progenitor cells

As emphasized before (see Introduction 1. 2.1) a mammary gland is a highly dynamic organ. Such notion is demonstrated during each menstrual cycle, where substantial growth of breast tissue occurs (Potten et al., 1988). Accordingly, during pregnancy, there is both a 10-fold increase in the number of alveoli per lobule as well as de novo formation of lobules by lateral budding from existing terminal ducts (Russo and Russo, 2004). These cellular dynamics led to postulate the existence of a population of precursor cells in the adult human breast that are capable of giving rise to new lobules. Several markers described in the literature are used for identification of human mammary stem cells (MaSC) (FIG 5). For instance, increased aldehyde dehydrogenase (ALDH-1) activity was associated with a population of human mammary epithelial cells which do not show estrogen receptor (ER) expression (Ginestier et al., 2007; Liu et al., 2008). Moreover, CD49+ and EpCam low markers are usually being used for sorting of the human mammary epithelial cells (Eirew et al., 2008). In mouse, enrichment of the MaSC compartment has, until now, been achieved by using a combination of cell surface markers ($Lin^{-}CD24^{+}CD29^{h}CD49^{fh}$) (dos Santos et al., 2013). As per localization of stem and progenitor cells in human mammary gland, the current view is that MaSC are present in mammary ducts and lobule, whereas progenitor cells are prevalent in the terminal ductal lobules (Villadsen et al., 2007). These data show that a candidate stem cell zone resides in ducts that are enriched in generally quiescent SSEA-4^{hi}/$K5^{+}$/$K6a^{+}$/$K15^{+}$/Bcl-2^{+}cells. The more frequently proliferating progenitors are found outside this region and are often surrounded by laminin-2/4 (Villadsen et al., 2007). Moreover, data from Peterson et al. provide evidence that a stem cell zone in ducts is marked by $K19^{+}$/$K14^{+}$ and can give rise to $K19^{+}$/$K14^{-}$, $K19^{-}$/$K14^{-}$, and $K19^{-}$/$K14^{+}$ lineage-restricted progenitors (FIG 5) (Petersen and Polyak, 2010).

In addition, RANK/RANKL signaling was described to be involved in proliferation and maintenance of normal and cancer breast stem cells. In mouse model, mammary glands of RANK- and RANKL-deficient mice develop normally during sexual maturation, but fail to form lobulo-alveolar structures during pregnancy due to defective proliferation and increased apoptosis of mammary epithelium (Fata et al., 2000). However, RANK/RANKL signaling has recently been proposed to be an important component in mammary carcinogenesis, specifically in the maintenance of breast cancer stem cells (Gonzalez-Suarez et al., 2010; Schramek et al., 2011; Tarragona et al., 2012). The distribution and function of mammary stem cells, as well as breast cancer stem cells in mature mammal remains to be fully elucidated.

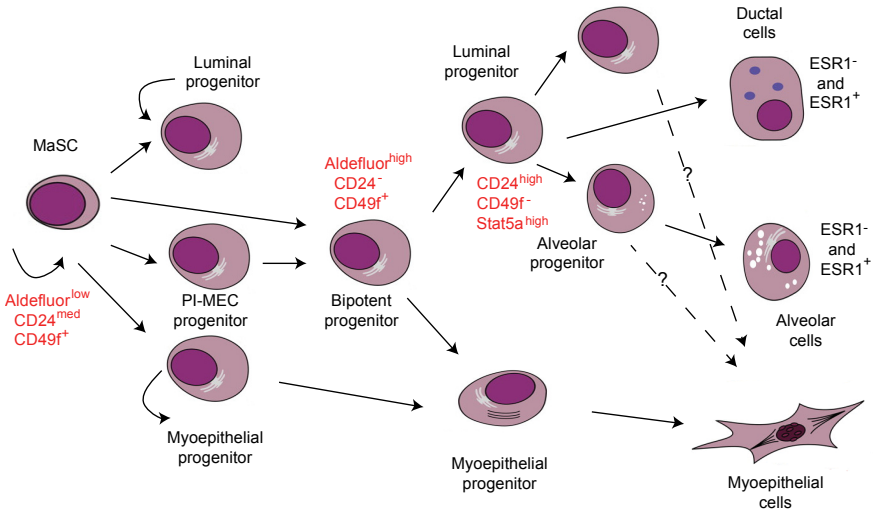


Figure 5. Schematic representation of mammary epithelial hierarchy based upon data from mouse. Multipotent, long-lived mammary stem cells (MaSC) give rise to a bipotent progenitor capable of generating cells in the luminal and myoepithelial lineages. MaSC also give rise to short-lived, pregnancy-identified mammary epithelial cells (PI-MEC). Recent lineage-tracing studies also suggest that unipotent luminal and myoepithelial progenitors provide much of the repopulating activity in an adult. Myoepithelial cells are ER^-, PR^-, $CK14^+$, $CD10^+$, SMA^+, while lobular and ductal luminal epithelial cells are $ER^{+/-}$, $PR^{+/-}$, $CK8/18^+$, $MUC1^+$, $CD24^+$. Modified from (Capusco and Ellis, 2013).

1.2.3 Hormonal regulation of mammary gland

Continuous mammary gland remodeling that occurs following puberty is under cyclical influence of reproductive hormones. These hormones include estrogens, progesterone, androgens, glucocorticoids, prolactin, thyroid hormone, insulin and insulin-like growth

factors (IGF-1 and IGF-2), fibroblast growth factors (FGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α) (Dickson and Lippman, 1995; Osborne and Arteaga, 1990). These hormones play an active role in breast epithelial cell growth, both during development and in the menstrual cycle. Since these hormones and their receptors regulate normal breast homeostasis, it is not surprising that malignant cells arising from breast tissue might also express receptors for many of these hormones and retain, to some degree, hormonal dependence.

Estrogen (E2), an ovarian steroid hormone, has a primary role in the establishment and maintenance of the reproductive function. It is actively involved in normal mammary cell growth and also modulates epithelial tumor growth (Yager and Davidson, 2006). Steroid hormones are hydrophobic molecules that diffuse across the plasma membrane (FIG 6). Inside the cell they bind to the intracellular receptors expressed by the hormonally responsive cells. Estrogen receptors (ER) are transcription factors that contain a domain of ligand-binding activity, a DNA-binding domain and a transactivation domain. Two ERs, ERα and ERβ have been described. These are encoded by different genes (*ESR1* and *ESR2* respectively) that are located in different chromosomes (Gorski et al., 1968; Toft and Gorski, 1966). In the absence of hormone, ERs are located in the cytosol. Upon estrogen binding, ERs change conformation, shuttle into the nucleus and form homodimers. The receptor-homodimers will then bind to specific DNA sequences known as Estrogen Response Elements (ERE).

In the breast, ERα receptor is expressed only in the subset of luminal epithelial cells (FIG 4). ERα-positive cells are located in the ductal epithelia and facilitate the development of a branched ductal tree, which functions as a scaffold for milk-producing alveoli. ERβ is expressed in the same cells as ERα and also in the myoepithelial cells. The ERα-null mice develop only a rudimentary mammary ductal tree and are unable to lactate, which indicates that ERα-positive cells make an essential contribution to mammary development (Couse and Korach, 1999). In contrast, the mammary glands of ERβ-null mice develop (Krege et al., 1998). Thus, ERα is a key transcriptional regulator in breast. Although the role of ERβ is still controversial, the scientific consensus is that it functions as a repressor of ERα transcriptional activity (Lindberg et al., 2003).

In order to mediate transcription, ERα requires the recruitment of coregulatory proteins, either coactivators or corepressors. The co-activator complexes enable ERs to respond appropriately to their ligands, to read extra- and intracellular signals, to modulate

chromatin structure and to cross-talk with transcriptional mediators at target gene promoters (Hall and McDonnell, 2005). The association with co-repressors provides an additional level of complexity in ER action. Ligand-independent transcription has also been described for the ER pathway. It occurs via phosphorylation of serine residues in ER, which is mediated by signaling events downstream of receptor tyrosine kinases, such as EGFR, ERBB2 and IGF receptor.

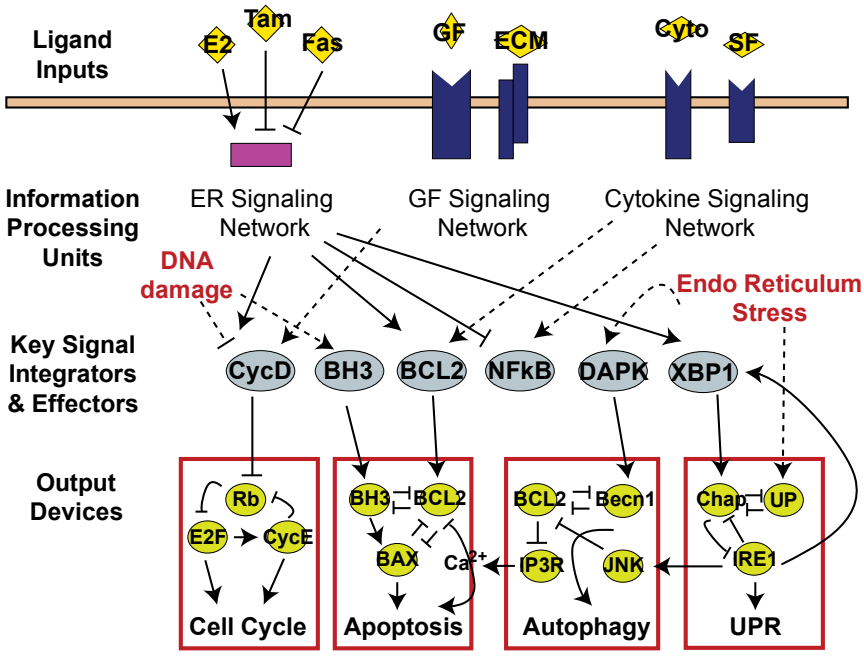


Figure 6. Estrogen signaling in breast. The estrogen receptor signaling network in breast epithelial cells. Extracellular signals, such as estrogen (E2), growth factors (GF), survival factors (SF), cytokines (cyto) and extracellular matrix (ECM), bind to receptor proteins which sum up the positive and negative signals drive responses in the downstream decision modules and stress modules. UPR, unfolded protein module involved in response to stress. Tamoxifen (Tam) and Faslodex (Fas) are inhibitors of the estrogen receptor (ERα). Modified from (Tyson et al., 2011).

Estrogen is synthesized primarily in the ovaries. Ovarian function and estrogen synthesis are regulated by the pituitary gonadotrophins, follicle-stimulating hormone and luteinizing hormone. Considerably lower levels of E2 and estrone are also synthesized by other organs, including mesenchyme of adipose tissue, osteoblasts and chondrocytes in bone, vascular endothelium, aortic smooth muscle and many regions in the brain. Ovarian synthesis of estrogen ceases at menopause. In post-menopausal

women, local synthesis is important for E2 functions in no-reproductive organs, such as the maintenance of bone density and cardiovascular protection.

The main function of ER signaling is to control growth and development of the mammary gland. This function is achieved by regulating the cell cycle, mainly G1 phase. Some important ERα target genes have a pro-proliferative function such as *c-MYC*, cyclinD1 (*CCND1*), cyclinE1 (*CCNE1*) and cyclinE2 (*CCNE2*). Moreover, such function is maintained in breast cancer cell lines. Namely, in these cells one of the earliest detectable transcriptional responses to estrogen is the induction of *c-MYC* via an atypical ERE (Dubik et al., 1987). Later, *CCND1* is induced coinciding with increased phosphorylation of retinoblastoma (pRb). These are necessary events for progression from G1 to S phase of the cell cycle.

Regarding the important role of estrogen in mammary gland, it is not surprising that estrogens are involved in breast cancer progression. There is considerable evidence that associates increased breast cancer risk with prolonged exposure to oestrogens. This includes the increased risk associated with early menarche, late first full-term pregnancy and late menopause. Why prolonged estrogen exposure should increase breast cancer risk it is still unknown. It is certainly clear that in a large proportion of cases E2 promotes breast cancer progression by stimulating malignant cell proliferation, but other mechanism are as well involved. Although only 15–25% of normal breast epithelial cells are ER-positive, two-thirds of breast cancers are ER-positive and most of these respond to endocrine therapy. In most cases breast cancer strikes post-menopausal women. Therefore, the non-ovarian sources of E2 are important in disease progression. Indeed, in post-menopausal patients, intratumoral concentrations of E2 are more than 20-fold higher than those present in the plasma. This is plausibly due to the fact that breast tumors produce high levels of aromatase, a cytochrome P450 enzyme complex that synthesizes oestrogens from adrenal steroids (Ali and Coombes, 2002). Similar to normal breast epithelium, in neoplastic breast cells estrogen signaling is contributing to control relative rates of cell proliferation and programmed cell death. However, pro-survival and proliferation signals are overwhelming pro-death and quiescence signals. Various transcriptional factors are found to closely interact with estrogen receptor signaling in breast cancer cells (Tyson et al., 2011). Therefore, estrogen signaling is plausibly involved in different processes, ‘modules’, in breast cancer cells. Such modules imply the ‘decision modules’ (cell cycle and apoptosis), the ‘stress modules’ (autophagy and the UPR) and the ‘signal

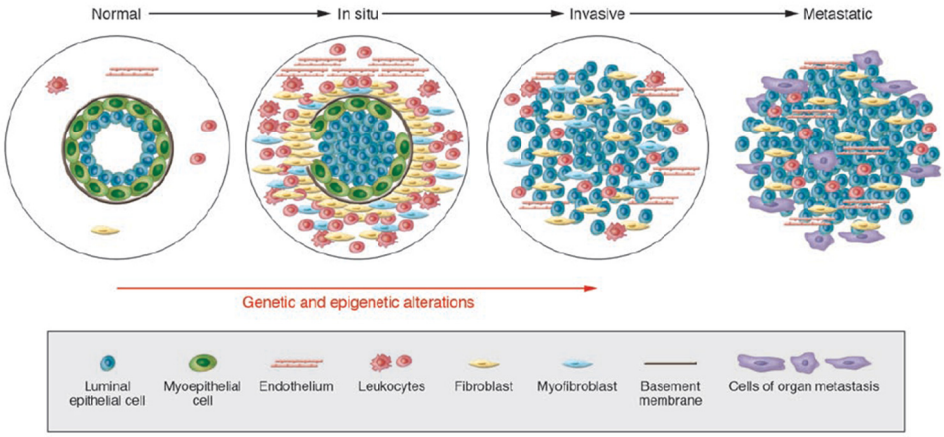
processing modules' (ER and growth factor signal transduction networks) (FIG 6) (Tyson et al., 2011).

1.2.4 Breast cancer development

Most of breast cancers are originating in the epithelial compartment of mammary gland. Therefore, 95% of breast cancers are carcinoma arising from luminal epithelial cell of ducts or lobes (Allred et al., 2001; Turashvili et al., 2005). Ductal subtype is the most frequent, accounting for 75%-80% of the cases. However, transcriptional profiling of breast cancer tumors has indicated that a proportion of breast cancers are of myoepithelial cell origin. In the initial phase of tumor progression breast cancers are confined to duct or lobe *in situ*. Later, tumor cells can invade the adjacent tissue and, in the most aggressive cases, colonize distant organs. Therefore, the hypothetical multistep model of carcinogenesis within the breast indicates that invasive carcinomas arise via a series of intermediate hyperplastic (with and without atypia) and neoplastic (*in situ* carcinoma) stages (Allred et al., 2001). As opposite to colon cancer, where there is a well-defined preinvasive lesion in the form of adenomas with defined genetic alterations, such well-defined lesion has not yet been identified in breast cancer (Allred et al., 2001; Fearon and Vogelstein, 1990). Rather, studies in the breast have been complicated by morphological heterogeneity of preinvasive lesions (FIG 7, FIG 8).
The initiation of breast cancer is due to transforming (genetic and epigenetic) events in a single cell. Multiple clinicopathological and genetic features distinguish breast carcinoma *in situ* from both normal breast tissue and other benign proliferative breast lesions (Burstein et al., 2004). For instance, chromosomal imbalances occur, with gain or loss at multiple loci, as hyperplastic lesions progress to invasive breast cancer. Moreover, loss of heterozygosity is noted in more than 70 percent of high-grade ductal carcinomas *in situ*, as compared to 35 to 40 percent of cases of atypical hyperplasia and none in specimens of normal breast tissue (Allred et al., 2001; O'Connell et al., 1998; Oesterreich et al., 2001). Furthermore, molecular markers associated with breast tumorigenesis have been identified. The estrogen receptor, normally expressed by luminal breast epithelial cells is expressed by over 70 percent of ductal carcinoma *in situ* lesions. The human epidermal growth factor receptor 2, ERBB2 (HER2/*neu*) proto-oncogene is overexpressed in a high percentage of ductal carcinoma in situ lesions but not in atypical hyperplasia. The *p53* tumor-suppressor gene is mutated in approximately 25 percent of all ductal carcinoma in situ lesions, but is rarely affected in

normal or benign proliferative breast tissue (Allred et al., 1992; Aubele et al., 2000; Burstein et al., 2004; Couse and Korach, 1999; Rudas et al., 1997).

A



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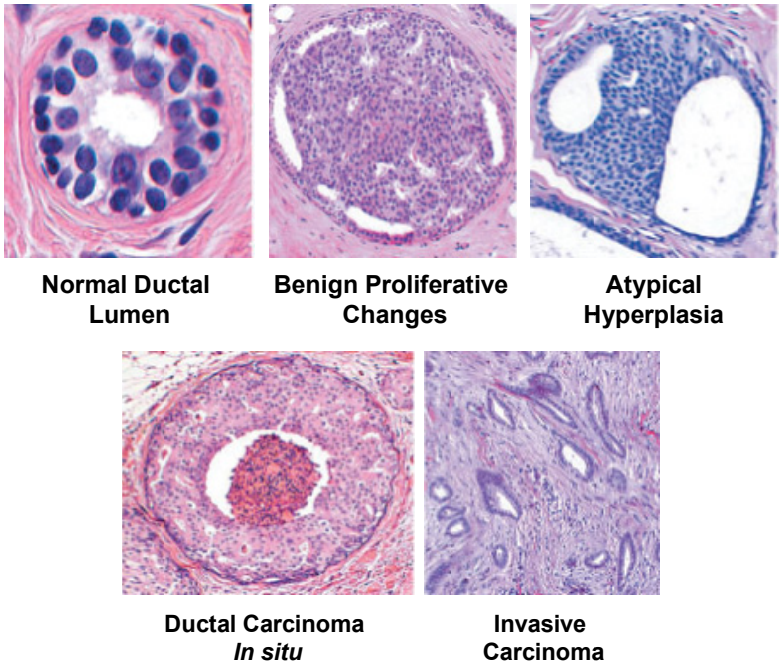
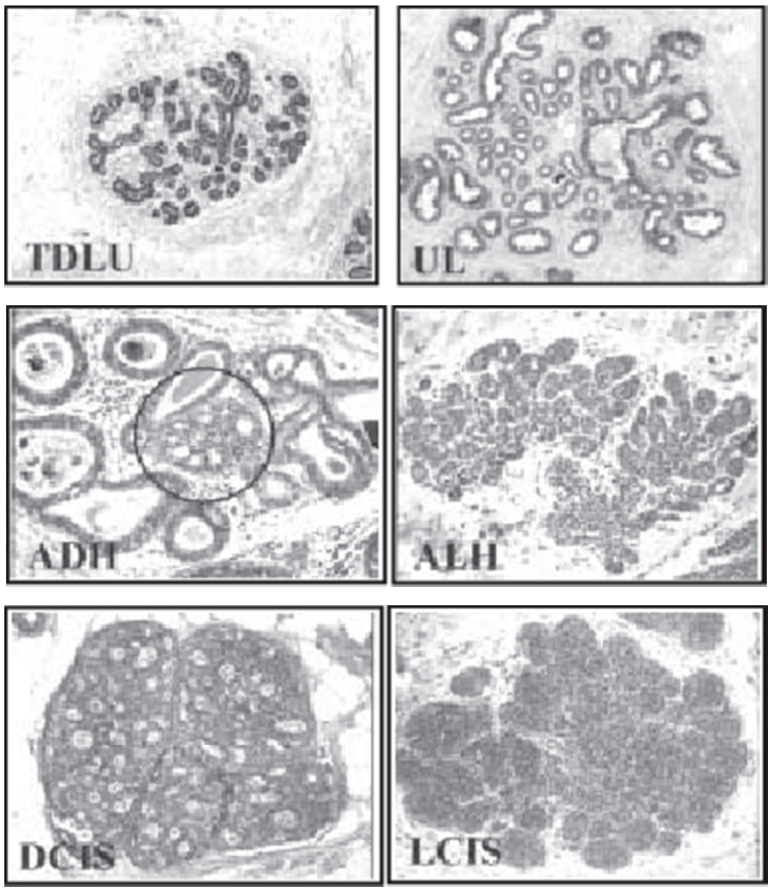


Figure 7. Hypothetical model of breast tumor progression. (A) Schematic view of normal, *in situ,* invasive, and metastatic carcinoma progression. Normal breast ducts are composed of the basement membrane and a layer of luminal epithelial and myoepithelial cells. Cells composing the stroma include various leukocytes, fibroblasts, myofibroblasts, and endothelial cells. In *in situ* carcinomas the myoepithelial cells are epigenetically and phenotypically altered and their number decreases, potentially due to degradation of the basement membrane. At the

same time, the number of stromal fibroblasts, myofibroblasts, lymphocytes, and endothelial cells increases. Loss of myoepithelial cells and basement membrane results in invasive carcinomas, in which tumor cells can invade surrounding tissues and can migrate to distant organs, eventually leading to metastases. From (Polyak et al., 2007). **(B)** The transformation from healthy tissue trough preinvasive lesions (ductal carcinoma *in situ*) to invasive breast cancer is shown. Modified from (Burstein et al., 2004).

In invasive breast cancers, subsequent tumor progression is driven by the accumulation of additional genetic changes combined with clonal expansion and selection. Various groups have performed comprehensive gene expression and genetic profiling studies comparing *in situ*, invasive and metastatic breast carcinomas but have failed to identify tumor stage-specific gene signatures. To date these studies focus mainly on tumor epithelial cells. However, involvement of other epithelial cells and myoepithelial cells and the stroma in tumor progression has not been explored in sufficient depth. Recent reports have shown that a significant fraction of genes is abnormally expressed in both tumor epithelial and stromal cells. These encode secreted proteins and receptors, implicating a role for abnormal autrocrine/paracrine signaling in breast tumor progression. Namely, chemokines expressed in stroma can enhance tumor cell proliferation, migration, and invasion and promote angiogenesis and metastatic spread. Several other chemokines including CXCL1, Il18, CCL5 and PCP1 have also been implicated in breast tumorigenesis (Polyak, 2007). Moreover, the work from Finak et al has shown that expression profiles of tumor stoma, based on 26-gene classifier, can predict clinical outcome in breast cancer. This signature implied that tumors that encompass more immune cells in their stoma, including T and NK cells, have a better clinical outcome. Consistently, the absence of immune cells is associated with worse outcome. Moreover, tumor stroma in these patients shows increased hypoxic and angiogenic response, where proliferation of endothelial cells and recruitment of monocytes and tumor associated macrophages is increased. Such tumor microenvironments is depicted with higher expression of pro-hypoxia gene *IL8*, monocyte and tumor associated macrophages attractants *CXCL1* and *ET-1*, as well as *MMP12* and *MMP1* genes, known to be included in tissue remodeling by macrophages (Finak et al., 2008).

A



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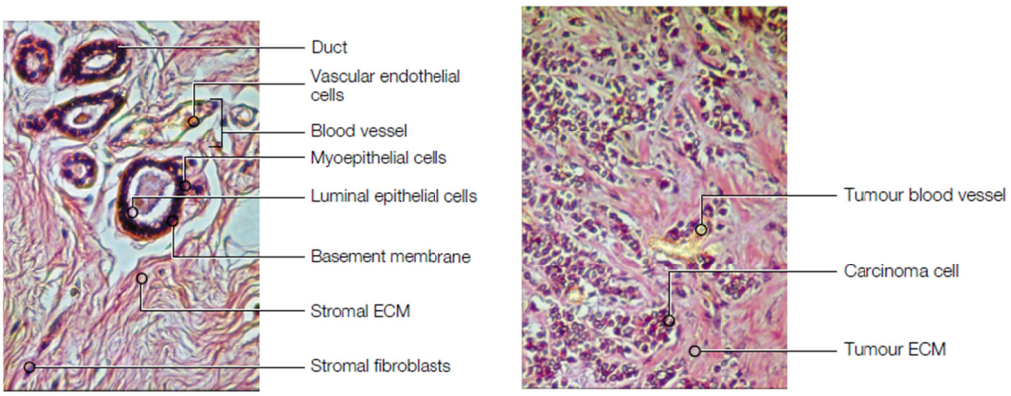

Figure 8. Histological model of breast cancer evolution. **(A)** Representative photomicrographs of important premalignant lesions. Terminal duct lobular units (TDLUs) are thought to be the major stem cell compartment giving rise to all types of premalignant breast lesions. Unfolded lobules (ULs) are TDLUs that are greatly expanded due to hyperplasia of their lining epithelium and may represent the earliest morphologically

recognizable premalignant change. Atypical ductal hyperplasias (ADHs) are small clonal outgrowths of low-grade epithelium (circle) that often arise in ULs. Ductal carcinoma *in situ* (DCIS) are large outgrowths of epithelium that greatly distend ductal and lobular spaces and vary on a histological continuum from low to high grade lesions. Atypical lobular hyperplasias (ALHs) represent relatively normal sized TDLUs that are partially filled by low grade neoplastic epithelium. Lobular carcinoma *in situ* (LCIS) represent TDLUs that are greatly distended by cells that are cytologically identical to those of ALH. ALH and LCIS are essentially the same disease on a quantitative continuum. From (Allerd et al., 2001). **(B)** The normal mammary gland shows a highly structured architecture. Lobular breast carcinoma is less organized. Tumor angiogenesis produces poorly defined blood vessels, and carcinoma cells intermingle with all the stromal elements. Modified from (Bissell et al., 2001).

1.2.5 Breast cancer classification and subtypes

Breast cancer is a heterogeneous disease with varied morphological appearances, molecular features, behavior, and response to therapy. Current routine management of breast cancer heterogeneity and corresponding treatment option is based on clinical and pathological factors. In early-stage breast cancer, where the use of systemic therapy has to be determined for every patient, the three main prognostic determinants used in routine practice are lymph node (LN) status, tumor size, and histological grade. The grading of a cancer in the breast depends on the microscopic similarity of breast cancer cells to normal breast tissue, and classifies the cancer as well differentiated (low, grade 1), moderately differentiated (intermediate, grade 2), and poorly differentiated (high, grade 3), reflecting progressively less normal appearing cells that have a worsening prognosis. The Nottingham (Elston-Ellis) modification of the Scarff - Bloom-Richardson grading system, also known as the Nottingham Grading System (NGS) is common clinical practice. NGS is based on the evaluation of three morphological features: (a) degree of tubule or gland formation, (b) nuclear pleomorphism, and (c) mitotic count (Rakha et al., 2010). Another widely used system or tumor classification is staging system. Staging is the process of determining how much cancer there is in the body and where it is located. The underlying purpose of staging is to describe the extent or severity of an individual's cancer, and to bring together cancers that have similar prognosis and treatment. The American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) recommend TNM staging. TNM describes the extent of the disease using three parameters: T, describes the size of the primary tumor and its invasive state; N,

describes involvement of regional lymph nodes; and M describes the presence of metastasis. Staging information that is obtained prior to surgery, by mammography, x-rays and CT scans, is called clinical staging and staging by surgery is known as pathological staging. Staging of breast cancer is one aspect of breast cancer classification that assists in making appropriate treatment choices, when considered along with other classification aspects such as estrogen receptor and progesterone receptor levels in the cancer tissue, the human epidermal growth factor receptor 2 (HER2/neu) status (see below), menopausal status, and the person's general health.

Hence, three biological markers, estrogen receptor, progesterone receptor and ERBB2/HER2 status, are systematically used to classify patients with breast cancer. Two of them, ER and PR, reflect the hormonal status and are quantified by immunostaining. Tumors are defined as ER and PR positive or negative, by counting number of positive cells (positive >1%, negative <1% of tumor cells). A third marker is ERBB2/HER2. Its immunohistochemical status grades tumors according to increase ERBB2 levels from 0 (negative), to 1+, 2+ and 3+. Tumors are further analyzed by Fluorescent *in situ* Hybridization (FISH) to determine *ERBB2/HER2* gene amplification. Hormone status and ERBB2/HER2 overexpression direct the strategy of targeted treatment due to their strong association with prognosis and outcome.

1.2.5.1 Breast cancer molecular subtypes

The application of microarray technology to profile human primary tumors allowed the introduction of a new concept in the classification of BC tumors, termed molecular subtypes. Tumors can be distinguished and classified by “intrinsic” differences in their gene expression patterns. Perou et al proposed that five main molecular subtypes of BC can be distinguished by gene expression profiling: Basal-like, ERBB2-positive, normal breast-like, Luminal A, and Luminal B BCs (Perou et al., 2000). Recently, a new subtype has been added to molecular portraits: claudin-low BCs (Prat et al., 2010). Interestingly, although the gene expression profiling classification system was not initially developed to predict clinical outcome, the different molecular BCs subtypes were found to be associated with distinct overall and relapse free survival rates (Sorlie et al., 2006). In fact, the six subtypes (basal-like, ERBB2, normal breat-like, luminal A, luminal B and claudin-low) not only express particular molecular markers and are associated differently with prognosis but they also display different physiopathological

and histological grade, and respond differentially to treatment. Basal-like and ERBB2 positive subtypes are associated with the shortest survival times.

Moreover, the recent scrutiny of breast cancer tumors, besides RNA profiling, included DNA copy number analysis, DNA methylation, exome sequencing, miRNA sequencing and reverse-phase protein array (2012). Such in depth profiling of human breast cancers confirmed the four main types of breast cancer tumors corresponding to luminal A, luminal B, ERBB2+ and basal-like tumors. Each of these subtypes showed significant molecular heterogeneity. Somatic mutations in only three genes (TP53, PIK3CA and GATA3) occurred at >10% incidence across all breast cancers. However, there were numerous subtype-associated and novel gene mutations. For instance the enrichment of specific mutations in GATA3, PIK3CA and MAP3K1 is present with the luminal A subtype. Moreover, protein expression defined new subgroups, possibly produced by stromal/microenvironmental elements. Integrate analyses have also identified specific signaling pathways dominant in each molecular subtype (2012).

To date, two diagnostical tests, MammaPrint and Oncotype DX that are based on molecular profiles of breast tumors are being in clinical practice. MammaPrint is a diagnostic test, used in paraffin embedded or fresh tissues of breast cancer patients, for the microarray analysis of 70-genes expressed in breast tumors (van 't Veer et al., 2002). This helps physicians determine whether or not each patient will benefit from chemotherapy. A low-risk test result means that the cancer has a 10% risk of relapse within 10 years without any additional treatments after surgery. With hormonal therapy alone this risk can be reduced to 5%. A high-risk test result means that the cancer has a 29% risk of coming back within 10 years without any additional treatments after surgery and these patients re recommended for both hormonal and chemotherapy (http://www.mammaprint.co.uk/).

Oncotype DX is a genomic test that analyzes the activity of a group of genes that can affect how breast cancer is likely to behave and respond to treatment. The Oncotype DX is used for two types of breast cancer patients. Firstly, it is used for to determine a risk of recurrence in woman with early stage ER positive cancer. Moreover, in these patients Oncotype DX can predict how probable it is that patients will benefit from chemotherapy after breast cancer surgery. Moreover, Oncotype DX can be used for patients with non-invasive breast carcinoma (ductal carcinoma *in situ*). In these cases, Oncotype DX can distinguish patients that can benefit from radiation therapy after surgery (http://www.oncotypedx.com/). In addition, recently developed, but still not in

clinical practice, PAM50 test may provide more accruable informations of patients at high risk of relapse that Oncotype DX. It measures 50 classifiers and five control genes and categorizes patients into five intrinsic breast cancer subtypes that confer prognostic information: luminal A, luminal B, HER2-enriched, basal-like, and normal-like. Unlike Oncotype DX, PAM50 does not predict a response to therapy. However, it provides more prognostic information in terms of recurrence and stratifies better the ER-positive node negative breast cancer patients at high or intermediate risk of relapse (Dowsett et al., 2013).

1.2.6 Breast cancer treatment

Although incidence of breast cancer is increasing due to better screening technologies (mammography) and an aging population, the mortality rates have been decreased because of improved treatment and earlier detection. Women diagnosed with invasive primary breast cancer receive both local and systemic treatment. Local treatment comprises surgery and radiation therapy. These treatments restrain the primary disease and reduce risk of recurrent cancer in the breast, chest wall and local lymph nodes. In some cases local treatment may prevent the dissemination of tumor cells, therefore reducing mortality of breast cancer disease. Two types of surgery can be applied depending on the grade and invasive phenotype of the tumor, and these are radical or conservative surgery. Postoperative radiotherapy is usually recommended after breast cancer surgery. It can reduce the risk of local recurrence by two-thirds. Furthermore, radiotherapy has beneficial effects on survival (Senkus et al., 2013).

However, systemic treatments are commonly given after local treatment, in order to reduce metastatic dissemination and diminish systemic recurrence and overall mortality. Systematic treatment implies cytotoxic chemotherapy and biological therapy (hormonal therapy in ER+ patients and anti-HER2 therapy, trastuzumab, in HER2 positive patients). Systemic treatment can be applied as neoadjuvant, adjuvant or palliative. Primary (neoadjuvant) systemic therapy is indicated in cases of locally advanced breast cancer, including inflammatory breast cancer, and for large operable tumors in order to reduce tumor size and possibly perform surgery. Chemotherapy is usually used for systemic neoadjuvant treatment. Trastuzumab should be added to primary chemotherapy in patients with HER2-positive tumors, whereas endocrine therapy in a neoadjuvant setting was not yet tested in clinical trials (Senkus et al., 2013). Adjuvant systemic therapy is given to patients after surgery to reduce the risk of

recurrence. Palliative treatment is used to improve survival and to control cancer after spreading.

Systemic treatment of breast cancer significantly improves disease-free and overall survival in the majority of patients, especially when used in adjuvant setting where the primary tumor has been removed. The magnitude of the effect of systemic treatment is greater in patients at high risk of relapse. Therefore, designing the strategy for systemic treatment of breast cancer patients require analysis of different clinical and pathological factors in order to discriminate patient at low- and high-risk of relapse. At present, internationally recognized prognostic parameters are: age, nodal status, tumor size, steroid receptor expression, HER2-*neu* status and histological grade. Hormonal receptor status is also a predictor for response to hormonal therapy. Gene expression profiles such as Mammaprint and Oncotype Dx Recurrence Score may be used to gain additional prognostic and/or predictive information to complete pathology assessment and help adjust the appropriate treatment for patient. This is particularly important in patients with ER positive early breast cancer patients (Guarneri and Conte, 2004; Senkus et al., 2013). The main goal of treatment planning is to avoid unnecessary treatments or underestimating the probability of relapse (Table 1).

Table 1. Systemic treatment recommendations for early breast cancer subtypes.

Subtype	Recomended therapy	Comments
Luminal A-like	**ET alone in the majority of cases.**	**Consider CT if (i) high tumor burden (four or more positive LN, T3 or higher) (ii) grade 3**
Luminal B-like (HER2-negative)	**ET + CT for the majority of cases.**	**If contraindications for the use of CT, one may consider ET + anti-HER2 therapy, altough no randomised data exist.**
Luminal B-like (HER2-positive)	**CT + anti-HER2 + ET for all patients**	
HER2-positive (non-luminal)	**CT + anti-HER2**	
Triple-negative (ductal)	**CT**	

For special histological types, we recommend following the St Gallen recommndation (ref) that propose ET for endocrine histologies (cribriform, tubular and mucinous) and CT for endocrine nonresponsive (apocrine, medullary, adenoid cystic and metaplastic). ET-endocrine therapy; CT-chemotherapy.

Modified from (Senkus et al., 2013).

Chemotherapy is administered in different regimens depending on the timing of administration and also on the combination of drugs. The chemical drugs more frequently used in breast cancer are paclitaxel, doxorubicin, docetaxel, 5 fluorouracil

and cyclophosphamide. These drugs are mainly cytotoxic acting via different mechanisms; disrupting microtubule function (e.g. taxanes), blocking replication machinery (e.g. anthracyclines) or intercalating DNA as anti-metabolites (e.g. fluorouracil). Combinatorial regimens in the adjuvant setting increase the survival compared to single-drug therapy. More recently, new agents are considered in systemic breast cancer treatment. Taxanes, such as paclitaxel and docetaxel, have been proved to have antitumor activity in advanced breast cancer.

Adjuvant chemotherapy causes many side effects and causes certain levels of toxicity in treated patients. The common side effect include myelosuppression, nausea and vomiting, neurological toxicity (comes mostly from taxanes), weight gain, ovarian failure, cardiac toxicity and fatigue. Although systemic chemotherapy reduces mortality in breast cancer patients, the optimal adjuvant treatment should be determined for individual patient in order to increase the efficiency of such treatment.
Tumors with an incomplete (some expression) and/or high degree of ER and PR expression are considered as endocrine responsive. Such patients are considered for endocrine therapy alone or for a combination of endocrine therapy and chemotherapy.
The systemic endocrine therapies include the selective oestrogen receptor modulators (SERM) and aromatase inhibitors (AI).

SERMs are competitive inhibitors of oestrogen-ER binding with mixed agonist and antagonist properties depending of the cell type. Tamoxifen is a SERM standardly used in treatment of breast cancer patients (Jensen and Jordan, 2003). Adjuvant systemic treatment with tamoxifen almost halves the rate of disease recurrence and reduces the annual breast cancer death rate by one-third. This effect made a significant contribution to the 25-30 % decrease in breast cancer mortality in the past two decades, Early Breast Cancer Trislists' Collaborative Group (EBCTTG) (2005). ER-positive breast cancers have high risk of metastatic recurrence for extended period of time following the surgery. Namely, in ER-positive breast cancer patients there are as many recurrences in years 6-15 after the surgery as in years 1-5. This surpasses the risk of recurrence of ER- negative breast cancer at 15 years follow-up. Therefore, the cumulative reduction in mortality is more than twice as big at 15 as at 5 years in standard setting of 5 years of tamoxifen treatment (EBCTCG) (2005).

The aromatase inhibitors are agents that block the synthesis of oestrogens. The development of third-generation AIs (e.g. letrozole, anastrozole, and exemestane) has provided an alternative to tamoxifen treatment in the management of postmenopausal

women with ER+ breast cancer. Unlike tamoxifen, AIs have no ER agonist activity. Furthermore, their differing mode of action may overcome tamoxifen resistance. However, they also show side effects reflected in bone loss and arthralgia that frequently develops in patients receiving AI.

The trastuzumab is used as systemic adjuvant treatment in HER2 patients with early stage disease. Moreover, trastuzumab is the currently first line treatment paradigm for HER2-positive MBC in combination with chemotherapy or as single agent (Yin et al., 2011). As a single agent trastuzumab can induce 30% response rate in HER2 overexpressign tumors, and the addition of trastuzumab to chemotherapy as compared with chemotherapy alone is associated with a significant improvement in objective response rate, duration of response and overall survival (median survival, 25.1 vs 20.3) (Slamon et al., 2001). However, trastuzumab has a cardiotoxiceffect and it should be avoided in patients with heart problems (Perez et al., 2011). Lapatinib is a reversible small molecule tyrosine kinase inhibitor that also targets HER2 by interfering with downstream signaling trough HER2 pathway. Lapatinib is currently approved as a first-line therapy in combination with letrozole for patients with metastatic breast cancer who overexpress HER2 and are estrogen receptor positive (Blackwell et al., 2010; Geyer et al., 2006).

Patients with triple negative breast cancer do not have options for targeted treatments. Hence, their treatment relay solely on chemotherapy. Recent reports have proponed novel targeted treatments that are under clinical trials. These imply poly ADP ribose polymerase (PARP) inhibitors, the mTOR pathway, insulin-like growth factor receptors, heat shock protein 90, and histone deacetylase (HDAC) inhibitors.

PARP inhibitors emerge as important targeted therapy for patients with BRCA1 and BRCA2, where this inhibitors of DNA machinery repair act as synesthetic lethal with defects in homologous recombination repair caused by BRCA1/2 mutation (Amir et al., 2010; Fong et al., 2009; Krishnakumar and Kraus, 2010). Moreover, studies have shown that activation of the mTOR/PI3K pathway promotes anti-estrogen resistance (Stoica et al., 2003). In 2012 mTOR inhibitors were approved for treatment of advance ER+ positive cancer in postmenopausal woman in combination with aromatase inhibitors (Baselga et al., 2009).

1.3 Metastasis

Metastasis, rather than primary tumors, is responsible for most cancer deaths. Metastasis arises following the spread of cancer from a primary site and the formation of new tumors in distant organs. Complex and redundant pathways involving the tumor cells and the microenvironment mediate tumor invasion at the primary site, survival and arrest in the bloodstream, and progressive outgrowth at a distant site. Although surgery and radiation therapy effectively control many cancers at the primary site, the development of metastatic disease signals a poor prognosis. Most metastatic lesions are not treated by surgery, as the presence of one lesion often signals a wider systemic disease. Chemotherapy, hormonal therapy and radiation serve palliative purposes in the metastatic setting, and some offer a modest but statistically significant extension of survival. Morbidity and mortality arising from metastatic disease can result from direct organ damage by the growing lesions, paraneoplastic syndromes, or from the complications of treatment. Mechanistic understanding of metastasis will help develop better therapies and improve patient outcome.

1.3.1 Metastatic patterns

Tumor metastasis consists of a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location, while cells go through process of clonal selection (FIG 1). Tumor cells must invade the tissue surrounding the primary tumor, enter either the lymphatics or the bloodstream, survive and eventually arrest in the circulation, extravasate into a tissue and grow at the new site. The term ‘colonization’ is herein used to reflect the combined influences of tumor cell proliferation, apoptosis, dormancy and angiogenesis in the formation of a progressively growing lesion in a distant site. One of the most enduring observations in metastasis research was published in 1889 by Stephen Paget. He proposed the “seed and soil hypothesis”, which postulated that tumor cells, the “seeds”, will only grow in a distant organ if they are competent to thrive in that particular microenvironment, the “soil” (Paget, 1989). This hypothesis was based on the preferential affinity of tumor cells for certain microenvironments. Almost forty years later, James Ewing challenged this theory proposing that metastatic preferences are dictated mainly by circulatory patterns (Fidler, 2003). Ewing hypothesized that tumor cells disseminate purely by mechanical factors to the organ to which they have the greatest vascular access. His proposal,

however, does not explain the observations of the metastatic spread made in patients or in experimental models. Although regional recurrences are highly dependent on the vascular perfusion efficiency, distant metastatic recurrence does not correlate with anatomically defined patterns of vascular or lymphatic circulation. For example, some organs such as the bone and the adrenal gland, which are frequent metastasis sites for certain cancers, are irrigated by a small fraction of the circulatory system. On the contrary, other organs with increased vascular supply, such as heart, muscle or kidney, are only sporadically colonized. Therefore, circulatory patterns alone provide only a partial explanation for preferred sites of metastasis.

In solid tumors, different tumor types have preferential sites for colonization. Although the brain is protected by the blood-brain barrier, several tumor types including lung, breast, and melanoma have a high incidence of brain metastasis (Gavrilovic and Posner, 2005). Sarcoma, colorectal, melanoma and breast tumors have a strong proclivity for dissemination to the lungs, while the liver, easily accessible through the portal-vein system, is preferentially colonized in colorectal and pancreatic cancer (Hess et al., 2006). Bone metastasis occurs in patients with breast, lung and prostate cancer with a high frequency (Hess et al., 2006).

The metastatic pattern of BC varies in the different subtypes. ER negative tumors show higher incidence of relapse in first years of follow-up but not after. Moreover, when looked at first site of relapse, ER-negative tumors preferentially colonize visceral organs (lung and liver) and soft tissue and afterwards bone. On the contrary, ER-positive status was associated with significantly higher rates of tumor metastasis to the bone. For patients with breast carcinoma, good prognostic factors for survival after the development of bone metastasis are good histological grade, positive estrogen receptor status, bone disease at initial presentation, a long disease free interval and increasing age. Interestingly, in ERBB2-positive tumors patients that progress through trastuzumab treatment have an increased rate of brain metastasis due to inaccessibility of the drug through the blood-barrier (Burstein et al., 2005).

1.3.2 Steps and molecular mechanisms of metastasis

Metastasis is a highly inefficient process. In experimental models only 0.001–0.02% of cancer cells that actually form metastatic foci (Fidler, 1970; Luzzi et al., 1998; Schneider et al., 2005). Primary tumors are known to release thousands of cells into

circulation daily, but metastatic frequencies are several orders of magnitude lower than that number. The success rate of metastasis depends on two main factors: 1) intrinsic properties acquired by cancer cells during transformation and 2) signals from the surrounding microenvironment (Joyce and Pollard, 2009; Polyak and Kalluri, 2010). An underlying concept in our study is that metastasis emerges from the somatic evolution of a genetically diversified cancer-cell population under the selective pressures of an environment that imposes tight rules on cell behavior (Gupta and Massague, 2006) (FIG 9). The enhanced proliferation or aggressiveness of tumor cells is challenged by multiple mechanisms that suppress tumor progression. Several mechanisms are intrinsic pressures such as genotoxic stress, telomere attrition, and engagement of apoptotic and senescence pathways (Lowe et al., 2004). Other mechanisms limiting tumor progression are external pressures from tumor microenvironment such as hypoxia, immune surveillance, and basement membranes.

In order to succeed, metastatic cells need to orchestrate very diverse cellular functions to overcome the difficulties. Independently of the colonization site, tumor cells must fulfill several prerequisites, including tumor initiating capacity (self-renewal), altered cellular adhesions, cell motility and survival.

The first step to colonize a distant organ is to escape from the primary tumor. Aggressive cells are able to disrupt the basement membrane and remodel the extracellular matrix to invade the subjacent stroma. Cancer cells secrete extracellular proteases that generate bioactive cleaved peptides, facilitating local invasion and, in later steps, modulating migration and survival (Kessenbrock et al., 2010).

The second step is to access circulation. In order to exit the tumor, cells must invade the tumor-associated vasculature. This process is facilitated by the production of new blood vessels through the activation of angiogenic mediators such as VEGF receptor (Hanahan and Folkman, 1996; Saharinen et al., 2011). Tumor cells penetrate into the blood vessels in a process called intravasation. The molecular mechanisms controlling intravasation are not well defined, but epithelial-to-mesenchymal transition (EMT) has been proposed to be a main contributor.

Once malignant cells enter systemic circulation they are subjected to several stresses including mechanical damage from the hemodynamic forces of the flow and the persecution by the immune system. Circulating cells take advantage of blood platelets to promote their survival (Gay and Felding-Habermann, 2011). Cell death induced by

loss of adhesive supports, also known as anoikis, might play a secondary role because it takes only few minutes for a tumor cells to exit the primary tumor to collide with a capillary bed and adhere to the vasculature wall (Nguyen and Massague, 2007). Mechanical shelter in capillaries is a prevalent form of tumor cell entrapment in distant organs. However, the initial homing of tumor cells to secondary organs may involve adhesive interactions between cell-surface receptors of malignant cells and ligands expressed in the host cells of distinct target sites.

To reach their final organ of destination, circulating cells adhered to the endothelium need to extravasate (Gupta et al., 2007; Hu et al., 2009) Different mediators are involved in this process, including angiopoietin-like-4 that disrupts vascular endothelial cell-cell junctions and increases permeability of lung capillaries (Padua et al., 2008). At the metastatic site, micrometastasis can remain in a dormant state for a prolonged period of time until reinitiating capacity is activated. Eventually, secondary tumors take advantage of the host stroma to empower their growth and achieve full colonization (Kaplan et al., 2005; Psaila and Lyden, 2009).

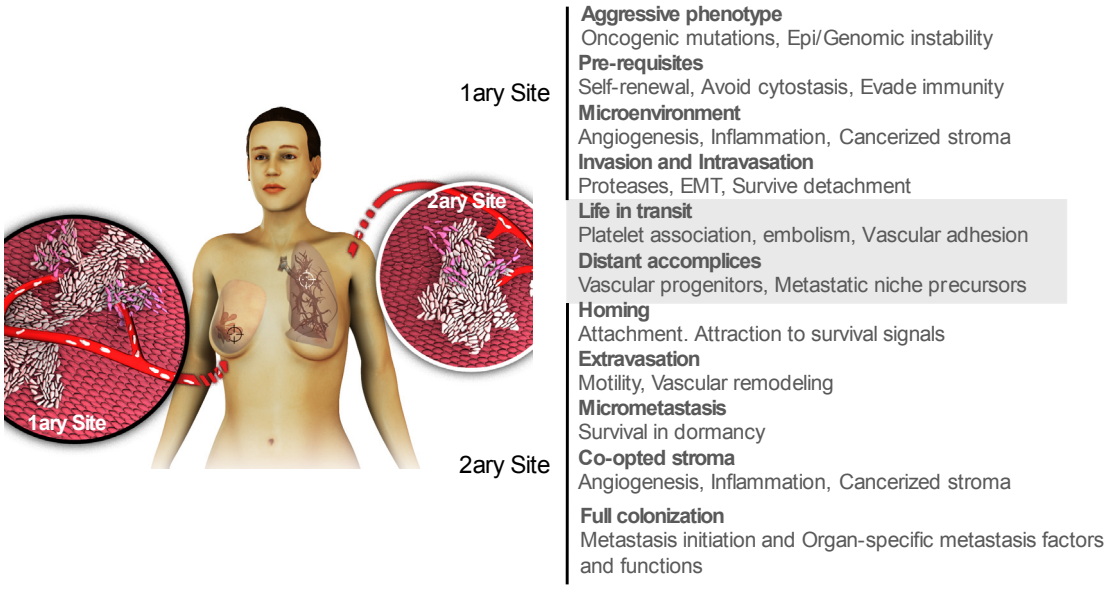

Figure 9. Stages of metastatic progression. Metastasis proceeds through the progressive acquisition of traits that allow malignant cells originated in one organ to disseminate and colonize a secondary site. Although these functions are depicted as a part of a continuous biological sequence, their acquisition during metastatic progression might not follow this particular order. Despite in some cases several factors may be necessary to implement a single step, other mediators of metastasis may facilitate execution of multiple stages

simultaneously. The specific steps of this sequence that are limiting for metastatic progression may also vary from one tumor type to another. Figure extracted from Gupta and Massagué, 2006.

1.3.3 Metastatic functions and its classification

How the functions required for metastasis are selected in the process of tumor progression is not well established. It is accepted that genomic instability and epigenetic plasticity are an important source of genetic heterogeneity in the bulk of the tumor(Baylin and Ohm, 2006; Fang et al., 2011; Feinberg et al., 2006). Genetic alterations in combination with a responsive microenvironment support the metastastic evolution of tumors. Transcriptomic analysis of primary tumors of BC patients revealed that genes expressed in the bulk of the primary tumor were sufficient to predict distant recurrence (van 't Veer et al., 2002). These observations raised the possibility that cells with metastatic potential within the primary tumor may not be as rare as it was originally believed. For example, Minn et al identified a set of genes that mediates BC metastasis to the lung.

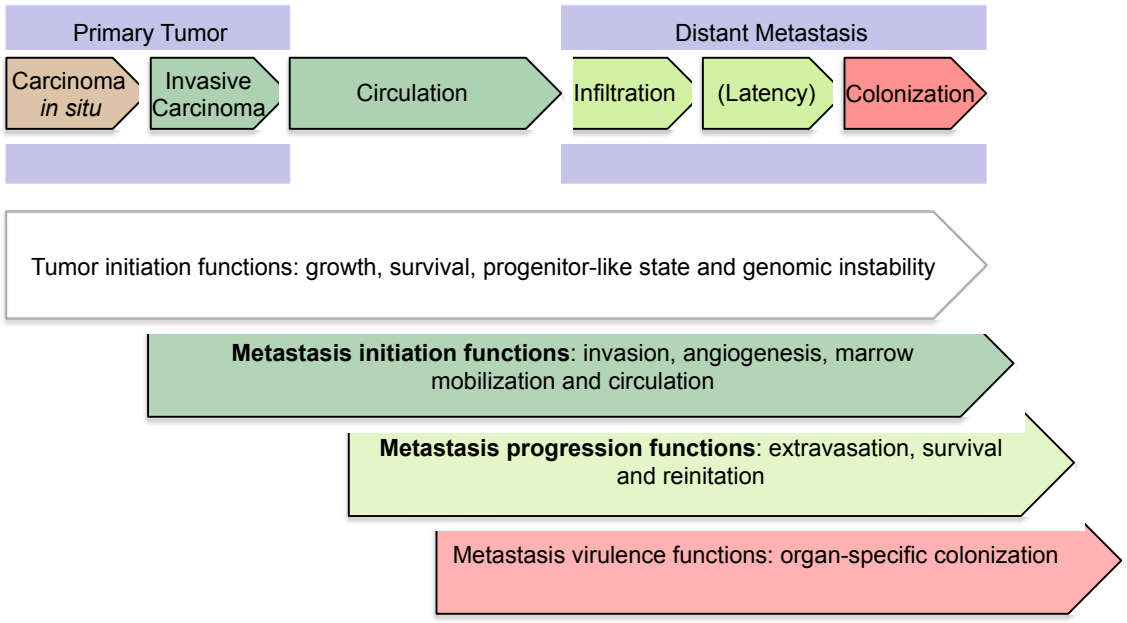

Figure 10. Basic steps of metastasis and classification of metastasic genes. The basic steps of metastasis include the progression of the primary tumor towards invasive carcinoma and the dissemination through the lymphatic or the blood vessels. Surviving circulating tumor cells colonize distant organs. After infiltration of the new microenvironment full colonization takes place with or without a period of latency. These steps are supported by both the cancer

cells and the tumor stroma. The metastasis specific genes endow circulating cells with distinct functions, infiltration, survival in dormancy, and colonization. These functions are classified as initiation functions (invasion, angiogenesis, marrow mobilization and circulation), progression functions (extravasation, survival and reinitiation) and virulence functions. Figure adapted from (Nguyen et al. 2009).

When being expressed in the primary tumors this set of genes clinically correlated with development of lung metastasis (Minn et al., 2005a). However, alterations in metastatic genes are acquired at individual steps along with metastatic progression. Of note, genes involved in tumor initiation, like ERBB2, are also mediators of different metastatic processes (Siegel et al., 2003).

Metastasis specific genes can be classified into three groups (FIG 10) (Nguyen and Massagué 2007). A first group includes genes associated with the initiation of metastasis that operate in the growth of the primary tumor and in invasion. These genes mediate functions related to angiogenesis, bone marrow mobilization, invasion and migration (Kalluri and Weinberg, 2009). Their expression may be lost upon exiting the primary tumor because their function is not anymore required. However, in some cases their expression might be retained (Nguyen and Massagué 2007). A second group includes genes that are associated with metastatic progression. These genes, so called metastasis progression genes, are involved in specialized activities required in specific steps of metastasis, yet they are already selected in the primary tumor. The third is composed of virulence genes which confer activities that are essential for the metastatic colonization of certain organs. Expression of virulence genes becomes only detectable in cancer cells that metastasize to such tissues. These genes enable the establishment of organ-specific metastasis rather than conferring an advantage to cancer cells at the primary tumor instead. Deregulation in the expression of these genes may result from stochastic alterations acquired for example, by genomic instability. In a particular microenvironment, their expression can be stabilized because it provides a selective advantage to malignant cells (Nguyen et al. 2009). Initiation and progression genes are the only groups that have prognostic value in BC primary tumors (Nguyen et al., 2009).

1.3.4 Bone metastasis

The skeleton is commonly affected by metastatic cancer. Breast, lung, prostate, kidney and thyroid cancer patients frequently develop bone metastasis (Coleman, 1997) (FIG 11). The prevalence of skeletal disease is greatest in breast and prostate carcinoma, reflecting both the high incidence and the relatively long clinical courses of bone metastasis in these tumors. Bone metastases are present in 80% of patients with advanced breast cancer disease and cause significant morbidity (Kozlow and Guise, 2005). Of great clinical importance is the observation that metastatic bone disease may remain confined to the skeleton, for extended period of time. In these patients, the decline in quality of life and eventual death is due almost entirely to the skeletal complications and their subsequent treatment. Furthermore, once tumors metastasizes to bone, they are usually incurable; only 20 percent of patients with breast are still alive five years after the discovery of bone metastasis (Coleman, 2001). Bone pain is the most common complication of metastatic bone disease resulting from structural damage, periosteal irritation, and nerve entrapment. Recent evidence suggests that pain caused by bone metastasis may also be related to the rate of bone resorption. Hypercalcemia occurs in 5-10% of all patients with advanced cancer but is most common in patients with breast carcinoma, multiple myeloma, and squamous carcinomas of the lung. Hypercalcemia can cause dysfunction of the gastrointestinal tract, kidneys and central nervous system. Pathological fractures of bone are relatively late complications of bone metastasis (Morton, 2000). The clinical courses of breast and prostate carcinoma patients with bone metastasis are relatively long, with a median survival of 2-3 years after bone metastatic disease occurred (Coleman, 1997).
When a tumor colonizes the bone, the metastasizing tumor cells mobilize and sculpt the bone microenvironment to enhance tumor growth and to promote bone invasion.
The understanding the bone microenvironment regulation of tumor localization, along with the tumor-derived factors that modulate bone in order to favor tumor expansion and invasion, is central to the pathophysiology of bone metastases. Basic findings of tumor-bone interactions have uncovered numerous therapeutic opportunities that focus on the bone microenvironment to prevent and treat bone metastases.

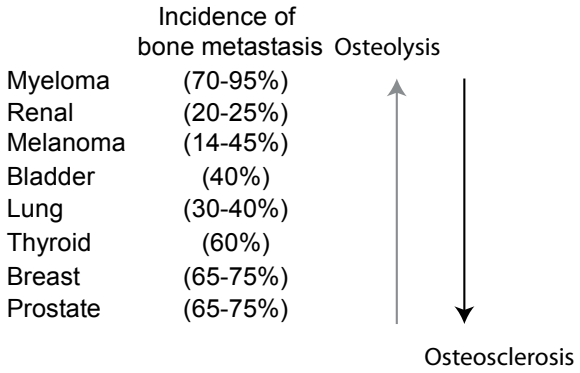

Figure 11. The nature of bone metastases. The incidence of bone metastases at autopsy and the typical radiographic appearance of the lesions are shown. Adapted from (Coleman et al.,2001).

1.3.4.1 Histology of bone

The bone is a highly vascular connective tissue that is constantly subjected to remodeling. The bone is the connective tissue distinguished by the fact that its matrix is mineralized by calcium phosphate in the form of crystals of hydroxyapatite. Therefore, bone serves as a structural tissue that acts as the levers for muscles, gives form to the soft tissues and provides protective cavities for vital organs. However, bone tissue is also a reservoir of minerals that can be redrawn upon hormonal stimulation (Hadjidakis and Androulakis, 2006). Bones can be classified based on their position, shape, size, and structure.

Bone matrix mainly consists of type I collagen fibers and noncollagenous proteins, and represents ~90% of the organic composition of the whole bone tissue. Within lamellar bone, the fibers form arches that allow the highest density of collagen per unit volume of tissue. The lamellae can run parallel to each other (trabecular bone and periosteum), or be concentric surrounding a channel centered on a blood vessel (cortical bone Haversian system) (FIG 12). Crystals of hydroxyapatite [$3Ca_3(PO4)_2 \cdot (OH)_2$] are found on the collagen fibers, within them, and in the matrix, and tend to be oriented in the same direction as the collagen fibers (Adler, 2000).

Osteocytes are osteoblasts trapped in the osteoid. Even though the metabolic activity of the osteoblast decreases once it is fully encased in bone matrix, these cells still produce matrix proteins. They form a network of thin canaliculi permeating the entire bone matrix. The osteocytes are finally phagocytosed and digested during osteoclastic

bone resorption (Elmardi et al., 1990). Despite the complex organization of the osteocytic network, the exact function of these cells remains obscure. It is likely that osteocytes enhance bone remodeling activity by recruiting osteoclasts to sites where bone remodeling is required (Lanyon, 1993).

A

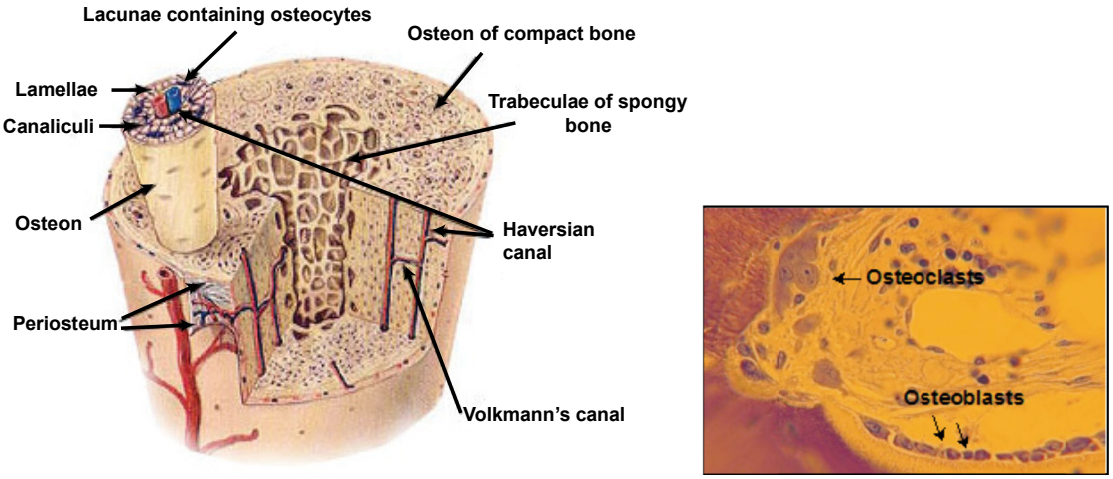

Figure 12. Histology of the bone. **(A)** Structure of compact and spongy bone. Compact or cortical bone is usually thick dense bone that forms the outer shell covering almost all bones in the body. The inner aspect consists of bone that is not as dense as cortical bone and has a honeycomb appearance. This is cancellous bone. The basic microscopic unit of bone is an osteon. Haversian canals run through the entire length of the bone carrying blood vessels. They are interlinked to each other through the Volkmans canals. On a transverse section of the bone each of these haversian canls is surrounded by a group of lacunae, which lodge an osteocyte. The entire group of osteocytes link to each other and to the centrally located haversian canal through cytoplasmic extensions that run through tiny channels called canaliculi. The osteocytes derive their nutrition through their cytoplasmic extensions from the vessels in the haversian canal. In mature bone the osteons are arranged in layers (Lamellar bone) while in developing bone they are arranged randomly (woven bone). **(B)** Histological image of bone with depicted osteoclast and osteoblast cells.

Osteoblasts are responsible for the production of the bone matrix constituents. Osteoblasts do not function individually but are found in clusters along the bone surface, lining on the layer of bone matrix that they are producing. They originate from multipotent mesenchymal stem cells, which have the capacity to differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, or fibroblasts (Bianco et al., 2001). Recent gene deletion studies have shown that absence of Runtrelated transcription factor 2 (Runx2) or of a downstream factor, osterix, is critical for osteoblast

differentiation. Osteoblasts produce osteoid by rapidly depositing collagen, followed by mineralization of matrix. Osteoblasts produce a range of growth factors under a variety of stimuli including the insulin-like growth factors (IGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β) and the bone morphogenetic proteins (BMP) (Canalis et al., 1993a; Canalis et al., 1993b; Chen et al., 2004; Globus et al., 1989; Rydziel et al., 1994).

The osteoclast, a giant multinucleated cell up to 100 mm in diameter, is derived from hematopoietic cells of the mononuclear lineage (Teitelbaum, 2000) and is the bone lining cell responsible for bone resorption. It is usually found in contact with a calcified bone surface and within a lacuna (Howship's lacunae) as a result of its own resorptive activity. Lysosomal enzymes such as tartrate-resistant acid phosphatase and cathepsin K are actively synthesized by the osteoclast and are secreted via the ruffled border into the bone-resorbing compartment.

1.3.4.2 Bone remodeling

In 1990 Frost defined the phenomenon of bone remodeling (Frost, 1990). This phenomenon is referred to the action of osteoclasts and osteoblasts where, in a homeostatic equilibrium, bone resorption and formation are balanced so that old bone is continuously replaced with new tissue. Osteoclasts and osteoblasts closely collaborate in the remodeling process in what is called a basic multicellular unit (BMU). During a cycle, approximately 10 osteoclasts dig a circular tunnel in the dominant loading direction (Petrtyl et al., 1996). They are followed by several thousands of osteoblasts that fill the tunnel (Parfitt, 1994). In this manner, between 2% and 5% of cortical bone is being remodeled each year with the trabecular bone being more actively remodeled.

The remodeling cycle consists of three consecutive phases: resorption, reversal and formation. In the resorption phase, partially differentiated mononuclear preosteoclasts migrate to the bone surface, form multinucleated osteoclasts and perform bone resorption. Their activation is achieved by the action of the cells of osteoblast linage. Namely, cells of the osteoblast lineage undergo changes in their shape, secrete enzymes that digest proteins on the bone surface and express receptor activator of NF-kappa B ligand (RANKL) (Hsu et al., 1999; Suda et al., 1999). RANKL is a 317 amino acid peptide, member of the tumor necrosis factor (TNF) superfamily. RANKL

interacts with a receptor on osteoclast precursors called RANK. The RANKL/RANK interaction results in activation, differentiation, and fusion of hematopoietic cells of the osteoclast lineage so that they begin the process of resorption. Additionally, it also prolongs osteoclast survival by suppressing apoptosis. In a reversal phase, mononuclear cells appear again and provide signals for osteoblast differentiation and migration. The formation phase follows with osteoblasts laying down bone until the resorbed bone is completely replaced.

Besides RANK and RANKL, other molecules have an important role in these processes. Namely, osteoprotegerin (OPG), a secretory dimeric glycoprotein belonging to the TNF receptor family with a molecular weight of 120 kDa, exhibits it effect by blocking RANKL. OPG acts as a decoy receptor, a soluble receptor acting as antagonist, for RANKL. It is mainly produced by cells of the osteoblast lineage, but it can also be produced by the other cells in the bone marrow (Hofbauer and Schoppet, 2004; Simonet et al., 1997). OPG regulates bone resorption by inhibiting the final differentiation and activation of osteoclasts and by inducing their apoptosis. Moreover, macrophage colony-stimulating factor (M-CSF), which binds to its receptor, c-Fms, on preosteoclastic cells, appears to be necessary for osteoclast maturation (FIG 13).

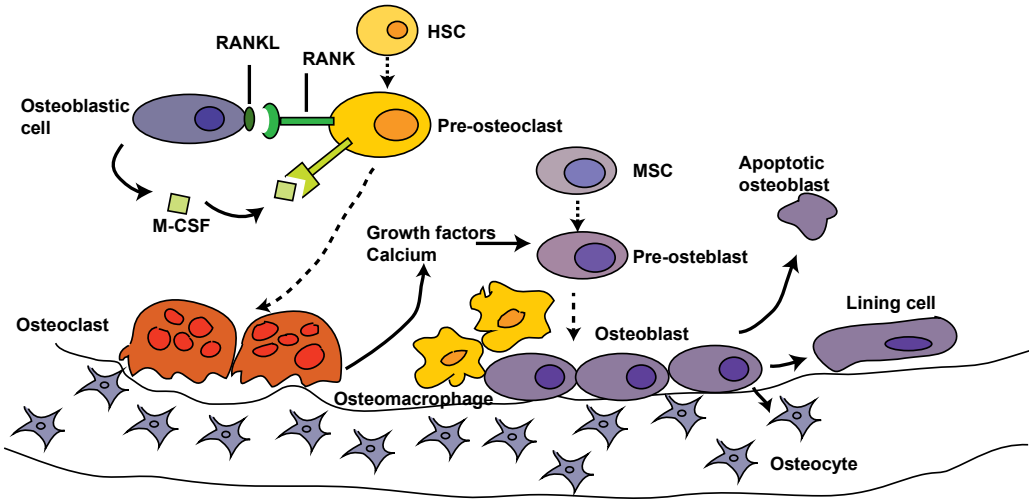

Figure 13. Bone remodeling process. In a balanced, coupled and sequential process (indicated by the dashed arrows), haematopoietic stem cell (HSC)-derived osteoclasts resorb bone (releasing growth factors and calcium) and mesenchymal stem cell (MSC)-derived osteoblasts replace the voids with new bone, a process that is dependent on osteoblast commitment, proliferation and differentiation coupled with osteoblast production of type I collagen and its subsequent mineralization to form the calcified matrix of bone. Osteocytes, which are terminally differentiated osteoblasts embedded in bone, sense

mechanical strain, signal to osteoclasts and osteoblasts, and participate in the remodelling process. Bone lining cells are osteoblastic in origin and have been proposed to form both a canopy over remodeling sites and a layer over bone surfaces, as well as a conduit to communicate with osteocytes. The endosteum and periosteum (the lining on the inne and outer bone surfaces) contain a population of tissue macrophages, termed osteomacs, which are likely to have important roles in bone remodelling. M-CSF, macrophage colony stimulating factor; RANK, receptor activator of NF-κB; RANKL, RANK ligand. Modified from (Weilbaecher et al., 2011)

The overall integrity of bone appears to be controlled by hormones and many other proteins secreted by both hematopoietic bone marrow cells and bone cells. Both systemic and local regulation of bone cells function is important for bone tissue integrity (Table 2).

Table 2. Regulation of bone remodeling.

Systemic regulation	
Parathyroid hormone	Stimulate bone resopbtion
Calcitirol	Enhance bone mineralization
Calcitonin	Cesation of osteoclast motility,inhibition of secretion of proteolitic enzymes
Growth hormone(GH)/IGF1/2	Skeletal growth
Glucocorticoids	Induce osteoblast maturation; decrease osteoblast activity
Thyrioid hormones	Stimulate both bone resorpion and formation
Estrogens	Decrease the responsivness of the osteiclast progenitor to RANKL; stimulate osteoblast proliferation and decrease their apoptosis
Androgens	Skeletal growth
Local regulation	
RANKl/RANK/OPG system	See in text
M-CSF	See in text
TNF-α, IL-10	Stimulate M-CSF production; directly increase RANKL production
IL-6	Stimulate osteoclastic bone resorption; promote osteoblast generation in conditions of high bone turneover
PTHrP	Promotes the recruitment of osteogenic cells; prevents the apoptotic death of osteoblasts,

1.3.4.3 Metastatic bone colonization

Metastatic bone colonization is a complex process that implies multiple steps. Initially, even before tumor cells reach the bone secondary site, the pre-metastatic niche in bone is being assembled. Further on, disseminated tumors cells go through process of bone homing, adaptation to bone microenvironment, formation of micrometastasis and activation of bone stroma in order to achieve successful colonization. Metastatic cells produce different growth factors which stimulate the stromal cells of the bone (mainly osteoclast and osteoblasts). These, in turn, proliferate and become activated, also secreting different growth factors. Moreover, osteoclasts induce release of growth factors trapped in the bone by mediating bone resorption process. Bone growth factors have great potential to stimulate tumor cells, hence, further busting metastatic growth.

Such interactions of tumor and bone stromal cells result in a vicious cycle of tumor growth.

Different reports show that primary tumors may condition the bone marrow via production of circulating factors (Weilbaecher et al., 2011). Examples of such factors include heparinase, osteopontin, matrix metalloproteinase and PTHrP. They are able to target cells in the bone microenvironment, thus rendering the bone to be susceptible to tumor cells localization and colonization (Kelly et al., 2005; Pazolli et al., 2009) (Anborgh et al., 2010; Guise, 2006; Lynch et al., 2005; McAllister et al., 2008). Besides this, tumor cells also show preference to adhere to the bone marrow endothelium. The mechanism mediating these functions could be similar to the one in hematopoietic stem cells. For instance, CXCL12 is expressed at high levels by osteoblasts and bone marrow stromal cells. On the other hand, cancer cells express CXCR4, the receptor of CXCL12. Therefore, homing of tumor cells to the bone is highly facilitated by this interaction (Kang et al., 2003; Muller et al., 2001; Smith et al., 2004; Sun et al., 2007). Moreover, expression of integrin αV β3 by breast and prostate cancer cells is associated with higher rates of bone metastasis, tumor associated osteolysis and colonization in bone (Clezardin, 2009; Schneider et al., 2010).

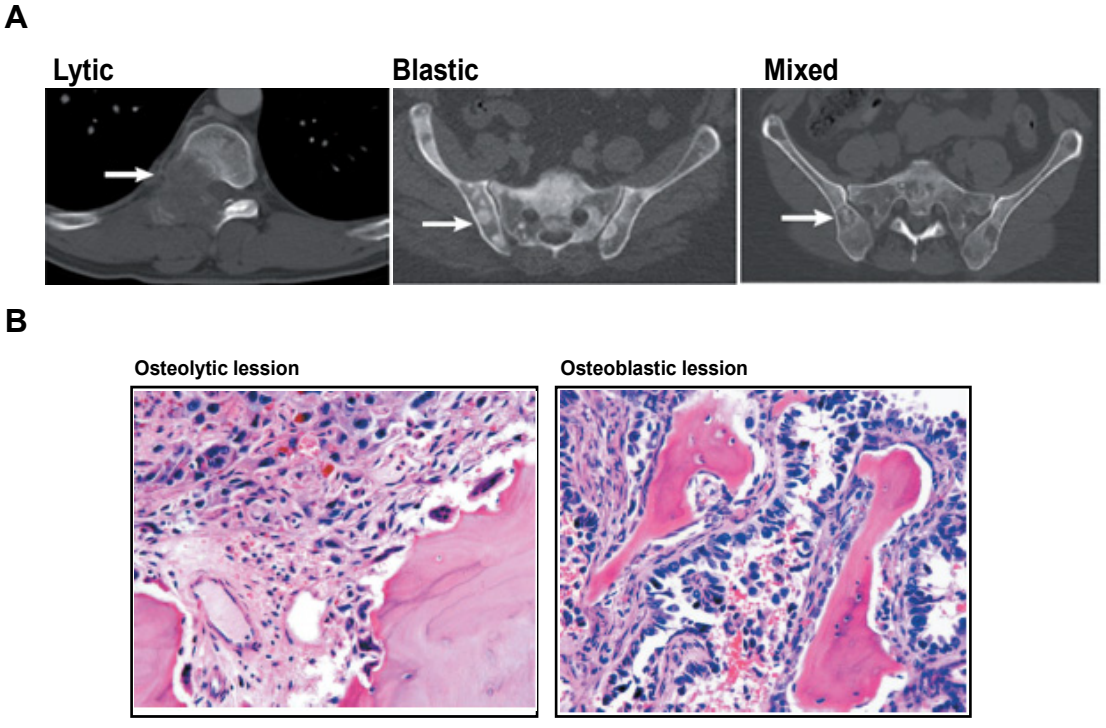

Figure 15. Clinical presentations of bone metastases. (A) Computerized tomography (CT) scans that show the different types of bone lesions: a lytic metastasis present in a

vertebral body from a patient with metastatic lung cancer; a blastic metastasis (deposition of new bone) in the pelvis of a patient with metastatic prostate cancer; and a scan that shows a patient with metastatic breast cancer who has both lytic and blastic metastases in the pelvis. **(B)** Histological images of osteolytic and osteoblastic bone metastasis. Modified from (Weilbaecher et al., 2011).

Once metastatic cells are in the bone microenvironment, they start the process of bone invasion. Bone invasion implies that tumor cell overcome the initial difficulties of new hostile environment and establish the interaction with bone stroma. As a result, successful colonization occurs and tumors grow as bone metastatic lesions. Tumor metastases in bone are defined as either osteolytic or osteoblastic. Osteolytic lesions are caused by osteoclast activating factors whereas in osteoblastic lesions cancer cell produce factors that stimulate osteoblast proliferation differentiation and bone formation. However, the osteolytic and osteoblastic lesions are two extremes since morphological analysis have revealed that, in most patients, bone metastases have both osteolytic and osteoblastic elements (FIG 14).

Multiple factors have been described to contribute to establishment of osteolytic bone metastasis. For instance, tumor derived PTHrP was characterized as mediator of local bone destruction and is associated with bone metastasis. Early reports have shown that transforming factor beta (TGFβ) induced tumoral PTHrP production in the bone microenvironment (Guise et al., 1996; Henderson et al., 2006; Yin et al., 1999). This, in turn, enhances bone resorption. Subsequent studies show that TGFβ in bone modulates many other pro-metastatic and osteolytic factors such as RANK, VEGF and CXCR4 (Dunn et al., 2009; Nannuru et al., 2010). Moreover, matrix metaloproteinases (MMPs) have been implicated in the general metastatic cascade, and more specifically in bone invasion and bone metastasis through an increase in locally active RANKL. Other autocrine-paracrine mechanisms that promote tumor osteolysis involve the Jagged 1-Notch signaling pathway. Jagged 1, expressed in breast cancer cells, mediates bone metastasis by activating the Notch pathway in bone cells. This results in increase of IL-6, which confers a growth advantage to tumor cells (Sethi et al., 2011). Expression of transcription factors such as GLI2, RUNX2 and hypoxia-induced growth factor 1 α (HIF1α) in tumor cells have been implicated in promoting tumor osteolysis (Dunn et al., 2009; Hiraga et al., 2007; Javed et al., 2005; Sterling et al., 2006) (FIG 15).

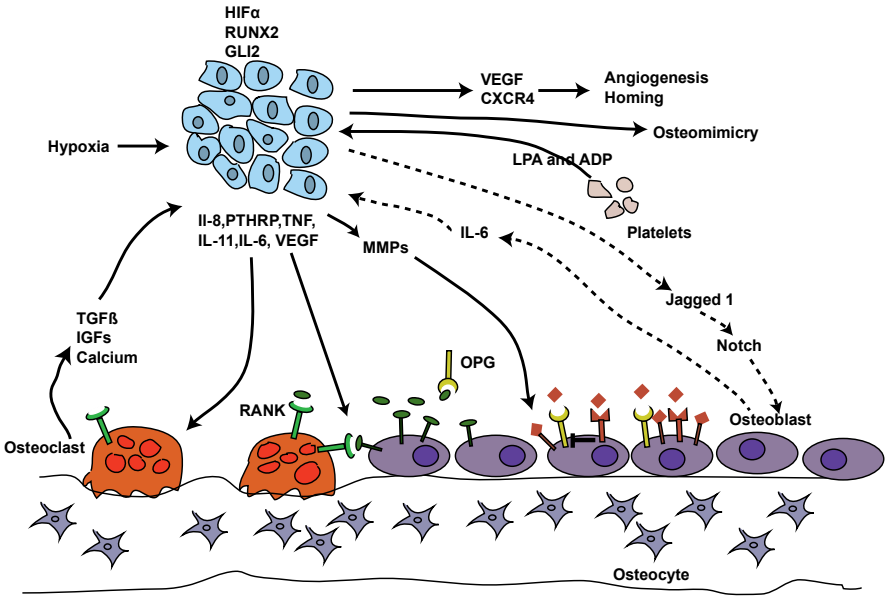

Figure 15. Mechanisms of tumour-associated osteolysis. Tumors secrete osteolytic factors (such as, parathyroid hormone-related protein (PTHRP), interleukin-11 (IL-11), IL-6, IL-8, vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF), Jagged 1 and epidermal growth factor (EGF)-like ligands) that stimulate osteoclastic bone resorption either directly (indicated by solid arrows) or indirectly (indicated by dashed arrows) by increasing the ratio of receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG). Osteoclastic bone resorption causes the release and activation of growth factors (transforming growth factor-β (TGFβ) and insulin-like growth factors (IGFs)) and ions (calcium) that are stored in mineralized bone matrix to further enhance the local milieu. Tumour-associated hypoxia and hypoxia-inducible factor 1α (HIF1α) in conjunction with TGFβ can increase tumour production of VEGF and the chemokine CXCR4 to increase angiogenesis and tumour homing. Tumour-produced matrix metalloproteinases (MMPs) can cleave membrane-bound RANKL (green balls) or EGF-like growth factors (red diamonds), which can increase the ratio of RANKL to OPG to favour osteoclastogenesis. Platelet-derived lysophosphatidic acid (LPA) and ADP act on tumour cells to induce growth and the release of osteolytic factors IL-8 and IL-6. Modified from (Weilbaecher et al., 2011).

Different factors have been described to contribute to tumor development in osteoblastic bone metastasis. These factors are mostly related to osteoblast proliferation, survival and activation. In return, osteoblasts stimulate tumor cells resulting in a codependent cycle (Mundy, 2002). One of the best studied factors involved in osteoblastic bone metastasis is growth factor endothelin-1 (Yin et al, 2000). It is commonly found to be expressed in osteoblastic prostate and some osteoblastic

breast cancers. Endothelin 1 is described to stimulate bone formation and osteoblast proliferation (Nelson et al., 1995). Moreover, TGFβ and bone morphogenic protein (BMP) family members are also shown to be important in osteoblastic bone metastasis. TGFβ growth factors induce osteoclast apoptosis, but stimulate osteoblast proliferation and chemotaxis (Marcelli et al., 1990). BMPs are reported to stimulate osteoblast differentiation and are involved in matrix production and mineralization (Harris et al., 1994). In prostate cancer, proteases such as serine protease urokinase (uPA) or serine protease prostate specific antigen (PSA) play important roles in bone metastasis (Mundy, 2002). uPA might mediate tumor invasiveness and growth factor activation. PSA cleaves the amino-terminus of PTHrP (Cramer et al., 1996; Iwamura et al., 1996). Moreover, these proteases are able to activate TGFβ. In addition, osteoblast proliferation is driven by mitogenic factors, such as insulin-like growth factors (IGFs), fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF). These factors are highly expressed by tumor cells and potentially have a role in osteoblastic bone metastasis (Chackal-Roy et al., 1989; Gleave et al., 1991) (FIG 16).

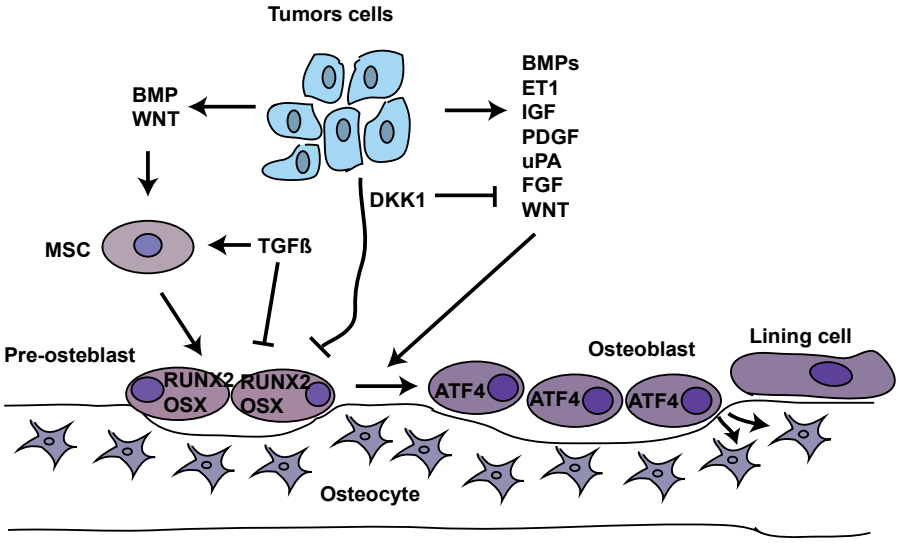

Figure 16. Tumour–osteoblast interactions. Tumours produce various factors that regulate bone formation at different levels of osteoblast development. Bone morphogenetic proteins (BMPs), WNTs and transforming growth factor-β (TGFβ) provide signals to mesenchymal stem cells (MSCs) to move to areas of bone formation and to differentiate to the osteoblast lineage. Osteoblast progenitors and pre-osteoblastic cells respond to positive osteoblastic factors that are produced by tumour cells, such as BMPs, endothelin 1 (ET1), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), urinary plasminogen activator (uPA) and fibroblast growth factors (FGFs), as well as the negative

regulator dickkopf 1 (DKK1). Osteoblast-associated transcription factors include RUNX2, osterix (OSX) and activating transcription factor 4 (ATF4). Once osteoblasts produce and mineralize a collagen matrix (shown in blue) they may undergo apoptosis, become lining cells or become sequestered in the bone matrix as terminally differentiated osteocytes. TGFβ can function at multiple levels that include recruiting stem cells and promoting stem cell renewal, coupling osteoclastic bone resorption to bone formation and inhibiting osteoblast differentiation. The BMP inhibitor, noggin, as well as endothelin A receptor antagonists can block osteoblastic metastases. Little is known of the potential interactions between tumors and osteocytes. Modified from (Weilbaecher et al., 2011).

In summary, bone tissue, rich in different growth factors, is a very potent secondary site for tumor growth. Successful metastatic growth is supported by active interaction between stroma and cancer cells. Besides osteoclast and osteoblasts other stromal cells also enhance vicious cycle of tumor growth. For instance, endothelial cells that reside in bone marrow can be recruited by tumor cells in a process of neoangiogenesis. Moreover, hematopoietic cells that reside in the bone marrow can influence cell communication by releasing pro-angiogenic factors or by creating permissive conditions in the tumour microenvironment that favor the growth of blood vessels (De Palma and Naldini, 2006; Silva et al., 2008). Also, adipose stem cells, myeloid and immune cell as well as platelets, influence tumor growth through various mechanisms. These include their ability to promote and protect tumor-initiating cancer stem cells, promote tumor growth or enhance antitumor immune responses or their angiogenic action (Bingle et al., 2002; De Palma and Naldini, 2006; Dirkx et al., 2006; Klopp et al., 2010; Yang et al., 2010).

1.3.4.4 Tumor cell dormancy in bone

Cancer recurrence after therapy and a long period of remission is frequent. In the case of breast cancer, 20-45% of patients will relapse years of decades later (Karrison et al., 1999; Weckermann et al., 2001). This latency period can be explained by cancer dormancy, a stage in cancer progression in which residual disease is present but remains asymptomatic. In many cancers, such as breast, prostate, gastric, colon, the minimal residual disease has been detected within the bone. Therefore, understanding tumor cell dormancy within the bone marrow is probably a key to inducing long-term remissions and overcoming resistance to cancer therapies (Aguirre-Ghiso, 2007).

Preclinical evidence suggests that metastatic tumor cells can home to and localize in the hematopoietic stem cell (HSC) niche and survive in a dormant state. The skeleton is the preferred site for many tumor cells to reside, and they can remain there in a dormant state for a long period of time. The presence of disseminated tumor cells (DTCs) in bone marrow is useful to predict the development of skeletal metastasis. Interestingly, it also predicts recurrence in distant organs such as lung or liver (Pantel and Brakenhoff, 2004). Another clinically important aspect of quiescent DTCs residing in bone marrow is their elevated resistance to cancer therapies (Aft et al., 2010; Braun et al., 2000; Clezardin, 2009). Therefore, understanding the biology of tumor cell dormancy in bone is of great importance for tackling metastasis recurrence in cancer patients.

Molecular characterization of disseminated breast cancer cells in the bone marrow has revealed the specific expression of a subgroup of transcripts, including the metastasis regulator *TWIST1*, and SRC protein activation (Watson et al., 2007; Zhang et al., 2009). Breast cancer cells that have active SRC are associated with an increased risk of bone metastases in humans, where SRC has a cell-autonomous pro-survival role. Increasing clinical evidence suggests that DTCs are influenced by the bone microenvironment. The treatment of women with localized breast cancer with the bisphosphonate zelondronic acid diminished the presence of DTCs in the bone marrow (Aft et al., 2010; Rack et al., 2010). Moreover, recent reports show that tumor cells might mimic HSC cells. Therefore, factors that are inducing HSC dormancy could be important for tumor cell dormancy in bone marrow niches (Shiozawa et al., 2010).

1.3.4.5 Treatment of skeletal metastasis

Bone metastasis is usually followed by complications such as pain, hypercalcemia, fractures, spinal cord compression and consequent deterioration in performance status. External radiotherapy, radiometabolic therapy, surgery and pain medications can control symptoms, but these treatments are not devoid of toxicities and their effects are usual shorter than disease course. Bone modifying agents like bisphosphonates (BP) and monoclonal antibody against RANKL emerged as putative agents for treatment of bone metastatic disease.

BPs are stable synthetic analogues of pyrophosphate (PPi). BPs have a carbon atom bridging the two phosphate groups instead, giving a fundamental P–C–P backbone

structure, that is essential for their biological activity (Winter et al., 2008). The P–C–P moiety acts as a 'bone hook' and is required for binding to bone mineral and calcium ion chelation (Rogers et al., 2000; Russell, 2006; Russell and Rogers, 1999). Zoledronic acid is the most potent commercially available nitrogen containing BP to date, characterized by an imidazole side ring containing two nitrogen atoms.

Following administration, BPs bind to and accumulate in mineralized bone matrix and are consequently released during resorption and selectively internalized by osteoclasts, therefore they effectively inhibit of bone resorption (Winter et al., 2008). These pharmacological compounds are widely used in the treatment of skeletal diseases associated with high osteoclast activity and accelerated bone turnover, such as osteoporosis. *In vitro* studies BPs are able to induce apoptosis of osteoclasts. Anti-tumor activity of different BPs has been demonstrated mainly in breast, prostate and myeloma cancer cell lines (Green, 2004). Namely, *in vitro* studies of zoledronic acid effect on tumor apoptosis, and inhibitory effects on tumor cell adhesion, invasion tumor cell viability and proliferation and angiogenesis weredemonstrated (Winter et al., 2008). Moreover, as by inhibiting bone resporption, BPs reduce the release of bone-derived cytokines and growth factor. In such manner bisphosphonates render bone to be less assessable for metastatic colonization by tumor cells. Zoledronic acid has also been shown to reduce expression of Cox-2 and subsequent prostaglandin expression in tumor cells. It inhibits the chemoattractant effect of stromal cell-derived factor 1 (SDF-1) downregulates expression of its receptor CXCR-4 on tumor cell (Denoyelle et al., 2003). Furthermore, other reports suggested that indirect anti-tumor effects of bisphosphonates include inhibition of angiogenesis (Winter et al., 2008).

Although radiotherapy is the treatment of choice for localized bone metastatic patients, the bisphosphonates prove an additional treatment indicated for patients with poorly localized bone pain or recurrence in bone pain (Coleman, 2008). Additionally, based on the results of large randomized controlled trials, the bisphosphonate have become the standard for the treatment and prevention of skeletal complications associated with bone metastasis in patients with breast cancer and multiple myeloma (Coleman, 2004). The use of bisphosphonates in managing bone metastasis had a profound beneficial effect on the frequency and severity of skeletal morbidity. The proportion of patients suffering from skeletal-related events (SREs) has been reduced by 30-50% and this reduction has resulted in significant and clinically important benefits in quality of life (Coleman, 2004; Coleman and McCloskey, 2011). Based on the preclinical evidence

that bisphosphonates may directly act on tumor cells, a number of studies evaluating the antitumor and antimetastatic potential of bisphosphonates in breast cancer have been initiated. Until now this clinical trials are somehow inconclusive.

Although for the past 20 years the bisphosphonates have been standard treatment for both benign and malignant bone diseases new bone modifying agents are being suggested in treatment of bone metastasis. The elucidation of the signaling pathways that regulate bone cell function and, in particular, recognition of the role of receptor activator of nuclear factor-kB ligand (RANKL) in bone resorption has provided potential therapeutic targets for inhibiting osteoclast activity. Denosumab, a fully human synthetic IgG2, monoclonal antibody against RANKL, has been approved for the treatment of postmenopausal osteoporosis as well as bone loss in patients with cancer and bone metastasis, and offer as an alternative treatment to bisphosphonates (Dougall and Chaisson, 2006). Denosumab binds to RANKL with high affinity, preventing its interaction with RANK in a way similar to that of osteoprogetenin (Brown and Coleman, 2012). Early development of drugs targeting RANK/RANKL started with recombinant OPG (AMGN-007). While OPG was active and well tolerated, AMG162, later named denosumab, a fully human antibody targeting RANK-ligand, reduced levels of markers for bone turnover markers to a greater extent (Bekker et al., 2004; Steger and Bartsch, 2011). As denosumab potently inhibits both mature osteoclast function and osteoclast differentiation, this agent was anticipated to inhibit interactions between tumor cells and osteoclasts, which would suppress bone turnover and potentially also inhibit the development of malignant bone lesions (Brown and Coleman, 2012). The clinical studies where denosumab was used in prevention of treatment-induced bone loss setting showed that it has a great potential in delaying the SRE. Some trial had shown it more potent than zoledronic acid (Lacey et al., 2012). The role of denosumab as anti-tumor agent in prevention or treatment of bone metastatic disease needs to be further clarified.

1.5 Transcription factor MAF

The MAF transcription factor belongs to the AP1 superfamily of basic leucine zipper (bZIP) proteins, which also include Fos, Jun, CREB and ATF families (FIG 17). Among this superfamily the contribution of the MAF family to tumorigenesis has been

underestimated although *MAF* gene was shown to be translocated in human cancers in the late 90's (Chesi et al., 1998; Nishizawa et al., 1989).

The MAF family derives its name from a founding member, v-maf, transduced in a retrovirus that induces musculo-aponeurotic-fibrosarcoma in chicken. The seven-member MAF family comprises large and small MAF (FIG 17). As dimeric partners of the bZIP NF-E2/Nrf family small MAF proteins might contribute to oncogenic processes by participating in antioxidant responses, but they have not yet been shown to be involved in human cancer. By contrast, large MAF proteins have been directly involved in carcinogenesis as demonstrated in cell culture, animal models and human cancers. The large MAF members MAFA (or L-MAF), MAFB, MAF (also known as c-MAF) and NRL differ from small MAF (MAFF, MAFG and MAFK) in their amino terminus by the presence of a transactivation domain (FIG 17). A single orthologue for each subfamily exists in *Drosophila melanogaster* (Eychene et al., 2008).

The AP-1 superfamily is characterized by the ability to bind TRE (12-O- tetradecanoyl phorbol 13-acetate (TPA) responsive element) or CRE (cAMP-responsive element) DNA sequence through their bZIP domain (Vinson et al., 2006). The leucine zipper domain allows the formation of homodimers or heterodimers, a prerequisite for DNA binding. As homodimers, these proteins recognize palindromic sequences, with the basic domain contacting DNA directly. The MAF family is defined by the presence of an additional homologous domain called the extended homology region (EHR) or ancillary domain, which also contacts DNA. Consequently, MAF proteins recognize a longer palindromic sequence than other AP1 family members (Kataoka et al., 1994b; Kerppola and Curran, 1994). This so-called MARE (Maf-recognition element) sequence is composed of a TRE or CRE core contacted by the basic domain and a TGC flanking sequence that is recognized by the EHR domain (Kataoka, 2007; Yang and Cvekl, 2007) (FIG. 17).

Although the TGC motif is crucial for MAF binding, the TRE or CRE core can be more degenerate. It is noteworthy that many natural *MAF* target genes contain only half of a MARE palindromic site. However, as homodimers, MAF proteins can also bind half MARE sites if they are flanked by 5′-AT-rich sequences (Yoshida et al., 2005). Alternatively, but not exclusively, some of these MAF target genes might be controlled by MAF containing heterodimers, with the MAF protein binding to the half MARE site. Small and large MAF proteins do not heterodimerize. Large MAF proteins can activate transcription by recruiting the co-activators p300 (EP300), CRE binding protein (CBP) and P/CAF (also known as KAT2B) or the TATA binding or repress transcription by

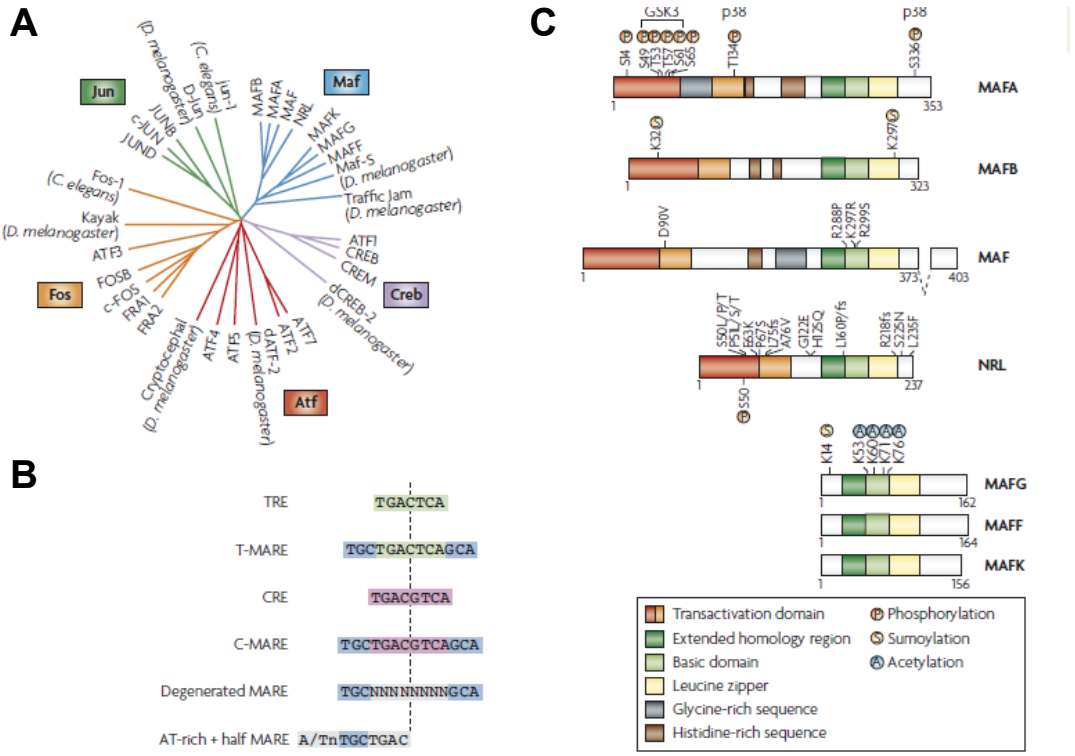

Figure 17. Maf proteins, members of the aP1 superfamily. (A) An unrooted phylogenic tree of the AP1 family of proteins. **(B)** Members of the AP1 family have different DNA recognition sequences: TRE, CRE and MAREs. **(C)** Schematic presentation of human Maf protein structures. *MAF* is alternatively spliced, resulting in two isoforms that differ in their carboxy termini. Crucial residues involved in post-translational modifications by glycogen synthase kinase 3 (GSK3) and p38 mitogen-activated protein kinase (p38) are indicated. The kinases responsible for serine (S)14 and S65 phosphorylation in MAFA and S50 in NRL remain to be identified. Mutations in human cataract (MAF) and retinitis (NRL) are shown using single-letter amino acid codes. MAFB has been shown to be sumoylated on lysine (K)32 and K297, as indicated. Small Maf proteins are devoid of a transactivation domain. MAFG has been shown to be sumoylated and acetylated on lysine residues as indicated. Adopted From (Eychene et al., 2008).

competing, notably with large MAF proteins, for binding to target gene promoters (Chen et al., 2002; Kataoka et al., 1996; Motohashi et al., 2002; Rocques et al., 2007). Therefore, the ratio between small MAF homodimers and large MAF-containing complexes might have important biological consequences (Motohashi et al., 2000).

The large *MAF* genes display a restricted expression which is tightly regulated in a spatio-temporal manner during development (Kataoka, 2007; Yang and Cvekl, 2007). Although these genes display some overlap in their expression profile, different

members can be expressed in a specific window of time in a developing tissue. This is exemplified in the lens and pancreas, where a cascade of expression of large *MAF* genes is observed. Therefore, the expression of the preceding gene could be directly involved in the expression of the next gene in the cascade. Accordingly, large MAF proteins can regulate their own expression as MARE sequences are present in *MAF*, *MAFB* and *MAFA* promoters (Raum et al., 2006).

Loss of function mutations in mice showed that large *MAF* genes are involved early in tissue specification, and later in terminal differentiation (Cordes and Barsh, 1994) (FIG 18). Within a given tissue, their roles appear to be highly specific and involve regulation of the expression of tissue-specific genes involved in terminal differentiation. Interestingly, in virtually every case their ability to regulate gene expression depends on their synergy with other transcription factors, some of which are direct binding partners (FIG 18). They often control the expression of a set of genes that are expressed at high levels, such as insulin in β-cells or crystallins in lens fibre cells. The biochemical and biological activities of large MAF proteins are regulated by various post-translational modifications including phosphorylation, ubiquitylation and sumoylation.

Recent reports described the novel roles of c-MAF in physiological processes. MAF has been shown to direct mechanoreceptor development by acting upstream of RET. RET is a receptor known to control development and formation of rapidly adapting mechanoreceptors (RAM) end organs (Wende et al., 2012). Moreover, MAF has an important role in differentiation of memory Th17 cells and influences cytokine production in these cells. By acting downstream of TGFβ and IL6 signals, MAF transcriptionally regulates expression of IL22, Il10, Il 17 and Il4 cytokines in Th17 cells (Lai et al., 2012; Rutz et al., 2011; Sato et al., 2011). Also, MAF exhibits a role in Th2 and follicular helper CD4 T cell differentiation, where this transcription factor is important for Il21 and CXCR5 production (Kroenke et al., 2012; Lee et al., 2011b).

Maf	Organ, tissue, cell type	Biological response(s)	Target gene(s)	Cooperating factor(s)	KO phenotype(s)
Mafa	Pancreas β-cells	Insulin transcription and production in β cells	Insulin (*Ins*)	PDX1, NeuroD	Diabetes mellitus[76]
Mafb	Rhomben-cephalon	Rhombomeres 5 and 6 identity	*Hoxa3* and *Hoxb3*	KROX20, Ets	Hindbrain segmentation problems in the kreisler (kr^{ENU}) mutant[37]; fatal apnea at birth due to lack of neurons from the PreBötC complex in the knockout mutant[95]
	Myeloid progenitors	Macrophage differentiation	Unknown	Unknown	Altered actin-dependent macrophage morphology[96]; F4/80 expression suppressed in non-adherent macrophages[97]
	Kidney podocytes	Podocyte differentiation	Unknown	Unknown	Aberrant podocyte foot process formation and proteinuria in the kr^{ENU} mutant[98]; tubular apoptosis and renal dysgenesis in the knockout mutant[97]
	Pancreas	Pancreatic endocrine cell commitment and maturation	Glucagon (*Gcg*)	PAX6	Reduced number of cells expressing insulin and glucagon in both kr^{ENU} and knockout mutants[99,100]
Maf	T cells	T helper 2 cell differentiation	*Il4*	NFAT	Lack of IL4 production[89] (embryonic and perinatal lethality)
	Lens	Lens differentiation	Crystallins *Cryaa*, *Cryab*, *Crybb1* and *Crygf*	PAX6, CREB, SOX1, SOX2, PROX1	Microphtalmia due to defect in lens fibre elongation[71,101,102]
	Endochondral bone	Chondrocyte differentiation	Collagen type II α1 (*Col2a1*)	SOX9	Decreased fetal bone length, abnormal terminal differentiation of hypertrophic chondrocytes[103]
Nrl	Neuroretina	Rod differentiation	Rhodopsin (*Rho*)	CRX	Lack of rods[104]

Figure 18. Physiological functions of MAF proteins discovered in knockout phenotype. Adopted from (Eychene et al., 2008).

Furthermore, *MAF* is involved in differentiation of adult cells of mesenchymal origin. In osteoblasts, *MAF* promotes differentiation by regulating genes osteoblast specific genes such as *Bglap1*. This function is in cooperation with osteoblast transcriptional factor RUNX2, and therefore MAF acts as modulator of bone formation. Also, MAF inhibits adipocyte differentiation indicating that MAF regulates the bifurcation of the mesenchymal cell lineage into osteoblasts and adipocytes. It is interesting to note that the *MAF* locus has been recently identified as one of the risk loci for obesity. Together with MAFB, MAF is enabling the entry in cell cycle followed by cell expansion in mature monocyte and macrophages, as shown in culture. Interestingly, the loss of differentiated phenotype and functions in these cell types is not observed under such circumstances (Aziz et al., 2009).

1.5.1 MAF in oncogenesis

Large MAF genes are *bona fide* oncogenes. Such observation is highlighted from their initial isolation where they were able to induce fibrosarcoma in chicken, to reports that prove their involvement in human cancer. MAF proteins can transform primary cells (Kataoka et al., 1994a; Kataoka et al., 1993; Pouponnot et al., 2006) and their oncogenic activity has been validated *in vivo* both in birds and mammals: the As42 avian retrovirus, expressing *v maf*, induces tumors in chickens (Kawai et al., 1992; Nishizawa et al., 1989) and transgenic mice expressing *MAF* in the lymphoid compartment develop T cell lymphomas (Morito et al., 2006). In humans, *MAF* genes are overexpressed in 60% of human angioimmunoblastic T-cell lymphomas (AITLs) and in 50% of cases of multiple myeloma (MM), and contribute directly to cancer progression (Hurt et al., 2004; Morito et al., 2006; Murakami et al., 2007). The oncogenic activities of all the large MAF proteins (except NRL) have demonstrated *in vitro.* NRL is the only member that has not yet been found to be deregulated in human cancer. On the contrary small MAF proteins have not shown oncogenic activity to date (Eychene et al., 2008). Interestingly, oncogenesis of large MAF proteins does not require an activating mutation (no such mutations have been found within large *MAF* genes in human cancers), although it can be enhanced by point mutation (Kataoka et al., 1996; Pouponnot et al., 2006). *MAFA* and *MAF* display the strongest oncogenic activity, whereas *MAFB* is less effective in transforming cells (Nishizawa et al., 2003; Pouponnot et al., 2006). This weaker activity of *MAFB* correlates with its reduced expression level compared to the other large MAF proteins, and likely reflects the fact that MAFB is a more unstable protein. Indeed, a threshold level of MAF expression might be required for oncogenic transformation, as only mice carrying a high copy number of the *MAF* transgene develop T-cell lymphoma (Morito et al., 2006). Moreover, *MAF* genes are also overexpressed in human cancers, suggesting that transformation requires a high level of MAF expression.

The transforming activity of the MAF proteins depends on their ability to act as transcription factors (Henderson et al., 2006; Kataoka et al., 2001). For instance, some of *MAF* target genes, such *cyclin D2*, *integrin β7* and *ARK5* (also known as *NUAK1*), have deregulated expression in MM, AITL and in *MAF* transgenic mice (Hurt et al., 2004; Mattioli et al., 2005; Monteiro et al., 2007; Morito et al., 2006; Murakami et al., 2007; Suzuki et al., 2005; Zhan et al., 2006). However, the capacity of the large *MAF*

genes to induce transformation does not strictly correlate with their transactivation strength. In summary, when expressed at high levels, non-mutated large *MAF* genes display oncogenic activity in cell culture, animal models and in human cancer.

The major contributions to the understanding of how *MAF* mediates oncogenesis originate from studies on MM. During development MAF proteins are not involved in the induction of cell proliferation, but are rather associated with a block of cell cycle progression. Paradoxically, oncogenic MAF proteins have been shown to increase cell proliferation which represents one of the mechanisms of its oncogenic action. (Hurt, 2004 Cancer cell). This effect on cancer cell proliferation is due to cell cycle progression rather than from anti-apoptotic activity (Eychene et al., 2008; Hurt et al., 2004). In multiple myeloma *MAF* positive group is characterized by a high proliferative index and it is tightly associated with overexpression of *cyclin D2*, which is *MAF* target gene. Accordingly, *cyclin D2* deregulation is also detected in T cell lymphomas in transgenic mice overexpressing *MAF* in the lymphoid compartment and in AITL expressing *MAF*.

Another characteristic of *MAF* oncogenic action is control of genes associated with invasion processes as *CXCL12* and *ARK5*. *ARK5* encodes a serine threonine kinase belonging to the AMP-activated protein kinase (AMPK) family, which is downstream of AKT. AKT has been previously shown to be involved in invasion and metastasis and MM cell lines overexpressing ARK5 exhibit increased invasiveness (Suzuki et al., 2004).

Multiple myeloma tumors that overexpress *MAF* demonstrate strong dependency of stroma for their growth. One of the main mechanisms underlining the tumor stroma interaction in multiple myeloma is mediated through the *integrin β7* gene that is controlled by *MAF*. Integrin β7/ αE heterodimer, the expressed on tumor cells, binds to E-cadherin that is found on the surface of bone marrow cells. Such adhesion of cancer cells to bone stroma provokes the expression of the proangiogenic cytokine, vascular endothelial growth factor (VEGF). *VEGF* facilitates tumor growth by adapting bone microenvironment and completing the tumor stroma vicious cycle (Hurt et al., 2004). The regulation of cell-cell interaction by MAF proteins is present in both physiological and pathological conditions. For instance, the pancreatic islets of *MAFA*-knockout mice display a disorganized architecture, characterized by intermingling of the different cell types (Zhang et al., 2005). The unique *D. melanogaster* orthologue of the large *MAF* genes, *traffic-jam* (*tj*) is expressed in somatic gondal cells that are in direct contact with

germline cells. Loss of *tj* function induces an improper intermingling between somatic cells and germline cells, causing a germ cell differentiation defect which is associated with deregulation of adhesion molecules, such as DE-cadherin, the *D. melanogaster* homologue of E-cadherin (Li et al., 2003).

CHAPTER 2. OBJECTIVES

OBJECTIVES

Hypothesis:

It has been suggested that cancer metastasis is supported by different genomic alterations and acquisition of specific genes during the process of tumor evolution. Therefore, we hypothesized that identifying genetic determinants of breast cancer metastasis to the bone should provide valuable clues for therapeutical intervention and reveal the mechanism of breast cancer cells homing, survival and colonization of the bone. Here, we followed an unbiased screening approach based on human breast cancer cells, experimental mouse model and patient data in order to identify genetic determinants of breast cancer metastasis to the bone. We hypothesized that some of these genetic determinants are present already in primary tumors and associate with metastatic relapse in patients, and therefore can be used as biomarkers. Additionally, they can mechanistically explain how the metastatic process to the secondary site occurs. Alternatively, some of the genetic determinants of metastasis are possibly acquired only in the metastatic site and not in primary tumors. These could support colonization of secondary site and underline bone metastatic specific processes.

Objectives:

- Identification of genes and genomic alterations associated with bone metastasis in human breast cancer cells and in patient samples.
- Functional validation of bone metastasis associated genes in experimental mice model of metastasis by means of gain and loss of function.
- Identification of bone metastasis mechanisms supported by previously validated candidate genes.
- Clinical relevance assessment of potential biomarkers of bone metastasis.

CHAPTER 3. RESULTS

RESULTS

3.1 16q22-24 DNA copy number gain specifically predicts and mediates, through *MAF* gene, breast cancer bone metastasis

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Summary

Bone is the most common site of metastatic disease in breast cancer patients leading to a poor quality of life and an eventual fatal outcome. Therefore, greater understanding of bone tropism and the biology of the metastasis to bone is needed. Biomarkers to identify patients at risk of bone disease would be clinically useful, particularly since bone microenvironment modifying agents such as bisphosphonates or the anti-RANK ligand antibody denosumab have the potential to prevent bone metastasis and improve survival (Brown and Coleman, 2012; Coleman et al., 2011; Gnant et al., 2009; Lee et al., 2011a). Herein, we described the identification of a 16q22-24 DNA genomic gain encoding the transcription factor MAF as a mediator of breast cancer bone metastasis through the control of PTHrP in an *in vivo* model. 16q22-24 genomic gain in breast cancer tumors specifically predicted risk of metastasis to bone. These results establish MAF as a novel target and 16q22-24 DNA genomic gain as a unique specific biomarker to identify patients at high risk for bone metastases, who may benefit from the inclusion of a bone targeted agent in their adjuvant treatment program.

Significance

Despite improvements in the treatment of early breast cancer, bone relapse remains common and the disease may be confined to bone throughout its clinical course. Greater understanding of the biology of bone-tropic metastatic processes and development of biomarkers to identify patients at risk of bone disease are needed, particularly since bone microenvironment modifying agents have the potential to prevent bone metastasis and improve survival. We have identified for the first time that 16q22-24 DNA genomic gain specifically predicts risk of breast cancer metastasis to the bone. This process is driven by *MAF*, a gene within this region, and its transcriptional control of PTHrP. 16q22-24 emerges as a specific biomarker to select patients that may benefit from bone targeted agents that can influence metastasis

Introduction

Women with primary breast cancer are at risk of distant metastatic relapse months, years or decades after surgery. Adjuvant (i.e. postoperative) systemic treatments such as chemotherapy, hormonal therapy (in oestrogen receptor positive tumors, ER+) or anti-HER2 therapy (in HER2 positive tumors) may eradicate micrometastatic disease and thereby reduce the risk of metastatic relapse. However, current adjuvant treatments are toxic (particularly chemotherapy), benefit a relatively small subset of women and the prevention of overt metastasis is non-organ specific. In contrast to this later point, breast cancer is a highly heterogeneous disease and there is clinical evidence of distinct patterns of disease relapse (Kennecke et al., 2010). In fact, the ability of metastatic breast cancer cells to grow in different environments may give rise to metastatic speciation, as suggested by the coexistence of tumor cells with different organ tropisms (bone, lung, liver and brain) in patients with advanced breast cancer disease (Nguyen et al., 2009).

Despite improvements in diagnosis and treatment of early breast cancer, both first and subsequent relapse in bone remain common, particularly in estrogen receptor positive (ER+) disease, and the disease remains largely confined to bone throughout its clinical course. Bone-targeted treatments, such as bisphosphonates and denosumab, modify the bone marrow microenvironment and significantly reduce skeletal complications in patients with overt bone metastasis. In addition, these agents may exert direct antitumor activity and clinical studies suggest they prevent metastasis. Clinical trials in early breast cancer have shown variable results, but for women who have undergone an induced menopause or have passed through menopause, consistently reduced relapse rates and improved survival has been observed in those receiving bisphosphonates (Coleman et al., 2011; Gnant et al.; Muller et al., 2001). This observation was not recapitulated in premenopausal women and as a consequence these agents are not approved in early breast cancer. Unfortunately, the benefit of these agents may be diluted due to the lack of specific predictors of bone metastasis (Lacey et al., 2012). For this reason, the discovery of biomarkers to select patients more likely to suffer bone metastasis, and that may benefit from bone-targeted agents, could have immediate clinical application (Coleman, 2011; Eckhardt et al., 2012).

Since the development of cDNA microarray technology, large breast cancer patient cohorts have been analyzed in order to identify prognostic and predictive biomarkers.

Gene expression profiling of primary tumors was successful in defining new molecular subtypes of breast cancer patients that correlate with histological subtype, ER status, HER2 status, lymph node status or BRCA1/2 mutations (CancerGenomeAtlasNetwork, 2012). However, to date only two-gene expression signatures are used in patients to predict chemotherapy benefit (Lo et al., 2010; van 't Veer et al., 2002). Interestingly, gene expression profiling analysis was useful in identifying potential metastasis regulating or initiating genes using experimental models (Eckhardt et al., 2012).

Here, we followed an unbiased screening approach to test the hypothesis that breast cancer metastasizes to the bone by selecting mediators for homing, survival and colonization of the bone by means of genomic alterations. Using this approach, we identified that the 16q22-24 DNA amplification specifically and independently predicts risk of bone metastasis in breast cancer patients, but not other sites. Moreover, we have functionally validated *MAF*, a gene within this genomic amplification, as the driver of breast cancer bone metastasis. The *MAF* gene is a member of the AP1 family of transcription factors and contributes to transformation in human angioimmunoblastic T-cell lymphomas (AITL) (Morito et al., 2006; Murakami et al., 2007) and multiple myeloma (MM) (Hurt et al., 2004). In MM and AITL patients several CNA in the 16q22-24 are observed (Eychene et al., 2008). MAF are basic region leucine zipper (bZIP) proteins that can switch from transcriptional repressors to activators, depending on the proteins with which they form heterodimers. The oncogenic activity of MAF proteins reported to date in hematological diseases is based mainly on their ability to enhance pathological interactions between malignant cells and the stroma (Eychene et al., 2008). Here, we show in breast cancer that MAF transcriptional activity controls PTHrP expression, a cytokine that strongly stimulates osteoclast differentiation and bone degradation, thus fueling metastasis-stroma interaction at the bone necessary for MAF mediated osteolytic lesions.

Results

Identification of CNA associated with Bone Metastasis in breast cancer cells

We developed an experimental xenograft mouse model to derive and select breast cancer cells prone to metastasize to the bone based on ER+ MCF7 breast cancer cells. We chose MCF7 xenografts since they have the potential to cause bone metastasis in only a small fraction of mice and are therefore well suited for detecting metastatic enrichment (Kang et al., 2003; Lu et al., 2011; Yin et al., 2003). To develop our model, luciferase expression was introduced to MCF7 cells to monitor the kinetics of metastatic spread by quantitative bioluminescence imaging. $5x10^5$ cells were inoculated in the left ventricle in BALB/c nude mice bearing 90 day-release estrogen pellets. After three rounds of *in vivo* passaging, we selected MCF7 cells, that we termed BoM2, with a significantly higher rate of bone metastasis compared to parental MCF7 cells (Figure 1A). The tumor growth at the primary site was however similar between parental MCF7 and BoM2 cells (Figure S1A). Parental and BoM2 metastatic derivatives both retained estrogen dependence for the induction of *MYC* and *CND1* expression and efficient tumor growth (Figures S1B and S1C).

We studied copy number alterations (CNA) associated with metastasis as recent studies suggest its relevance in breast cancer (Andre et al., 2009; Curtis et al., 2012). We used comparative genomic hybridization (CGH) analysis between BoM2 and parental cells and focused on genomic changes affecting the expression of potential bone metastasis gene mediators. This uncovered significant losses in chromosomes 6, 12, 19, 20 and 21 and one significant copy number gain (16q22-q24) in BoM2 bone metastatic cells compared to the initial parental population (Figures 1B and S1D). We confirmed the gain in the 16q chromosomal region in BoM2 cells (88.6% of the cells) compared to parental MCF7 cells (32.7%) by means of a 16q23 Fluorescence In Situ Hybridization (FISH) probe (Figure 1C).

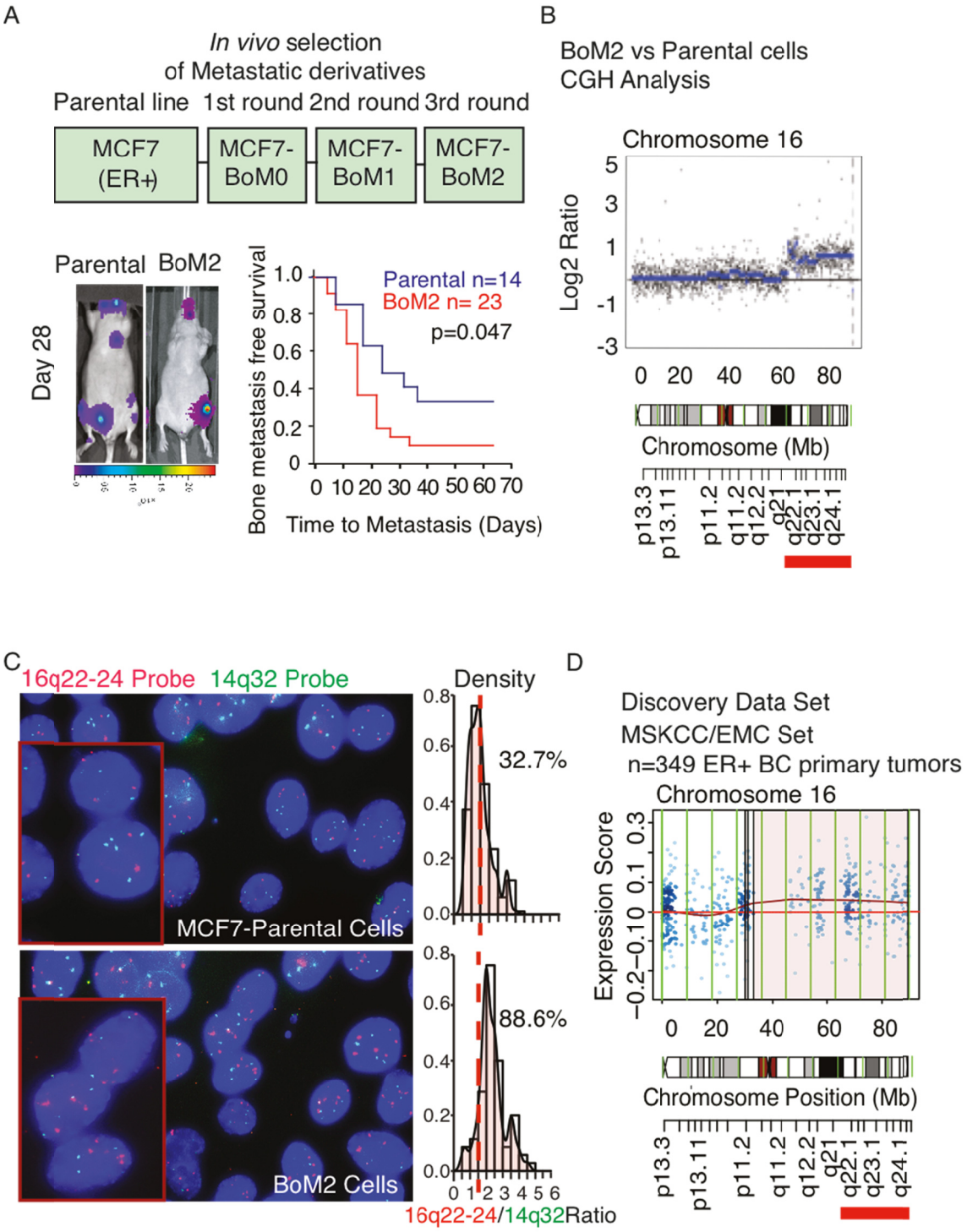


Figure 1. Amplification of 16q22-24 genomic DNA region is associated with breast cancer bone metastasis. See complete figure legend in page 69

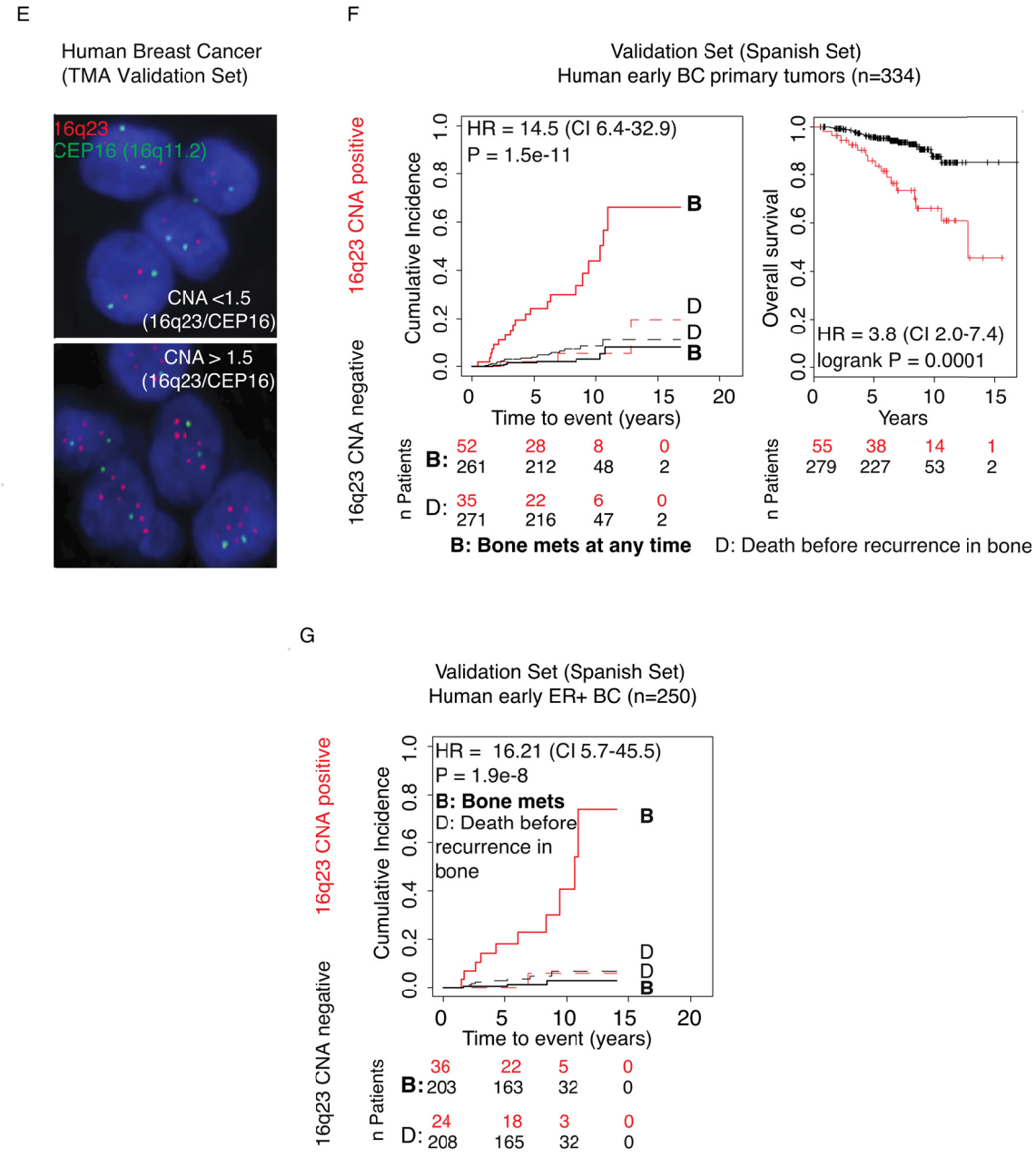
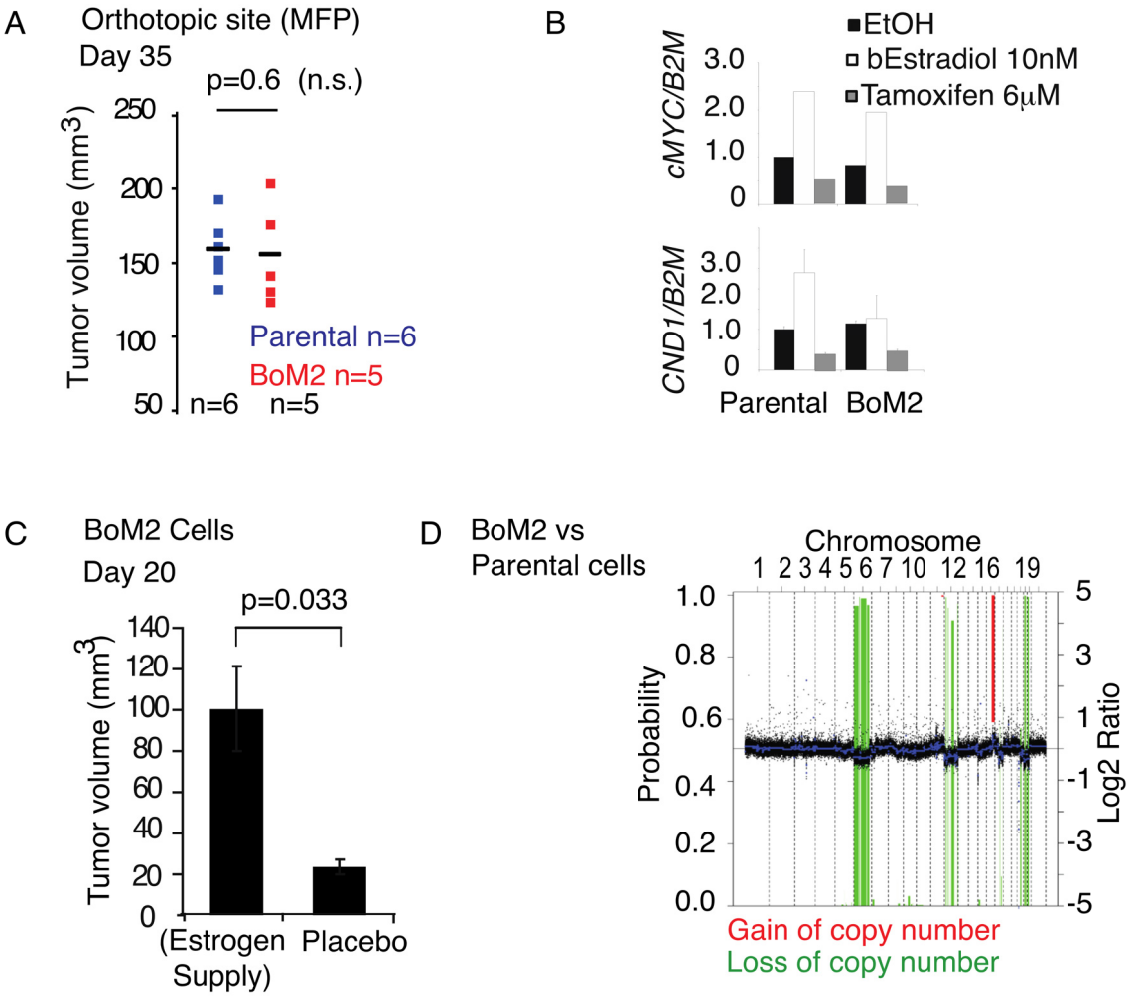
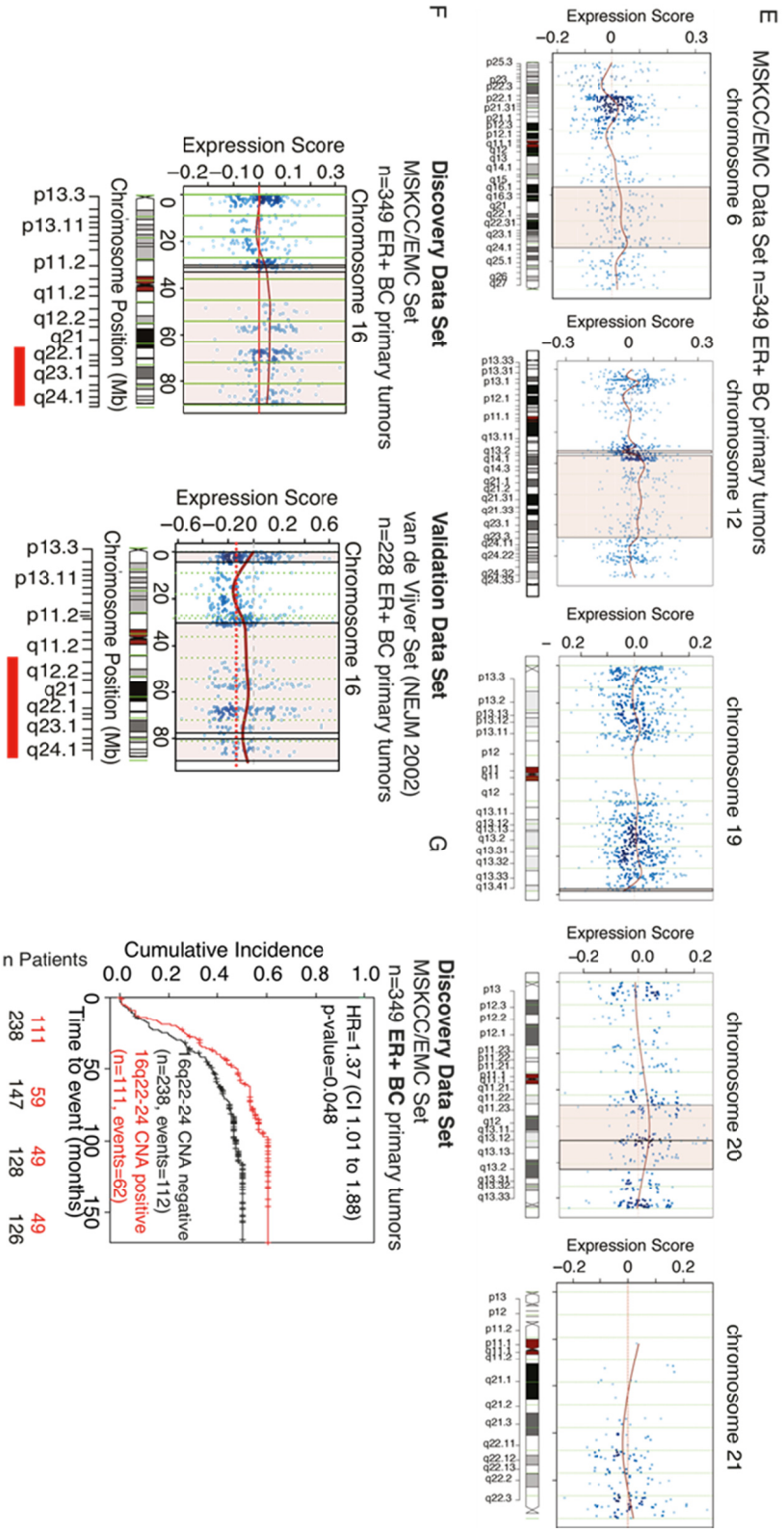


Figure 1. Amplification of 16q22-24 genomic DNA region is associated with breast cancer bone metastasis. Continuation (A) Schematic representation of in vivo selection of subsequent bone metastatic derivatives (BoM0, BoM1 and BoM2) originating from MCF7 Parental cell line with representative bioluminescent images. Kaplan-Meier curve shows bone metastasis-free survival of mice injected with Parental or BoM2 via left ventricle. **(B)** For chromosome 16, black dots and blue horizontal lines represent normalized log2 intensity ratios and segments, respectively. BoM2 are compared over MCF7 parental cells. At the bottom, in red,the 16q22-24 DNA genomic amplification is highlighted. **(C)** Panel depicting percentage of Parental and BoM2 cells with 16q23 region amplification based on ratio between 16q23 region

copies and 14q32 region copies. Representative images of FISH stained Parental and BoM2 cells. n= number of cells scored in each group. **(D)** Analysis of copy number alteration based on gene expression (ACE-like Algorithm, R phenoTest package). Colored area depicts genomic areas where increased gene expression is significantly ($p<0.05$) associated with metastasis in ER+ breast cancer tumors (discovery MSKCC/EMC data set). Dark red line reflects differences in gene expression between subjected populations. Clear red line implies the expected lack of variation between populations. This plot focuses on chromosome 16. **(E)** FISH using a 16q23 region probe, within the 16q22-24 DNA amplification identified in 1B, and CEP16(16q11.2) to measure DNA amplification in primary breast cancer tissues. Representative images of patient sample without 16q23 amplification (Case1, 16q23/CEP16 < than 1.5 copies) and with 16q23 amplification (Case2, 16q23/CEP16> than 1.5 copies). **(F)** Cumulative incidence (Left) of bone metastasis at any time, using death before recurrence in bone as a competing event and Kaplan-Meier curve of overall survival (Right) in the validation Spanish breast cancer human primary tumor data set (n=334). Patients were stratified according to 16q23/CEP16 CNA negative (<1.5) and 16q23/CEP16 CNA positive (> or = 1.5) group based on cut-off of 1.5 16q23/CEP16 copies per cell. A minimum of 50 cells per core and 3 cores per tumor were scored. HR-hazard ratio.CI-Confidence Interval. **(G)** Cumulative incidence plot of bone metastasis at anytime, using death before recurrence in bone as a competing event, for ER-positive patients in Spanish data set. Patients were divided between 16q23 CNA negative and 16q23 CNA positive group based on cut-off of 1.5 16q23/CEP16 copies per cell. A minimum of 50 cells per core and 3 cores per tumor were scored. HR-hazard ratio.CI-Confidence Interval.



Supplemental Figure 1 (Related to Figure 1) (A) Tumor growth from parental and BoM2 cells injected orthotopically in mouse mammary fat pad was scored. Data is presented as tumor volume for individual tumors from each experimental group. **(B)** *CCND1 and cMYC* mRNA expression levels measured by qRT-PCR in the presence of estradiol or tamoxifen for 24h, as indicated, and normalized to *B2M* levels. Data is presented as mean of three independent experiments with sd. **(C)** Tumor growth of tumors from BoM2 cells with or without estrogen supply was scored. Data is mean with sd. (n=3) **(D)** CGH analysis of BoM2 and Parental cells. Genetic gains and losses in BoM2 comparing to Parental cells, for each chromosome, are depicted with red and green color bars respectively.



Supplemental Figure 1. Continuation (Related to Figure 1) **(E)** Analysis of copy number association with metastasis based on gene expression (ACE-like Algorithm, R phenoTest package). Colored area depicts genomic areas were increase or decrease gene expression is

significantly ($p<0.05$) associated with metastasis in ER+ breast cancer tumors (discovery MSKCC/EMC data set). These plots focus on potential deletion on chromosome 6, 12, 19, 20 and 21. **(F)** Analysis of copy number alteration on Chromosome 16 based on gene expression (ACE-like Algorithm, R phenoTest package). Colored area depicts genomic areas were increased or decreased gene expression is significantly ($p<0.05$) associated with metastasis in ER+ breast cancer tumors are illustrated for the discovery (MSKCC/EMC data set) and validation (van de Vijver data set) cohorts side by side. **(G)** Cumulative incidence plot of metastasis using ER+ BC primary tumors from the MSKCC/EMC data set. Patients were divided between 16q22-24 CNA negative or positive group based on significant gain in this region compared to mean of all tumors. HR-hazard ratio. CI-95% Confidence Interval.

Analysis of selected CNA in a discovery-training set of breast cancer patients

Next, we studied whether any of the genomic changes found in our experimental model were detected in primary breast cancer specimens and associated with metastasis risk by means of an ACE-like algorithm (Analysis of CNAs by Expression data (Hu et al., 2009), details in supplemental data). The ACE algorithm uses the strong correlation between genomic alterations and aberrant expression of genes in affected loci (Hu et al., 2009), which was adapted to correlate expression and risk of metastasis. To parallel our model of MCF7 cells, that are ER+, our clinical analysis focused on ER+ primary breast cancer specimens including 349 patients with annotated clinical follow up (discovery MSKCC/EMC breast cancer tumor data set from an original series of 560 cases (211 were ER-) (Bos et al., 2009) (details on the data set in supplemental data). None of the five sites of chromosome loss reported above were concordantly lost and significantly associated with metastasis in patients (Figure S1E). In contrast, DNA amplification in the 16q22-24 region detected in our experimental model was significantly associated with metastatic risk in this clinical series (Figures 1D). The reproducibility of this association was confirmed by applying the ACE-like algorithm to an independent breast cancer primary tumor expression validation cohort with clinical annotation for metastasis (van de Vijver data set)(van de Vijver et al., 2002)(Figure S1F). Interestingly, the 16q22-24 CNA posive ER+ breast cancer tumors in the discovery MSKCC/EMC data set had a higher cumulative incidence rate of metastasis (Hazard Ratio (HR)= 1.37, 95% C.I. 1.01-1.88, p=0.048)(Figure S1G).

Validation of the 16q22-24 genomic gain in breast tumors that develop bone metastasis

Following our preclinical and clinical findings we tested and validated the ability of 16q22-24 chromosome region genomic gain to specifically predict bone metastasis in an independent patient sample set and using an alternative technical approach. Since our ultimate goal was to evaluate whether our finding could be implemented in clinical practice, we tested 16q22-24 by FISH (using 16q23 and centromeric 16q11.2 (CEP16) probes) in paraffin-embedded samples in an independent validation set of primary breast cancer specimens from patients with stage I, II or III and annotated follow up, including site of eventual metastasis (validation Spanish data set (Rojo et al., 2011), MARBiobanc. Details provided in supplemental materials). 14% of 334 primary breast cancers analyzed with complete clinical information and successfully hybridized were classified as 16q23 chromosome CNA positive, defined when an average of at least 1.5 copies of the 16q23 normalized to the CEP16 centromeric probe per cell (a minimum of 50 cells per specimen were scored) were detected (Figure1E). 16q23 CNA positive tumors were at a very high cumulative incidence rate of bone metastasis at any time (Hazard Ratio (HR)(bone mets)=14.49, 95%C.I. 6.4-32.9, p=1e-11; HR was calculated considering death before recurrence in bone as a competing event) (Table 1, S1 and S2; Figure1F). Of note, the size of the cohort used makes the estimates imprecise, but if the true value of the HR is only 6.4 (corresponding to the lower 95% boundary) this would still be clinically relevant. Moreover, this genomic amplification significantly predicted poor overall survival (HR(OS)=3.8, 95% C.I. 2.0-7.4, p=0.0001, Figure 1F) and there was a non-significant trend towards less risk of soft tissue and visceral metastasis (Figure S2A). 16q23 CNA positive tumors were significantly associated to high grade tumors, increasing tumor size and high proliferation (assayed by Ki67, >15%) but not with age, menopausal status, ER, PR, HER2 and lymph node involvement at diagnosis (Table S1). The factors significantly associated with higher bone metastasis rate were 16q23 amplification and high Ki67 index (Table 1). In a multivariate competing risk Cox analysis combining tumor size, grade, proliferation and lymph node parameters and 16q23CNA, the latter retained the strong association with the higher bone metastasis rate shown in the univariate analysis with a hazard ratio of cumulative bone metastasis at any time using death before recurrence in bone as a competing event of 18.02 (95% C.I. 6.69-48.53, p=3e-11) (Tables1 and S2). Thus, 16q23 amplification determined by FISH was an independent marker of bone

metastasis at any time (Sensitivity (Se)=0.71, Specificity (Sp)=0.88 for cumulative bone metastasis ever; median follow up is similar in both categories, Table S1) (Figures S2B and S2C) with a 97% negative predictive value (NPV) for cumulative bone metastasis (very low risk of false negative readouts). The relationship between 16q23 CNA positive and bone metastasis was significant in clinically relevant breast cancer subtypes, including ER+ tumors (Figure 1G), triple negative (TN) and HER2+ (Figure S2D).

Table 1.
Cumulative incidence of recurrence in bone at any (measured from date of primary tumor surgical resection). Analysis in patients with 16q23 CNA. This analysis is done considering competing events (death before recurrence in bone). The risk of the competing event is reported in Table S2

	Univariate (n=313)			Multivariate (n=313)		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Menopausal status			0.597			-
Premenopausal	1.00			-		
Postmenopausal	0.81	0.37 to 1.74		-	-	
Tumor size, mm			0.12			0.299
≤20	1.00			1.00		
21-50	1.86	0.87 to 3.93		1.56	0.63 to 3.81	
>50	1.73	0.60 to 5.01		0.56	0.12 to 2.57	
Tumor grade			0.174			0.160
I	1.00			1.00		
II	0.87	0.41 1.83		2.75	0.32 to 23.33	
III	1.58	0.75 to 3.33		1.12	0.12 to 10.27	
Lymph nodes			0.120			0.255
None	1.00			1.00		
1-3	0.94	0.41 to 2.14		1.20	0.46 to 3.11	
4-9	1.27	0.38 to 4.24		1.79	0.33 to 9.54	
>9	3.39	1.17 to 9.81		4.57	1.16 to 18.01	
Hormonal receptor status			0.414			-
Negative	1.00			-		
Positive	0.71	0.32 to 1.57		-	-	
HER2 status			0.580			-
Negative	1.00			-		
Positive	1.30	0.52 to 3.22		-	-	
Proliferation (Ki-67)			0.010			0.263
Low proliferation (<15%)	1.00			1.00		
High proliferation (≥15%)	2.81	1.37 to 6.34		1.65	0.68 to 3.98	
16q23 (FISH CNA)			1e-11			3e-10
Below 1.5	1.00			1.00		
Equal or More 1.5	14.48	6.37 to 32.94		18.02	6.69 to 48.53	

Abbreviations: HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2

Table S1. Baseline Characteristics According to 16q23 CNA

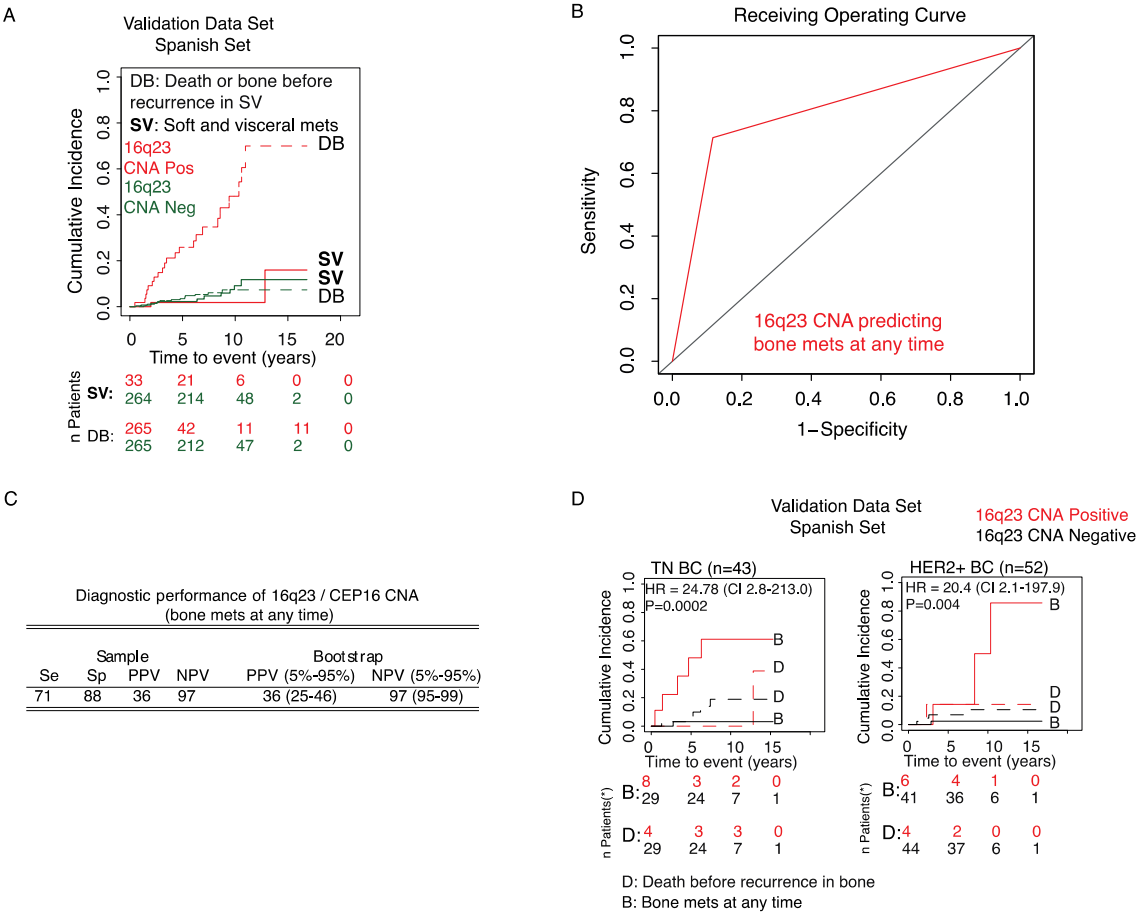
Characteristics	Complete series (n=334)		16q23 CNA < 1.5 (n=279)		16q23 CNA > or =1.5 (n=55)		*P*
	No. of patients	%	No. of patients	%	No. of patients	%	
Age (median, range)	58, 26-90		58, 31-90		58, 26-90		
Menopausal status							0.632
Premenopausal	104	31.1	85	30.5	19	34.5	
Postmenopausal	230	68.9	194	69.5	36	65.5	
Tumor size, mm							0.008
≤20	204	61.0	179	64.2	25	45.4	
21-50	100	30.0	80	28.7	20	36.4	
>50	30	9.0	20	7.1	10	18.2	
Tumor grade							0.011
I	57	17.0	52	18.6	5	9.1	
II	159	47.6	138	49.4	21	38.2	
III	118	35.3	89	32.0	29	52.7	
Lymph nodes							0.091
None	203	60.7	175	62.7	28	50.9	
1-3	86	25.7	72	25.8	14	25.4	
4-9	29	8.6	21	7.5	8	14.6	
>9	16	4.7	11	4.0	5	9.1	
Estrogen receptor status							0.174
Negative	84	25.1	66	23.7	18	32.7	
Positive	250	74.9	213	76.3	37	67.3	
Progesterone receptor status							0.282
Negative	118	35.3	95	34.0	23	41.8	
Positive	216	64.6	184	66.0	32	58.2	
HER2 status							0.850
Negative	271	81.2	227	81.4	44	80.0	
Positive	63	18.8	52	18.6	11	20.0	
Bone mets at any time							9e-12
Negative	306	91.6	271	97.13	35	63.64	
Positive	28	8.4	8	2.87	20	36.36	
(median follow up, months)			7.1		6.9		
Soft and visceral metastasis before bone mets or death							0.998
Negative	318	95.2	265	94.9	53	96.3	
Positive	16	4.7	14	5.0	2	3.6	
Proliferation (Ki-67)							0.005
Low proliferation (<15%)	229	68.5	200	71.7	29	52.7	
High proliferation (≥15%)	84	25.1	62	22.22	22	40.0	
Proliferation (n.a.)	21	6.2	17	6.1	4	7.3	

Abreviations: HER2, human epidermal growth receptor 2, n.a. not avaliable

Table S2 (Continuation Table 1).
Competing risk (death before recurrence in bone) survival analysis complementing table S2

	Univariate (n=306)			Multivariate (n=306)		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Menopausal status			0.550			-
Premenopausal	1.00			-		
Postmenopausal	1.32	0.51 to 3.43		-	-	
Tumor size, mm			0.038			0.150
≤20	1.00			1.00		
21-50	1.63	0.68 to 3.91		2.23	0.71 to 6.94	
>50	3.20	1.16 to 8.81		4.08	0.99 to 16.80	
Tumor grade			0.039			0.069
I	1.00			1.00		
II	0.40	0.15 to 1.05		1.47	0.17 to 12.54	
III	2.97	1.22 to 7.21		4.39	0.52 to 36.43	
Lymph nodes			0.107			0.626
None	1.00			1.00		
1-3	0.74	0.27 to 2.02		0.74	0.22 to 2.48	
4-9	1.68	0.47 to 5.97		0.70	0.14 to 3.42	
>9	4.73	1.57 to 14.18		1.76	0.41 to 7.43	
Hormonal receptor status			0.081			-
Negative	1.00			-		
Positive	0.45	0.18 to 1.08		-	-	
HER2 status			0.105			-
Negative	1.00			-		
Positive	2.20	0.88 to 5.47		-	-	
Proliferation (Ki-67)			0.347			0.163
Low proliferation (<15%)	1.00			1.00		
High proliferation (≥15%)	0.57	0.16 to 1.97		0.41	0.13 to 1.55	
16q23 (FISH CNA)			0.936			0.748
Below 1.5	1.00			1.00		
Equal or More 1.5	0.95	0.27 to 3.28		0.59	0.16 to 2.18	

Abbreviations: HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2



Supplemental Figure 2 (Related to Figure 1) (A) Cumulative incidence soft and visceral tissue metastasis using death or bone metastasis before recurrence in soft and visceral metastasis as a competing event was used in Spanish data set. Patients were divided between 16q23 CNA negative and 16q23 CNA positive group based on cut-off of 1.5 16q23/CEP16 copies per cell. A minimum of 50 cells per core and 3 cores per tumor were scored. HR-hazard ratio. CI- 95% Confidence Interval. **(B)** Receiver Operating Characteristic (ROC) curves for diagnostic performance of 16q23 amplification for bone metastasis ever and as a first site. In a ROC curve the true positive rate (Sensitivity) is plotted in function of the false positive rate (100-Specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. **(C)** Table represents bone metastasis diagnostic performance of 16q23 amplification (bone metastasis ever) measured by FISH. Se-Sensitivity, Sp-Specificity, PPV-positive predictive value and NPV-negative predictive value. **(D)** Cumulative incidence plots of bone metastasis at anytime using death before recurrence in bone as a competing event for TN and HER2+ BC patients in Spanish data set. Patients were divided between 16q23 CNA negative and 16q23 CNA positive group based on cut-off of 1.5 16q23/CEP16 copies per cell. A minimum of 50 cells per core and 3 cores per tumor were scored. Please note that the numbers are small and make the estimates

imprecise. HR-hazard ratio. CI-95% Confidence Interval.

Expression of the MAF gene, within the 16q22-24 amplified region, predicts bone metastasis

To identify genes in the 16q22-24 genomic amplified region that specifically mediate bone metastasis (Figure S3A), we took advantage of BoM2 bone metastatic experimental derivatives. The only genes in the chromosome 16q22-q24 amplification that were differentially expressed more than 2 fold (p-value<0.05) in BoM2 derivatives compared to parental cells were *MAF* and *SLC9A5* (Figures S3A-S3D). We then evaluated the clinical significance of the expression of *MAF* and *SLC9A5* in the breast cancer discovery MSKCC/EMC data set. The Cox hazard ratio for the association of *MAF* mRNA levels as a continuous variable and bone metastasis was statistically significant (p=0.032) while the *SLC9A5* mRNA levels were not associated with bone metastasis (p=0.95). Those breast cancer tumors expressing high MAF levels (above average plus one standard deviation) were at a higher cumulative incidence of bone metastasis than the rest (HR(bone mets)= 2.5, C.I. 1.7-3.8, p-value=2e-5) but not of brain or lung metastasis in patients (Figures 2A and S3E). Following, we confirmed that the MAF gene was amplified in 6.1% of the 773 tumors evaluated in copy number variation studies by the TCGA breast cancer project (Details in supplementary material (CancerGenomeAtlasNetwork, 2012)). This percentage was smaller but within the same range of that described above for 16q23 gain, using FISH, in our validation data set given the different sensitivity of the technologies used. This clinical association suggested *MAF* function as the potential genetic driver of bone metastasis linked to the 16q22-24 DNA amplification. Interestingly, a log-linear relationship between *MAF* expression and bone metastasis hazard ratio is observed only when a given level of *MAF* expression is achieved (data not shown). This observation is concordant with the fact that several copies of *MAF* gene are required in human AITL and MM transgenic mouse models to induce transformation and an aggressive phenotype (Hurt et al., 2004; Morito et al., 2006; Murakami et al., 2007).

The *MAF* gene can be transcribed in two spliced isoforms and contributes to transformation in 60% of AITL (Morito et al., 2006; Murakami et al., 2007) and 50% of MM cases (Hurt et al., 2004). Besides this role in hematological malignancies, *MAF* genes also support tumor suppressor-like activities in specific cellular contexts, reflecting their function in terminal differentiation. However, to date MAF has never been associated with breast cancer transformation, tumor progression or metastasis

(Eychene et al., 2008).

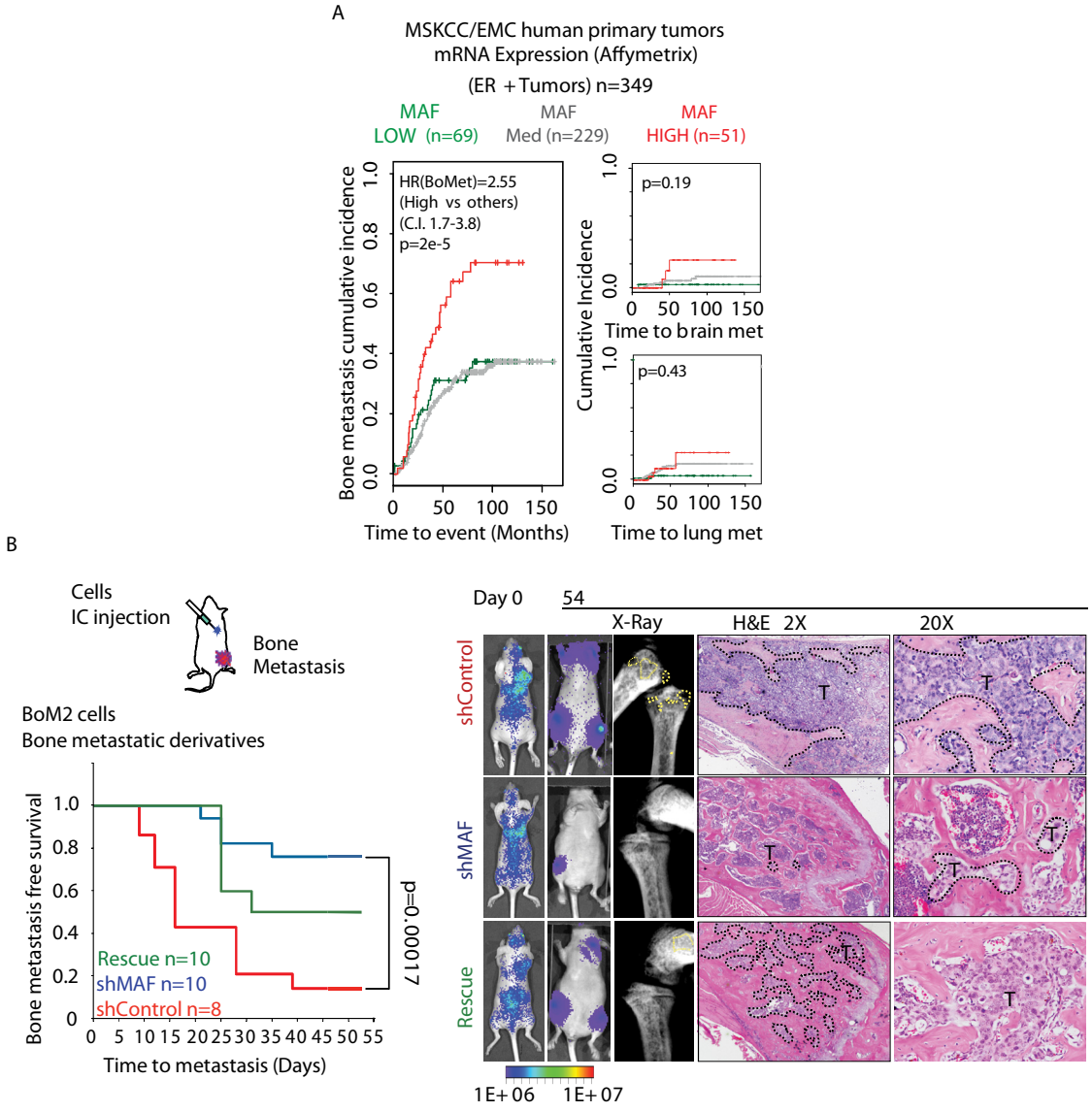


Figure 2. MAF mediates osteolytic bone metastasis in breast cancer cells. (A) Cumulative incidence plot of bone (Left), brain (Right upper) and lung (Right lower) metastasis in ER+ primary breast cancer patients (discovery MSKCC/EMC data set). Low, Med and High represent MAF expression levels in the following way: low (<mean - SD), medium (≥ mean - SD and ≤ mean + SD) and high (> mean + SD). **(B)** Schematic representation of intracardiac injection. (Left) Kaplan-Meier curve of bone metastasis-free survival for BoM2 shControl, shMAF and Rescue cells. (Right) Representative bioluminescent images at day 0 and at endpoint, day 54, representative X-Ray images and H&E staining of bone metastasis for each

group are shown. Scale bar, 100 μm. Osteolytic area is depicted by a yellow dashed line. Tumor (T) area is highlighted with a white dashed line.

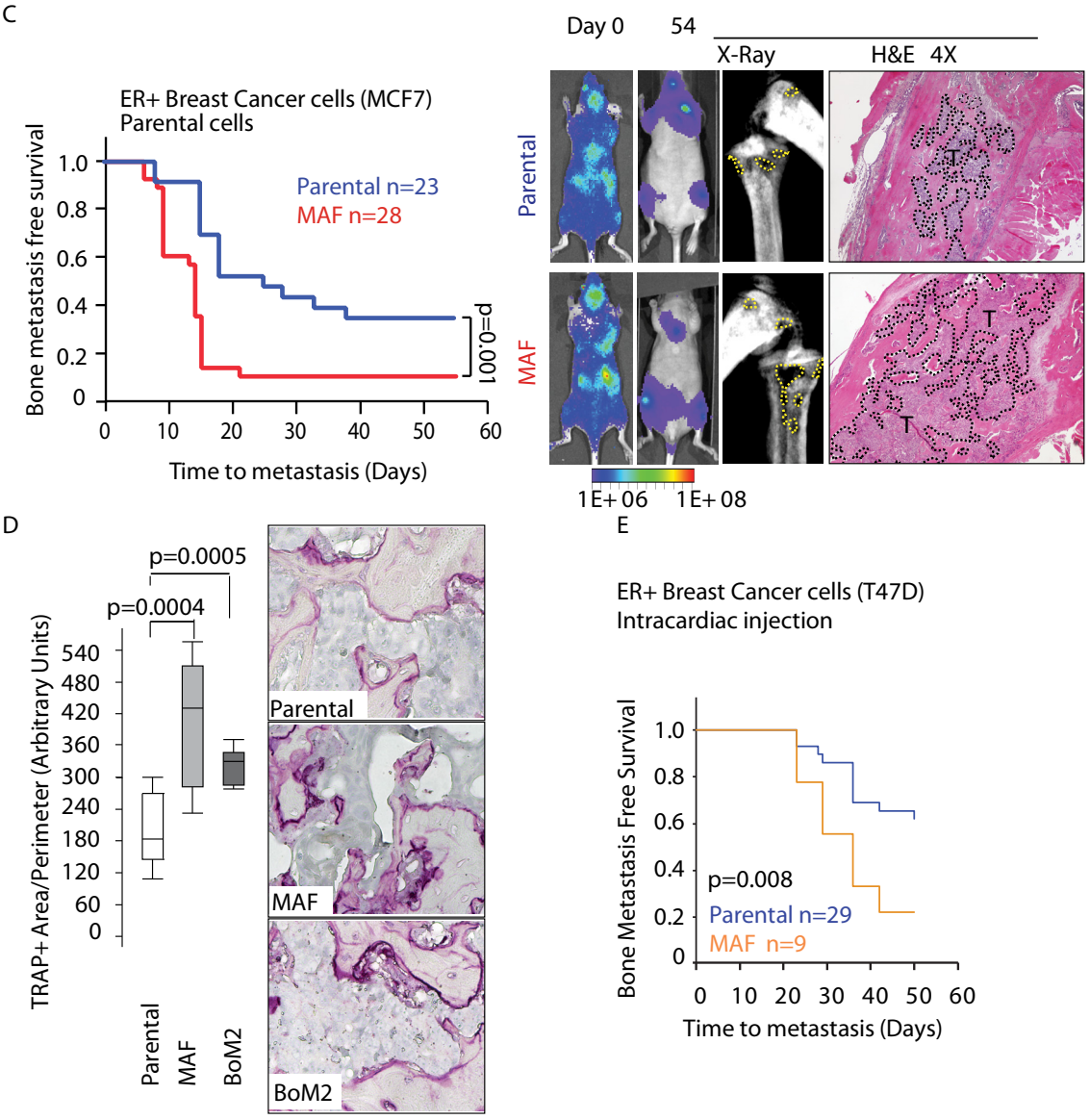
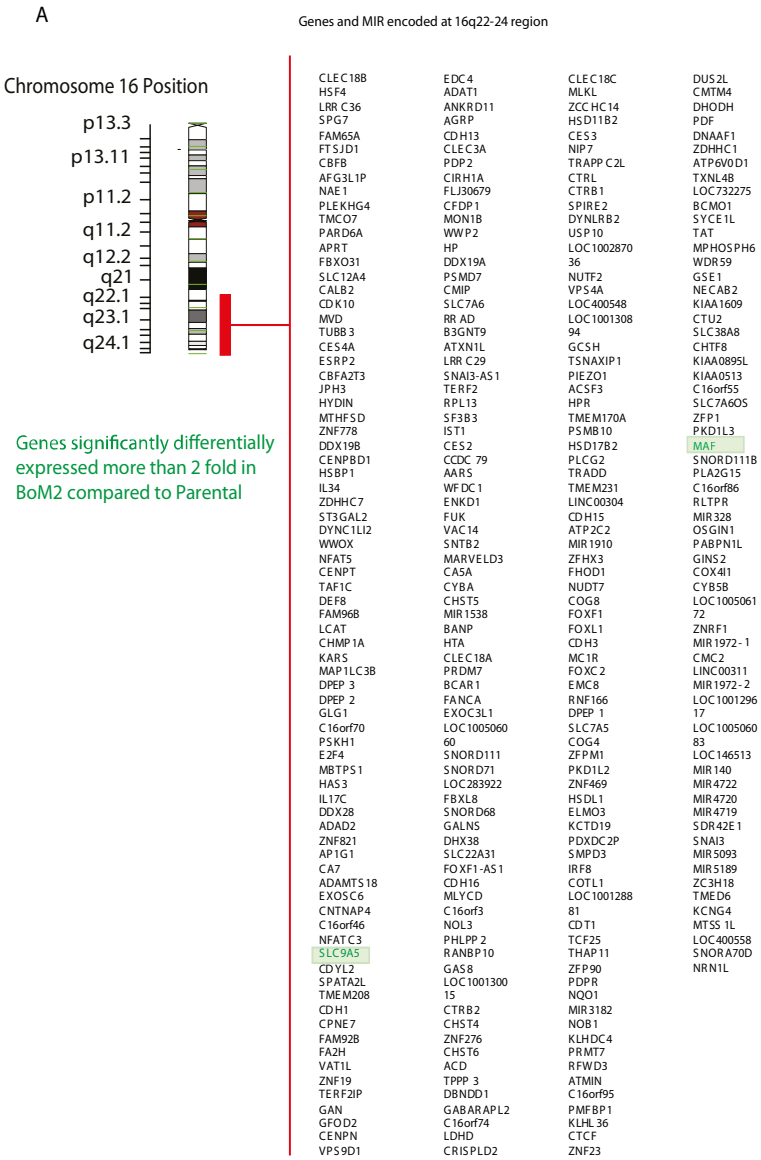


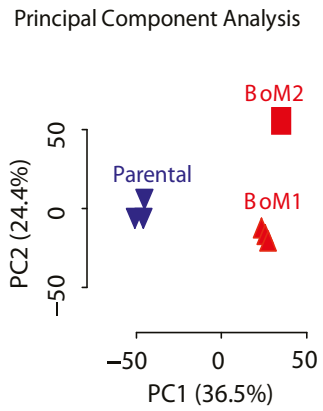
Figure 2. MAF mediates osteolytic bone metastasis in breast cancer cells. Continuation (C) (Left) Kaplan-Meier curve of bone metastasis-free survival for control Parental or MAF Short and Long spliced isoform (concomitantly) expressing cells. (Right) Representative bioluminescent images at day 0 and at endpoint, day 54, representative X-Ray images and H&E staining of bone metastatic lesions at end point for each group are shown. Scale bar, 100 μm. Osteolytic area yellow dashed line. Tumor (T) area is highlighted with a white dashed line. **(D)** (Left) Quantification of TRAP+ positive area per bone perimeter along the bone-tumor interface of metastasis from each experimental group in B). Minimum of 10 random fields per tumor, covering the whole tumor area, were captured. Data is presented as box plot with

median, IQR and min and max values. (Right) Representative images of TRAP stained bone lesions are shown for each experimental group. (E) Kaplan-Meier curve of bone metastasis-free survival. T47D cells were transfected with Control or MAF Short and Long isoform overexpressing vector (simultaneously) and injected into the left ventricle of nude mice. Bone metastasis was determined by bioluminescence.

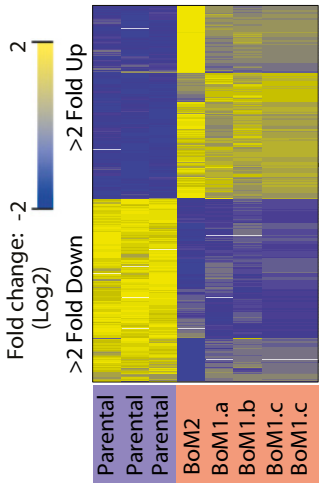


Supplemental Figure 3 (Related to Figure 2) (A) Plot depicts a schematic representation of the chromosome 16. In red highlighted the region and genes encoded within the 16q22-24. Among them, in green those genes that are significantly differentially expressed (absolute fold change bigger than 2 and Bayesian FDR below 5%) in BoM2 compared to parental MCF7 cells.

B



C



D

Genes differentially expressed in BoM2 vs Parental and located at Chr16

"Chromosome"	"Postion" Start	End	Entrez id	Symbol
16	17196183	17564738	64131	XY LT1
16	18995432,5	19075260	79905	TMC7
16	30542780	30546194	65988	ZNF747
16	56691855	56693214	4494	MT1F
16	67282855	67306093	6553	S LC9A5
16	79627746	79634622	4094	MAF

E

Expression ER+ samples

Univariate analysis with all variables:

	HR	CI.low	CI.up	Pvalue
MAF.HighvsRest	2.55	1.71	3.82	2e-5
pr.1vs0	1.19	0.79	1.80	0.39
her2.1vs0	1.14	0.70	1.85	0.59
age (+1SD)	1.07	0.89	1.28	0.46
size (+1SD)	1.09	0.92	1.31	0.33

pr= Progesteron receptor
her2= HER2 receptor
1=positive
0=negative
MAF.High= MAF levels above mean+sd

Multivariate analysis

	HR	CI.low	CI.up	Pvalue
MAF.HighvsRest	2.59	1.59	4.21	3e-4
pr.1vs0	1.31	0.81	2.11	0.26
her2.1vs0	1.36	0.77	2.39	0.30
age (+1SD)	1.01	0.82	1.25	0.91
size (+1SD)	1.14	0.91	1.43	0.26

Supplemental Figure 3 Continuation (Related to Figure 2) Continuation (B) Principal component analysis (PCA) based on gene expression profiles segregated parental MCF7 cells from BoM1 and BoM2 populations based on the genetic variability. The Principal component 1 (PC1) captured the bone metastasis tropism phenotype. **(C)** Comparative genome-wide expression analysis demonstrated that 511 genes were differentially expressed at least 2-fold in highly bone metastatic derivatives. **(D)** Gene list highlighting the 6 genes differentially expressed with a Bayesian FDR below 5% in BoM derivatives compared to parental cells located at the Chromosome 16. In green, genes located in 16q22-24. **(E)** Univariate and multivariate analysis of different covariates association, including MAF gene expression, with

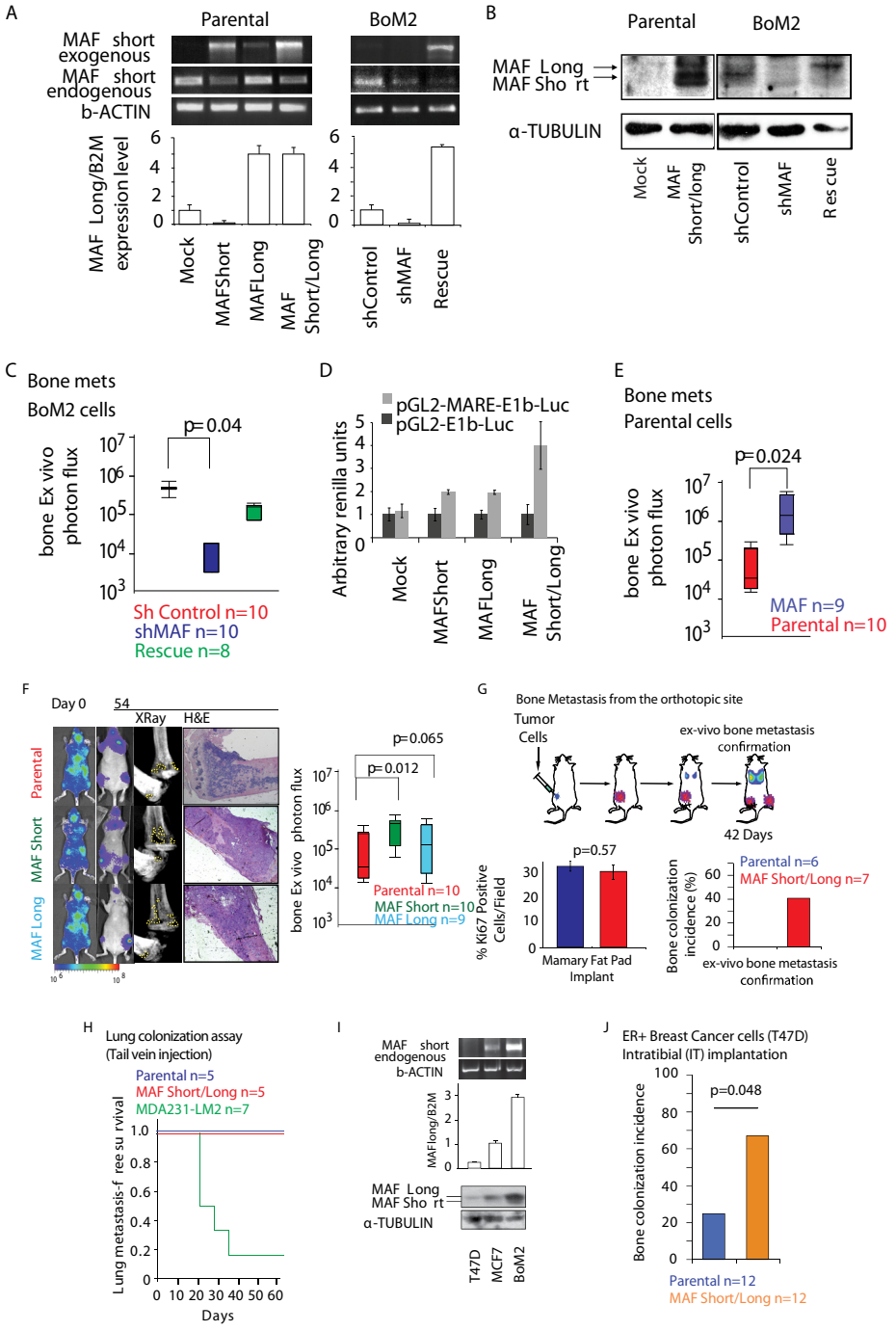
bone metastasis using the discovery MSKCC/EMC data set.

MAF promotes breast cancer bone metastasis

To test whether *MAF* mediates bone metastasis in breast cancer cells, we induced shRNA-mediated *MAF* knockdown in BoM2 cells that reduced the level of endogenous MAF by more than 90% (Figures S4A and S4B). This could be rescued by exogenous over-expression of both *MAF* spliced isoforms (Figures S4A and S4B). Only 23% of mice inoculated with BoM2 MAF knockdown cells developed bone metastasis detectable by luciferase activity, compared to 90% in shControl BoM2 cells or 50% in the rescue group (Figures 2B and S4C) at day 52 post injection. Bone metastasis reduction in MAF-depleted cells was accompanied by a reduction in the extent of the hind limb osteolytic lesions determined by X-Ray (Figure 2B). Of note, the marked reduction of MAF levels in MAF-depleted BoM2 cells (Figure S4A and S4B) resulted in fewer bone metastatic events than the parental population (Figures 2B and 2C).

On the contrary, MAF overexpression (each isoform independently or collectively) enhanced the capacity of MCF7 to metastasize to bone after intracardiac injection (Figures 2C, S4A, S4B, S4D-S4F) and increased the number of tartrate-resistant acid phosphatase (TRAP+) multinucleated osteoclasts at the perimeter of the metastatic lesions (Figure 2D). Interestingly, 3 out of 7 mice bearing MAF expressing orthotopic-implanted tumors that grew to>300 mm^3 in size developed bone metastasis compared with none out of 6 in the control group (Figure S4G). High levels of MAF expression did not support lung colonization when cells were delivered via tail vein (Figure S4H).

Further, to determine whether MAF mediates breast cancer bone metastasis in another cellular model, we used T47D breast cancer cells, that are poorly metastatic (Lu et al., 2011; Yin et al., 2003) and express lower levels of MAF than parental MCF7 cells (Figure S4I). MCF7 parental and BoM2 cells develop bone metastasis in almost 60% and 90% of the mice 30 days post inoculation respectively (Figure 1A and 2B,C) while T47D cells only caused bone metastasis in 15% of the mice 30 days post intracardiac inoculation, which increased to 40% 50 days post injection (Figure 2E, parental group). MAF overexpression in T47D cells significantly reduced bone metastasis free-survival (Figure 2E) and increased bone metastasis incidence and colonization (Figure 2E, Figure S4J) compared to parental T47D cells, thus confirming MAF as a mediator of bone metastasis in the two breast cancer models tested.



Supplemental Figure 4. (Related to Figure 2) (A) *MAF* expression levels in Parental cells transfected with Control, *MAF Short*, *MAF Long* or *MAF Short* and *Long* spliced isoform expression constructs (left) and in BoM2 Control, shMAF or Rescue BoM2 cells (Right). *MAF* long expression levels were determined by qRT-PCR using TaqMan probe and normalized to *B2M* levels. *MAF* short endogenous levels were determined by qRT-PCR using Syber Green reaction with indicated primers and normalized to *b-ACTIN* levels. Presence of ectopically expressed MAF short isoform was detected by qRT-PCR using using Syber Green reaction

with indicated primers described at the material and method section. (B) Western Blot depicting MAF protein levels in Parental Control, MAF Short and MAF Long spliced isoform overexpressing cells (simultaneously) and in BoM2 Control, shMAF or Rescue BoM2 cells. α-TUBULIN was used as loading control. **(C)** Quantification of *ex vivo* bioluminescent signal at hind limbs of mice from Fig. 2b in each experimental group. Black line depicts average intensity. **(D)** Renilla activity of C-MARE reporter plasmid in Parental cells transiently transfected with Control, MAF Short, MAF Long or MAF Short and Long spliced isoform expressing vectors. Activity of C-MARE promoter was normalized to Control condition and presented in arbitrary units. Data are mean of three independent experiments with S.D. **(E)** Quantification of *ex vivo* bioluminescent signal at hind limbs of mice from Fig. 2c in each experimental group. **(F)** (Left) Kaplan-Meier curve of bone metastasis-free survival for control Parental or MAF Short or Long spliced isoform expressing cells. (Right) Representative bioluminescent images at day 0 and at endpoint, day 54, representative X-Ray images and H&E staining of bone metastatic lesions at end point for each group are shown. Scale bar, 100 μm. (Right) Quantification of *ex vivo* bioluminescent signal at hind limbs of mice from in each experimental group. **(G)** (Upper) schematic representation of the experimental approach used herein: bone metastasis was scored from MAF expressing and control MCF7 cells implanted in the mammary fat pad. Only mice bearing size matched tumors (>300 mm3) at day 42 where considered. Ex vivo photon flux was used *postmortem* for lesion confirmation. Bottom left, values represent the mean with sd. Percentage of Ki67-positive cells in mammary fat pad tumors from Control or *MAF* Short and *MAF* Long isoform overexpressing cells (simultaneously). For each tumor a minimum of 10 random fields were counted for Ki67-positive cells. Values are mean with sd. (n=4). Bottom right bone colonization incidence was plotted. **(H)** Kaplan-Meier curve of lung metastasis-free survival for Parental, *MAF* Short and Long isoform overexpressing (simultaneously) and MDA231-LM2 cells after tail vein injection. **(I)** *MAF* expression levels in BoM2, MCF7 Parental and T47D cells. *MAF* long expression levels were determined by qRT-PCR using TaqMan probe and normalized to *B2M* levels. *MAF* short endogenous levels were determined by qRT-PCR using Syber Green reaction with indicated primers and normalized to *b-ACTIN* levels. Western Blot for MAF and α- TUBULIN were shown. **(J)** Bone colonization incidence of T47D cells transfected with Control or *MAF* Short and Long isoform overexpressing vector (simultaneously) and injected intratibiae in balb-c nude mice. Bone metastasis was determined by bioluminescence. Fisher test was used to score significance.

PTHrP contributes to MAF-induced bone metastasis-stroma interactions

Next, we investigated the possible mechanism by which MAF may drive bone metastasis. In size-matched bone metastatic lesions derived from different MCF7 cell derivatives, MAF expression resulted in a larger area of activated osteoclasts (TRAP+ cells) along the lesion perimeter (Figure 2D). These results suggest that MAF supports the aggressive outgrowth of BoM2 cells by modifying their bone marrow microenvironment conditions. In the absence of direct cellular metastasis activity, MAF may instead transcriptionally control the activity of genes promoting homing and bone remodeling capabilities to colonize the bone. To this end, we examined known mediators of bone stroma-tumor interactions (Guise et al., 2005; Lacey et al., 2012). Interestingly, *PTHLH,* commonly known as *PTHrP* (parathyroid hormone-related protein), expression appeared to be under the control of MAF, as determined by qPCR analysis, while other potential suspects such as RANK or RANKL were not (Figures 3A and S5A).

In the *PTHrP* distal promoter (P1) we observed a consensus MAF responsive element. Thus, to investigate the role of MAF in *PTHrP* induction, we used two transcriptional reporter constructs based on the -3401/-2421bp (P1 promoter) and -1424/-342bp (P2/P3 promoter) region of the human *PTHrP* promoter (Figure 3B). The activity of the P1 construct, containing the MARE site, was increased by overexpression of MAF (Figure S5B) while no effect was observed in the P2/3 promoter (Figure S5B). Evidence of a MAF-direct interaction with the endogenous promoter was obtained by chromatin immunoprecipitation (ChIP) analysis of relevant promoter regions (Figure 3C). MAF binding to the P1 promoter region was observed (Figure3C). This binding was not observed at the proximal region (Figure 3C). Further, patient breast cancer metastasis growing in the bone retained *MAF* expression compared to metastasis elsewhere (n=58, MSKCC breast cancer Metastasis Data Set GSE14020 (Zhang et al., 2009), Figure 3D). Moreover, 77% of breast cancer metastases expressing *MAF* and *PTHrP* above the average (represented by the zero axis) were in bone (Figure 3D). PTHrP plays a fundamental role in humoral hypercalcaemia of malignancy and development of osteolytic bone metastasis (Guise et al., 2002). In our studies, conditioned media from cells expressing MAF enhanced the induction of osteoclast differentiation from bone marrow derived cells *in vitro*, a process that was prevented upon co-incubation with a PTHrP antagonist (7-34Aa, PTHrP-AN) (Figure3E).

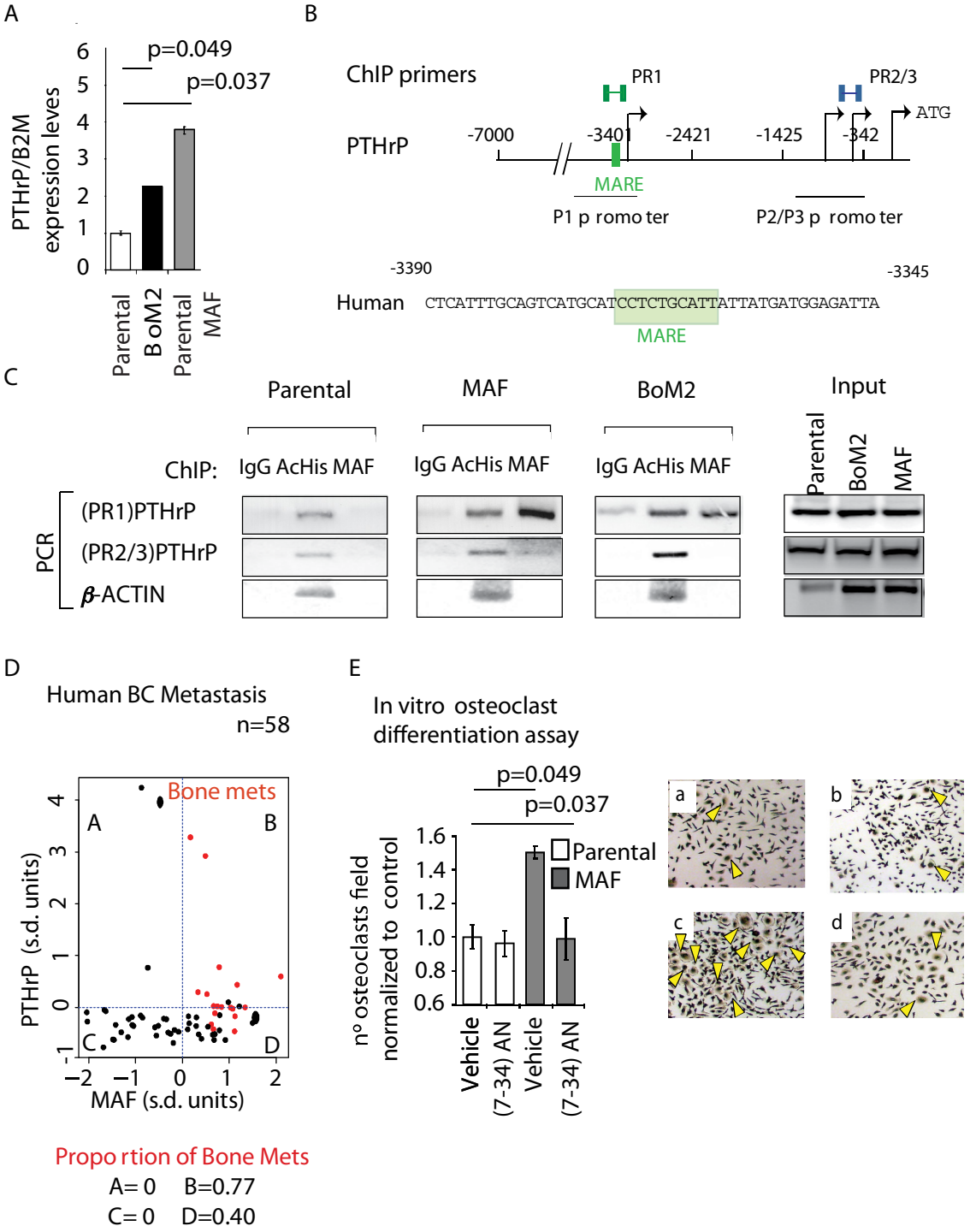


Figure 3. Bone metastatic promoting effect of MAF is mediated by PTHrP. See complete figure legend in page 89.

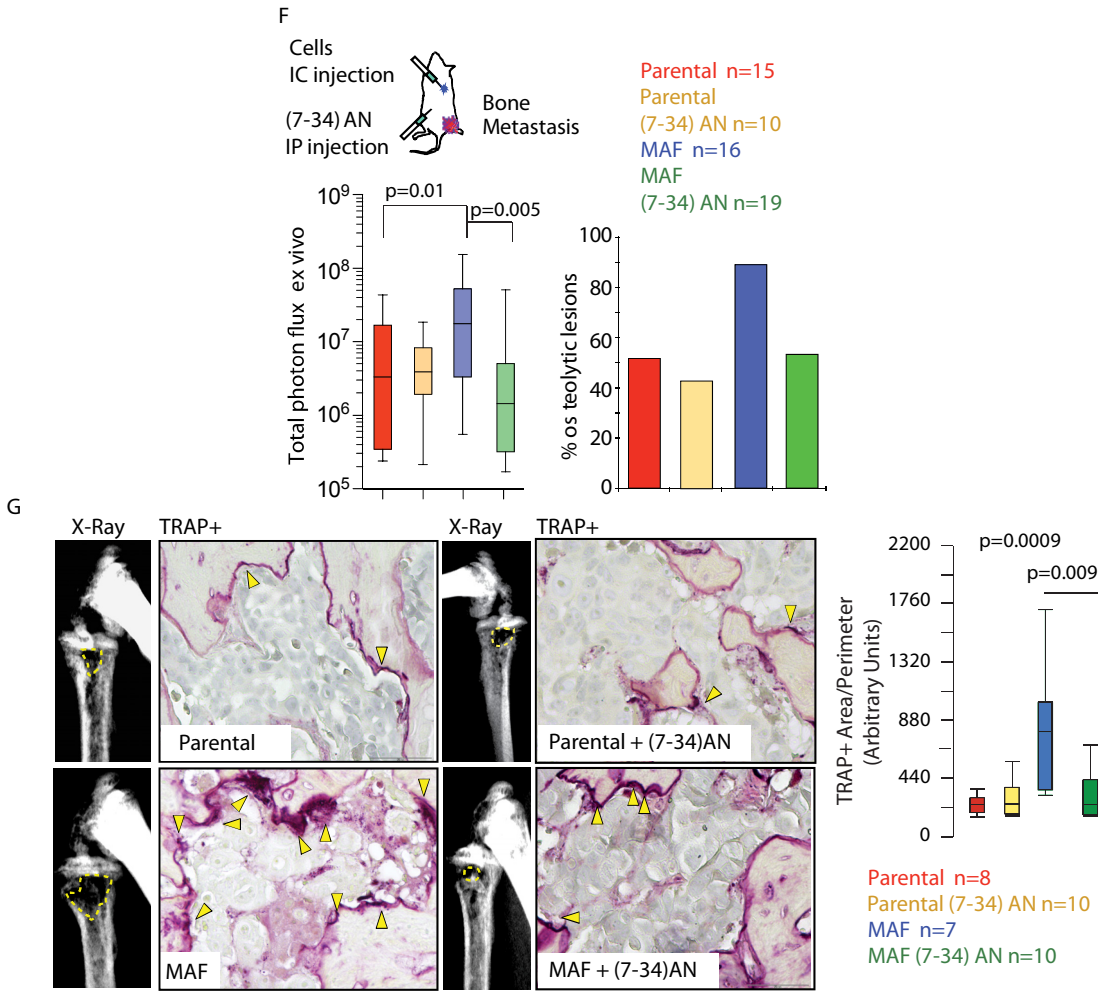
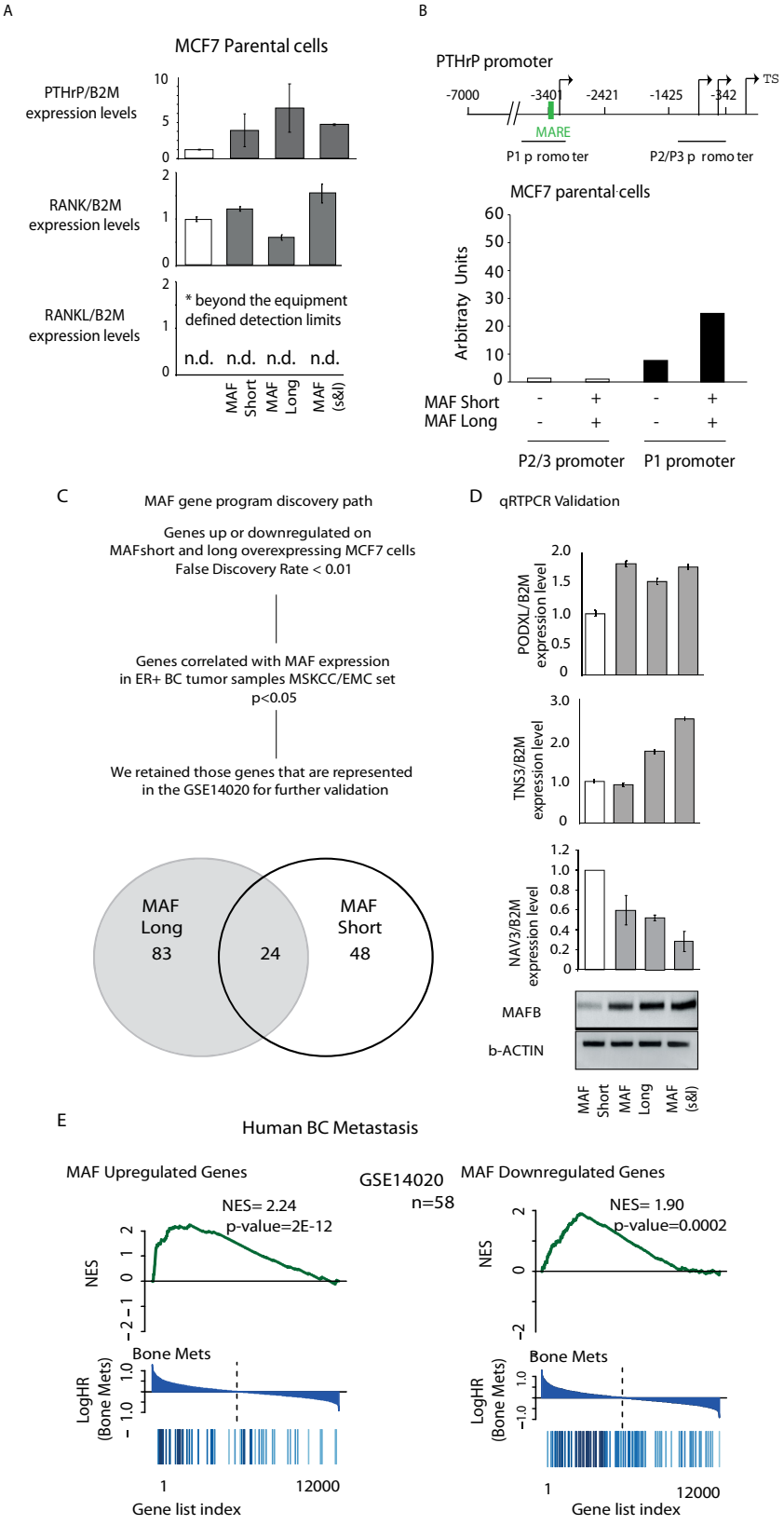


Figure 3. Bone metastatic promoting effect of MAF is mediated by PTHrP. Continuation (A) PTHrP expression levels measured by qRT-PCR in Parental Control, BoM2 and Parental MAF Short and Long (simultaneously) overexpressing cells. Expression is normalized to B2M as endogenous control. Data is presented as mean of three independent experiments with sd. **(B)** Schematic representation of the *PTHrP*promoter regions (P1 and P2/P3) and regions amplified in ChIP assays (primer sets, PR1 and PR4). The nucleotide sequence of the human *PTHrP*promotercontaining binding elements for MAF (MARE, MAF response element; green box) are shown. **(C)** Parental, MAF-expressing and BoM2 MCF7 derivatives cells were subjected to ChIP assays with the indicated antibodies and PCR primers. The proximal region of the*PTHrP* promoter (P2/P3) and the *β-ACTIN* promoters were used as a negative control. **(D)** Dot chart of the standardized expression of *MAF* against the standardized expression of *PTHrP* in human breast cancer metastasis from GSE14020 metastasis data set. The red dots depict bone metastasis while the black dots depict other soft tissue metastasis. The blue dotted lines depict the average *MAF* or *PTHrP*

expression in metastasis samples. Bottom table shows the proportion of bone metastases in each quadrant above. **(E)** (Top) Quantification of the number of TRAP+ differentiated multinucleated (>3 nuclei) osteoclast per field, normalized to control, is presented for indicated experimental conditions. Data is mean of three experiments with sd. (bottom) Representative images of osteoclast differentiation assay in vitro for Control condition (a), Control treated with PTHrP antagonist (b), MAF Short/Long overexpressing condition (c) and MAF Short/Long overexpressing condition incubated with PTHrP antagonist (d). Yellow arrows highlight multinucleated large cellular entities. **(F)** Schematic representation of cancer cells intracardiac injection and PTHrP antagonist intraperitoneal inoculation. (Left) Quantification of *ex vivo* bioluminescent signal at hind limbs of mice bearing a bone metastasis in each experimental group at end point. (Right) Percentage of osteolytic bone lesions measured as visible X-Ray lesions for each experimental group. **(G)** (Left) Representative image of X-Ray and TRAP straining of bone metastasis from each experimental group. Scale bar, 500 μm. (Right) Quantification of TRAP+ positive area per bone perimeter along the bone-tumor interface of metastasis from each experimental group. Minimum of 10 random fields per tumor covering the whole tumor area were captured. Data are presented as box plot with median, IQR and min and max values.

To test whether PTHrP mediates MAF driven bone metastasis in breast cancer cells, we injected MAF over-expressing MCF7 cells into the left ventricle of BALB/c nude mice and evaluated their capacity to establish and grow bone metastases in the presence or absence of PTHrP-AN. MAF expressing cells in mice treated with PTHrP-AN or vehicle produced bone metastasis with similar penetrance, yet treatment with PTHrP-AN caused a significant reduction in the extent of the hind limb osteolytic lesions similar to that observed in parental cells (Figure 3F). This reduction was accompanied by a reduction in the area of osteoclast (TRAP+ cells) at the perimeter of the metastatic lesions (Figure 3G). These results suggest that MAF mediated PTHrP expression is an important factor for MAF driven osteolytic breast cancer bone metastasis.



Supplemental Figure 5 (Related to Figure 3) (A) Expression levels of *PTHrP, RANK* and

RANKL in Parental Control, MAF Short and MAF Long spliced isoform overexpressing cells (independently or collectively) measured by qRT-PCR using TaqMan probe genes and normalized to *B2M* levels. n.d. below detection limits. **(B)** Schematic representation of the *PTHrP* promoter regions (P1 and P2/P3). The nucleotide sequence of the human *PTHrP* promoter containing binding elements for MAF (MARE, MAF response element; green box) is shown. Reporter assays using P2/3 or P1 promoter region of the *PTHrP* gene in front of a luciferase reporter gene. **(C)** Identification of genes whose expression is higher or lower in MAF short and long expressing than in parental MCF7 cells. **(D)** Expression levels of some of the above identified genes *NAV3, TNS3, MAFB* and *PODXL* in Parental Control, MAF Short and MAF Long spliced isoform overexpressing cells measured by qRT-PCR using TaqMan probe genes and normalized to *B2M* levels or SybrGreen. **(E)** Gene Set Enrichment Analysis (GSEA) to score the association of the genes in C) with the HR for bone metastasis.

A multifunction transcriptional program contributes to MAF-mediated bone metastasis

Besides PTHrP, MAF may also transcriptionally control the activity of other genes promoting cell survival, homing and other bone remodeling capabilities to colonize the bone. To identify relevant bone metastasis genes transcriptionally controlled by MAF, we focused on genes that significantly correlate with MAF gene expression in ER+ patient breast cancer samples ($p<0.05$, MSKCC/EMC data set) and whose expression changed accordingly to MAF overexpression in MCF7 cells (Figure S5C). These analyses provided a list of putative MAF target genes in breast cancer, named MAF program. Among the MAF positively correlated genes there were some well-known MAF target genes, including MAFB (Eychene et al., 2008). MAFB is the less effective MAF large family member at transforming cells and contains MARE binding sites on its promoter that regulate their expression by large MAF proteins (Eychene et al., 2008). Interestingly, a large set of other genes are also expressed or repressed according to changes in MAF including functions such as survival/proliferation (FGF18, EFEMP1, PDGFC, NR3C1, RERG and others), migration (NAV3, EFEMP1, WIPF1 and others), growth (PDGFC), stress/metabolism (ABCG2, HSPD1, STIP1 and others), cell signaling (ANXA9, MAPK8IP3, MALL, CBL and others), RNA processing (EIF2S1, EIF3B and others), cell differentiation (FAT1, MAFB and others) and adhesion (PODXL, ITGB5 and others). The expression pattern for some of these genes in MAF expressing cells was further confirmed by qPCR analysis (Figure S5D). Strikingly, GSEA analysis highlighted a strong association between the expression of the MAF

program upregulated genes and breast cancer bone metastasis (Figure S5E). These results might underline that besides PTHrP, MAF is transcriptionally driving a collection of events that might broadly support other relevant functions necessary for bone metastasis and explain its dominant effect.

MAF predicts breast cancer bone metastasis in a validation series

To further validate the ability of MAF to specifically predict bone metastasis, we applied again a training-validation schema as previously used for 16q22-24 CNA. To this end, we analyzed the MAF protein in the validation Spanish data set (Rojo et al., 2011). We set up an immunohistochemistry (IHC) assay and an optical computerized analysis system that allowed us to classify tumors according to the optical density (OD) of MAF protein expression (Figures 4A and S6A). Scoring of MAF OD was set to only measure nuclear tumor cell staining. Tumors with an IHC signal above background (>1000 OD, cut off determined based on the ROC curve) were considered high MAF (Figure 4A, experimental procedures). MAF protein levels by IHC and 16q23 DNA amplification by FISH were significantly correlated (Figure S6B). High MAF protein staining in breast cancer primary tumors predicted a high cumulative risk of metastasis to bone at any time but not to soft tissue and visceral sites (HR(bone mets)=4.7, 95% C.I. 2.3-9.6, p=5e-5; using death before recurrence in bone as a competing event, Figure 4B; Tables 2, S4 and S5). MAF protein expression by IHC enabled better assignment of positive cases in the validation set than MAF mRNA expression (measured by affymetrix arrays) in the discovery set (Figure 4C). The association of bone metastasis and MAF protein overexpression was significant in both ER+ and triple negative breast cancer subtypes (Figures S6C-S6E), in line with the results with 16q23 FISH assay. ER+ breast cancer patients with MAF OD above 25000 developed bone metastasis with almost complete penetrance (Sensitivity=0.36; Specificity=0.99, HR(bone met at any time)=21.3, 95% C.I. 8.3-54.7, p=3e-7 using death before recurrence as a competing event, Figure S6E). In triple negative disease, MAF levels above 1000 OD were sufficient to identify patients at high risk of bone metastasis (Sensitivity=0.75, Specificity=0.83; HR(bone met at any time)=10.7, 95% C.I. 2.2-53.3, p=0.001 using death before recurrence as a competing event, Figure S6E). In HER2+ patients no statistical significance was achieved (Figure S6D). The predictive value of MAF protein expression for cumulative bone metastasis was independent of any of the tested routine clinico-pathological parameters (Table 2, S4 and S5; multivariate analysis,

using death before recurrence in bone as a competing event, HR(bone mets)=5.28, 95% C.I. 2.5 to 11.2, p=0.00002)).

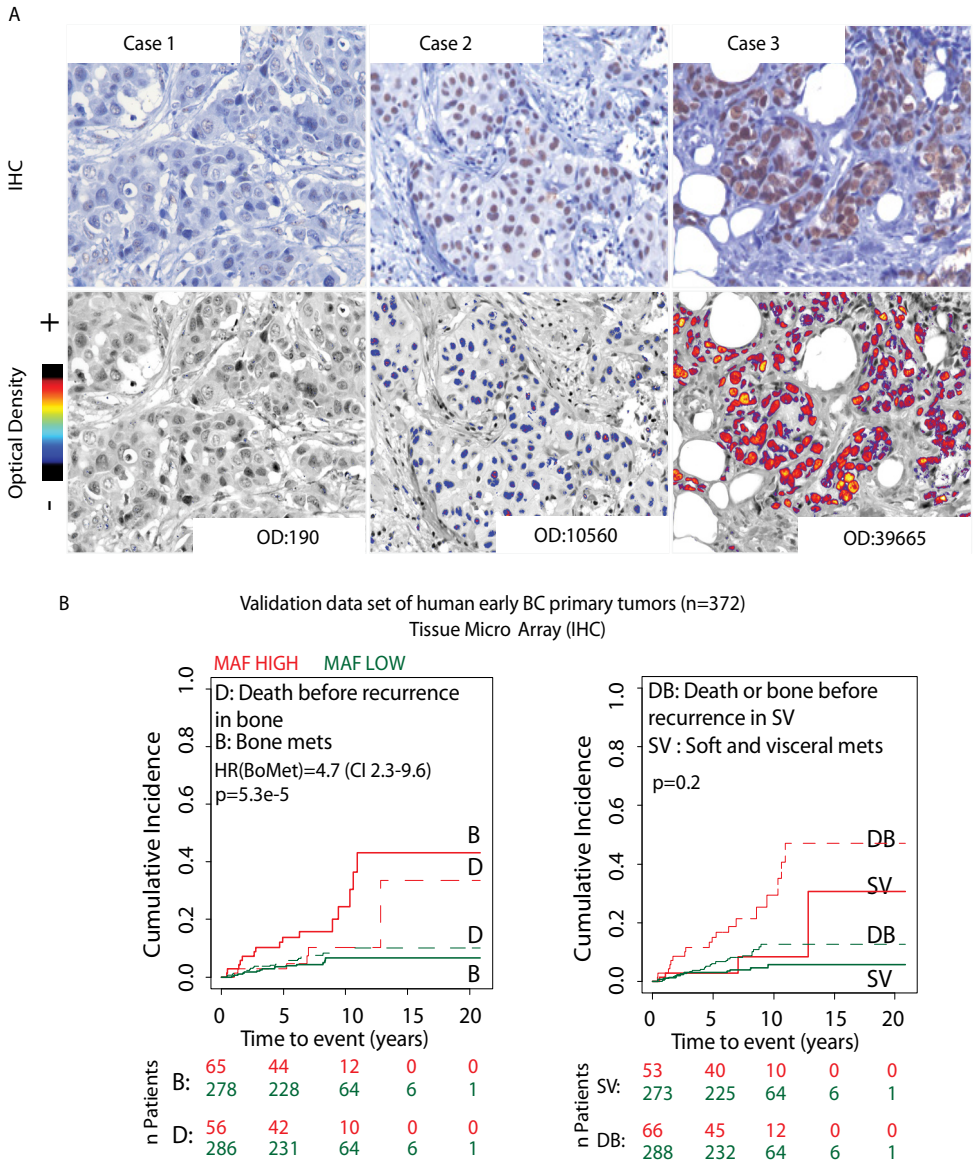


Figure 4. MAF is a clinical biomarker for breast cancer bone metastasis. See complete figure legend in page 95.

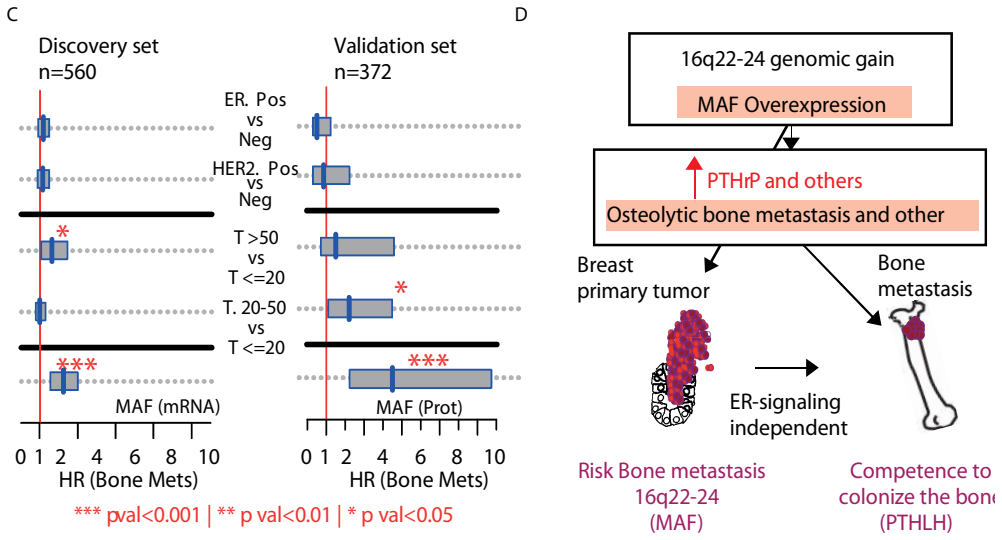
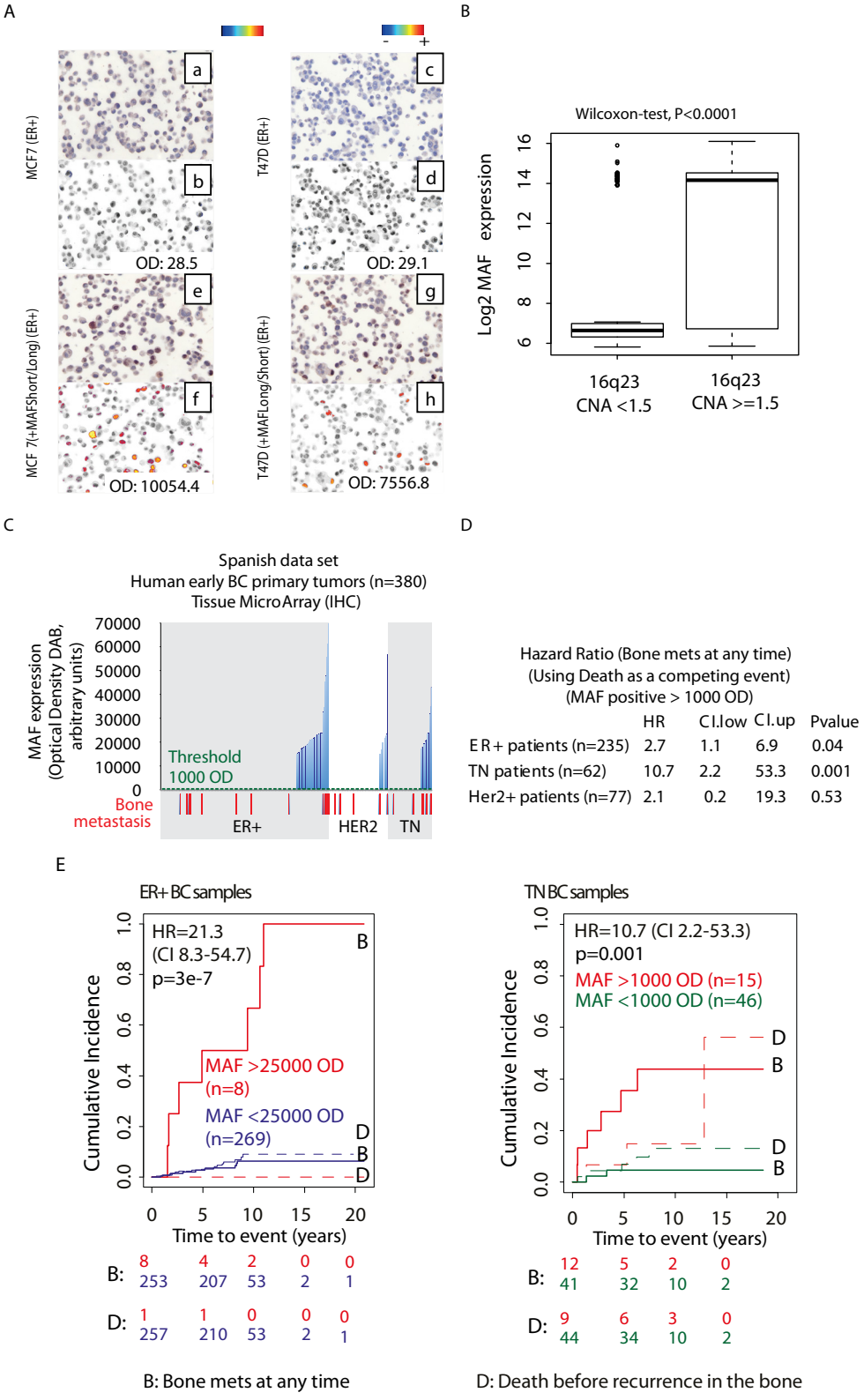


Figure 4. MAF is a clinical biomarker for breast cancer bone metastasis. Continuation **(A)** Representative MAF immunostainings of primary breast cancer tissues. Case 1 represents MAF negative tumors (OD<1000). Case 2 and 3 are MAF positive tumors (OD >1000 and >25000 respectively). **(B)** Cumulative incidence plot of bone metastasis at any time, considering death as a competing event, (Left) and non-bone metastasis (Right) in Spanish data set. MAF high group (red line, OD >1000); MAF low group (green line, OD<1000). **(C)** 95% confidence intervals for the Cox proportional hazard ratios are illustrated for the discovery and validation cohort for selected values of covariates represented in both cohorts. MAF is measured at the mRNA level and Protein level at the discovery and validation data sets respectively. **(D)** Model showing how 16q22-24 DNA genomic amplification identifies breast cancer primary tumors that will metastasize to the bone. This amplification drives overexpression of MAF transcriptional factor which, in turn, controls the expression of *PTHrP* and others and increases competence of breast cancer cells to colonize bone. The indicated mechanism is suggested to be independent of ER- signaling since is driven by DNA amplification at least in ER+, and TN breast cancer.



Supplemental Figure 6 (Related to Figure 4) (A) To set up the automatic system for the

analysis of MAF protein expression by immunohistochemistry (IHC) in primary breast tumor tissue samples, MCF7 and T47D cells transfected with Control vector were used as poor expressing control cells (a, c) and MCF7 and T47D cells transfected with MAF Short and Maf Long (simultaneously) expressing vector were used as a positive control (e,g). Digitally analyzed images with calculated optical densities for each cell pellet in a,c,e and g are shown (b,d,f,h respectively). **(B)** Boxplot with the MAF protein log2 score (IHC, OD values) on the vertical axis and amplified vs not-amplified categories on the horizontal axis is depicted. A Wilcoxon signed-rank test has been used. **(C)** Plot depicts MAF protein expression (OD) in a cohort of 372 primary breast cancer tumors (validation Spanish data set). Tumors are segregated according to BC subtype (ER+, HER2+ and TN). Red tick depicts tumors from patients that eventually developed bone metastasis. OD-optical density based on MAF immunostaining. 15 MAF positive tumors developed bone metastasis (PPV=21), while 14 did in the MAF negative group (PPV=4.5) **(D)** Table shows Hazard Ratio for bone metastasis at any time, using death before recurrence in bone as a competing event, and its p-value of high MAF protein expression (OD>1000) group compared to MAF low group in different BC subtypes in Spanish data set. **(E)** Cumultaive incidence plot of bone metastasis at any time, considering death before recurrence in bone as a competing event, in ER+ (Left) and TN (Right) BC patients in Spanish data set. MAF cut off is indicated in each plot.

Table 2.
Cumulative incidence of recurrence in bone at any (measured from date of primary tumor surgical resection). Analysis in patients with MAF (IHC). This analysis is done considering competing events (death before recurrence in bone). The risk of competing events is reported in Table S4

	Univariate (n=343)			Multivariate (n=343)		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Menopausal status			0.676			-
Premenopausal	1.00			-		
Postmenopausal	0.85	0.40 to 1.80		-	-	
Tumor size, mm			0.007			0.151
≤20	1.00			1.00		
21-50	2.13	1.03 to 4.37		2.21	0.95 to 5.13	
>50	1.56	0.54 to 4.47		2.34	0.65 to 8.41	
Tumor grade			0.046			0.121
I	1.00			1.00		
II	1.42	0.69 to 2.93		4.27	0.55 to 32.99	
III	1.27	0.61 to 2.65		2.37	0.28 to 19.68	
Lymph nodes			0.007			0.047
None	1.00			1.00		
1-3	1.06	0.47 to 2.39		1.22	0.49 to 3.02	
4-9	0.63	0.15 to 2.65		0.67	0.14 to 3.23	
>9	5.14	2.20 to 11.99		4.04	1.46 to 11.15	
Hormonal receptor status			0.167			-
Negative	1.00			-		
Positive	0.58	0.27 to 1.22		-	-	
HER2 status			0.670			-
Negative	1.00			-		
Positive	0.81	0.31 to 2.13		-	-	
Proliferation (Ki-67)			0.014			0.044
Low proliferation (<15%)	1.00			1.00		
High proliferation (≥15%)	2.52	1.23 to 5.18		2.23	1.03 to 4.84	
cMAF (IHC)			5e-05			2e-05
Non-overexpression	1.00			1.00		
Overexpression	4.68	2.29 to 9.57		5.28	2.50 to 11.20	

Abbreviations: HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2

Table S3. Baseline Characteristics According to MAF IHC expression

Characteristics	Complete series (n=372)		MAF non-overexpression (n=301)		MAF overexpression (n=71)		*P*
	No. of patients	%	No. of patients	%	No. of patients	%	
Age (median, range)	58, 26-90		58, 31-90		59, 26-90		
Menopausal status							0.772
Premenopausal	109	29.3	87	28.9	22	31.0	
Postmenopausal	263	70.7	214	71.1	49	69.0	
Tumor size, mm							0.429
≤20	206	55.3	165	54.8	41	57.7	
21-50	130	35.0	109	36.2	21	29.6	
>50	36	9.7	27	9.0	9	12.7	
Tumor grade							0.799
I	67	18.0	56	18.6	11	15.5	
II	178	47.8	144	47.8	34	47.9	
III	127	34.2	101	35.5	26	36.6	
Lymph nodes							0.369
None	222	59.7	177	58.8	45	63.4	
1-3	90	24.2	78	25.9	12	16.9	
4-9	37	9.9	28	9.3	9	12.7	
>9	23	6.2	18	6.0	5	7.0	
Estrogen receptor status							0.764
Negative	95	25.5	76	25.2	19	26.8	
Positive	277	74.5	225	74.8	52	73.2	
Progesterone receptor status							0.679
Negative	132	35.5	105	34.8	27	38.0	
Positive	240	64.5	196	65.2	44	62.0	
HER2 status							0.326
Negative	296	79.6	236	78.4	60	84.5	
Positive	76	20.4	65	21.6	11	15.5	
Bone mets at any time							6e-5
Negative	342	91.9	286	95.0	56	78.9	
Positive	30	8.1	15	5.0	15	21.1	
(median follow up, months)			7.6		6.9		
Soft and visceral metastasis before bone or death							0.355
Negative	354	95.1	288	95.7	66	92.9	
Positive	18	4.8	13	4.3	5	7.1	
Proliferation (Ki-67)							0.100
Low proliferation (<15%)	272	73.1	226	75.1	46	64.8	
High proliferation (≥15%)	100	26.9	75	24.9	25	35.2	

Abreviations: HER2, human epidermal growth receptor 2

Table S4 (Continuation Table 2)
Competing risk (death before recurrence in bone) survival analysis in patients

	Univariate (n=342)			Multivariate (n=342)		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Menopausal status			0.12			-
Premenopausal	1.00			-		
Postmenopausal	1.94	0.78 to 4.79		-	-	
Tumor size, mm			0.002			0.054
≤20	1.00			1.00		
21-50	1.96	0.94 to 4.09		2.62	1.06 to 6.50	
>50	2.59	1.05 to 6.39		3.55	1.08 to 11.69	
Tumor grade			0.024			0.263
I	1.00			1.00		
II	0.67	0.31 to 1.43		1.08	0.29 to 4.01	
III	2.03	0.97 to 4.22		2.05	0.55 to 7.61	
Lymph nodes			0.203			0.671
None	1.00			1.00		
1-3	1.11	0.49 to 2.51		1.32	0.53 to 3.25	
4-9	1.40	0.48 to 4.05		0.84	0.24 to 2.88	
>9	2.70	0.94 to 7.78		1.85	0.55 to 6.15	
Hormonal receptor status			0.029			-
Negative	1.00			-		
Positive	0.43	0.20 to 0.89		-	-	
HER2 status			0.163			-
Negative	1.00			-		
Positive	1.79	0.81 to 3.94		-	-	
Proliferation (Ki-67)			0.282			0.080
Low proliferation (<15%)	1.00			1.00		
High proliferation (≥15%)	0.60	0.23 to 1.58		0.43	0.15 to 1.18	
cMAF (IHC)			0.624			0.637
Non-overexpression	1.00			1.00		
Overexpression	1.25	0.51 to 3.09		1.24	0.50 to 3.06	

Abbreviations: HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2

Discussion

We provide novel evidence on the role of 16q22-24 genomic region to predict a high risk of bone relapse in patients with early breast cancer. We also have shown that *MAF*, a gene within this genomic amplification, is a key driver of breast cancer bone metastasis.

MAF gene overexpression encoded within the 16q22-24 region supports the molecular processes that affect the metastatic course from the primary site to distal colonization. Interestingly, the acquisition of high levels of MAF expression parallels that observed in MM and AITL, where several copies of the *MAF* genomic region or a t(14,16) translocation are gained leading to transformation and aggressive osteolytic bone colonization (Eychene et al., 2008). The biology of different metastasis mechanisms to the bone might rely on a common mediator that exerts similar or different functions depending on the tumor of origin. Moreover, the observation that the bone metastatic site imposes MAF selection in both malignancies of the breast and the hematopoietic system may create opportunities for the development of common targeted therapies to prevent disease dissemination.

Within the 16q22-24 region gain other genes besides *MAF* could potentially contribute to breast cancer bone metastasis. The FISH probes used in our analyses cover approximately 2.2 Mb and 0.2 Mb gaps, respectively. The first probe flanks five genes *VAT1L, CLEC3A, WWOX, 5srRNA* and *MAF* (ordered from centromer to telomer) while the latter only flanks *MAF*. We narrowed *MAF* as the causal mediator of breast cancer bone metastasis based on its clinically relevant association combined with the use of our metastatic experimental cell system, loss-and gain-of-function experiments in animal models and the consistency of our CNA results when reducing the size of our region of analysis. Another gene in this region, *WWOX* (Bednarek et al., 2000), has been suggested to be a relevant tumor suppressor in cancer. However, it is difficult to reconcile this with reports whereby 16q deletion, implying loss of *WWOX,* is associated with good prognosis in breast cancer patients (Andre et al., 2009; Hansen et al., 1998) and 16q gain, implying *WWOX* gain, with bad prognosis and bone metastasis. However, we cannot exclude that other genes in this region might have a partial contribution to the phenotype observed (Nguyen and Massague, 2007).
Guided by the osteolytic bone metastasis phenotype observed in MCF7 and T47D MAF expressing cells, we focused on potential downstream gene candidates based on

the literature and showed that PTHrP levels depend on MAF activity. While a role for PTHrP has been well established in bone metastasis (Guise et al., 2005), it was unknown that MAF positive tumors select for PTHrP expression. We showed that PTHrP was a major contributor to MAF mediated osteolytic bone metastasis in experimental models and identified the molecular mechanisms by which MAF controls PTHrP transcription. Using reporter assays and chromatin immunoprecipitation (ChIP), we determined that MAF controlled the expression of PTHrP P1 promoter region, including a MARE (MAF response element) binding site, and showed direct binding of MAF to this MARE region of PTHrP P1 promoter. Furthermore, we demonstrated that MAF transcription factor function could control the expression of other genes that could also support bone metastasis. The data showed that MAF provides new biological functions that could contribute to cellular survival, homing and bone remodeling capabilities to colonize the bone. As opposed to normal epithelia, most carcinoma cells present diverse alterations in survival, growth and homing, including adhesive properties that allow cells to disobey tissue architecture. These changes, concomitantly with dynamic cytoskeletal changes, cell-matrix interactions, actin-myosin contractions and focal contact disassembly, allow cells to move from one site to another and finally establish at a distant site (Friedl and Wolf, 2003). Our results suggest that MAF gene program supports breast cancer cells bone metastasis steps through a series of both cell autonomous and also niche related functions (Nguyen and Massague, 2007). Some of these activities might be necessary for the early steps of dissemination and might explain why MAF upregulation is selected for in breast cancer human primary tumors that will metastasize to the bone. Clinical data showed that MAF high expression in breast primary tumors is associated with risk of bone metastasis and MAF high expression is retained in bone metastasis compared to other sites. This opens the possibility to use MAF as a molecular target for the treatment and prevention of bone metastasis (Eckhardt et al., 2012).

These preclinical findings as well as results from a clinical discovery series, led us to test 16q22-24 chromosome region genomic gain by FISH in paraffin-embedded samples from patients with primary breast cancer and long term follow up. In fact, FISH assays are routinely used to detect genomic alterations with clinical utility (*i.e.* HER2 (17q2) amplification (Lebeau et al., 2001) and ALK translocation(2,5)(Martelli et al., 2009)). Using our FISH assay, we detected 16q23 CNA in 14% from a total of 334 primary breast cancer specimens and showed that it had a significant and independent

association with the risk of bone metastasis. This observation is in agreement with the expected frequency of breast cancer patients that develop bone metastasis (10-20%) after 15 years follow up, assuming variations depending on the standard of care used in the cohort of analysis, (Kennecke et al., 2010; Yerushalmi et al., 2010). Of note, conflicting data on the role of 16q region alterations in human cancers had been reported, such as 16q loss (Hansen et al., 1998) as well as 1q gain/16q loss (Andre et al., 2009), associated with good outcome in breast cancer, while 16q loss was associated with prostate cancer osteoblastic bone metastasis (Harkonen et al., 2005). In addition, we also developed an immunohistochemical assay to detect MAF expression. Consistently, MAF protein levels measured by IHC and 16q23 DNA amplification by FISH were significantly correlated and, in a similar manner to the FISH assay, high MAF protein staining in primary tumors predicted a high cumulative risk of metastasis to the bone at any time but not to soft tissue and visceral sites.

Based on the mechanistic and clinical data presented above, we propose that the 16q22-24 genomic amplification selectively predicts bone metastasis risk in early stage breast cancer and that the *MAF* gene encoded within this region mediates breast cancer bone metastasis (Fig. 4d). This novel finding may enable the identification of patients at high risk of bone metastasis in a timely fashion. Clinical trials involving thousands of patients have, or are testing, the capacity of bisphosphonates or denosumab to prevent bone metastasis. The results of these trials to date have yet to influence routine clinical practice (Coleman et al., 2011; Gnant et al.; Muller et al., 2001). In the era of personalized medicine, the incorporation of a biomarker to enrich a population for those most likely to benefit from bone targeted agents is urgently needed (Coleman, 2011). 16q23 genomic amplification and MAF expression will be now tested in archival tumor samples from patients included in completed or ongoing international phase III trials. Furthermore, our data establishes MAF as a novel target to prevent or treat breast cancer bone metastasis.

EXPERIMENTAL PROCEDURES

Cell culture

The MCF7 and T47D breast cancer cell lines were purchased from the ATCC. MDA231-4175 (LM2) is a lung metastatic subline derived from MDA231 breast cancer cell line at Massagué laboratory(Minn et al., 2005a). The BoM2 bone metastatic subline is derived from MCF7 following a similar procedure as described in(Kang et al., 2003). These cell lines and their genetically modified derivatives were maintained as described before(Tarragona et al., 2012).

Stable cell lines expressing the shRNA MAF or a non-silencing shRNA were generated as described(Tarragona et al., 2012):

MAF-
CCGGTGGAAGACTACTACTGGATGACTCGAGTCATCCAGTAGTAGTCTTCCATTTTT

Control-
CCGGCTGTTGCTATCGGGTCAACAACTCGAGTTGTTGACCCGATAGCAACAGTTTTTG.

For MAF overexpression in cells, MAF isoforms cDNA sequences were cloned into the retroviral vector pBabePuro. All cell lines were stably tansfected with TK-GFP-Luciferase construct and sorted for GFP.

Animal studies and xenografts

All animal work was approved by the institutional animal care and use committee of IRB Barcelona. Female BALB/c nude mice of 11 weeks of age were used for all studies. For intracardiac and tail vein injections, cells were resuspended in PBS and injected into the left cardiac ventricle or tail vein of mice using 26G needle as previously described (Tarragona et al., 2012). Prior to injection, mice were anesthetized with ketamine (100mg/kg body weight) and xylazine (10mg/kg body weight) and immediately after injection of tumor cells, mice were imaged for luciferase activity. 90-day release estrogen pellets were subcutaneously provided unless otherwise indicated. Mice continued to be monitored weekly using IVIS imaging, unless otherwise indicated. For injection of orthotopic site tumor cells, mice were treated as described above and tumor cells mixed with matrigel before inoculation. Upon becoming palpable, tumors were measured with a digital caliper. Tumors were resected before reaching the size of 300 mm^3. In order to block PTHrP activity *in vivo*, animals were administered, twice a day, with 6 □g of (7-34Aa) PTHrP antagonist

peptide (Bachem, Switzerland) dissolved in PBS.Control groups were treated with PBS.For all ER-positive breast cancer cells implanted in mice, estrogen supply was provided by using subcutaneously implanted estrogen pellets (90-day release)(Innovative Research of America, USA).

For intra-tibial injections, BALB/c nude mice were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The injection site was prepped with betadine scrub followed by a 70% alcohol wipe. A 1-cm skin incision was made on the antero-medial part of the leg and the muscle was moved using blunt forceps. The bone was drilled using a 26G syringe-needle (BD Syringes). Single-cell suspensions ($2x10^4$ cells) in a final volume of 25 µl were injected into the upper half of the tibia medullary cavity, as felt by a lack of resistance when pushing cells into the cavity. The skin was sutured back and inoculations were confirmed by BLI. Development of bone lesions was followed by weekly BLI imaging and X-ray radiography.

Oligonucleotide array assays

RNA sample collection and generation of biotinylated complementary RNA (cRNA) probe were carried out essentially as described in the standard Affymetrix (Santa Clara, CA) GeneChip protocol. Ten micrograms of total RNA was used to prepare cRNA probe using a Custom Superscript Kit (Invitrogen). For expression profiling 25ng of RNA per sample was processed using isothermal amplification SPIA Biotin System (NuGEN technologies). Each sample was hybridized with an Affymetrix Human Genome U133APlus2.0 microarray at the IRB Barcelona Functional Genomics Laboratory. All microarray statistical analyses were performed using Bioconductor(Gentleman et al., 2004). Background correction, quantile normalization and RMA summarization was performed as implemented in bioconductor'saffy package(Irizarry, 2009). Biases due to experimentation date and family relations between mice were removed with ANOVA adjustment. A semi-parametric empirical Bayes(Rossell, 2008) procedure based on moderated t-tests(Smyth, 2005) as implemented in the limmapackage was performed to select the bone metastasis enriched genes setting the Bayesian FDR at 5%(Rossell, 2008). Additionally, only genes with an absolute fold change value bigger than 2 were considered differentially expressed.

Patient gene expression data sets

The patients' information is publically available and has been downloaded from GEO (Barrett et al. (2007)). The following cohorts were used: A) MSKCC/EMC. Pooled

GSE2603, GSE2034 and GSE12276. This union cohort has 560 patients. In order to remove systematic biases, the expression measurements were converted to z-scores for all genes prior to merging. ER+ patients were selected based on the bimodality of gene ESR1 (figure S1e). B) GSE14020. ER+ patients were selected based on the bimodality of gene ESR1. C) As described in van de Vijver et al N Engl J Med 2002; 347:1999-2009, patient expression profiles and clinical annotations were downloaded following instructions.

Alteration analyses in the copy number in data expression

The detection of copy number alterations (CNA) by means of expression profile analysis is based on strong correlation between the genomic alterations and the aberrant gene expression in the affected genomic regions. Specifically, the exact detection of CNAs using gene expression analysis is possible and its difficulty stems from the type of starting expression data(Hu et al., 2009). We used the function findCopyNumber form the bioconductor'sphenoTest package which implements an approach similar to the one of Hu et al. 2009, to find regions with copy number alterations in the cohort MSKCC/EMC. Given enrichment scores (in our case log hazard ratios) and the chromosomal positions of those enrichment scores we found areas where the enrichment is bigger/lower than expected if the positions where assigned at random.

Within each chromosome a smoothed score for each gene was obtained via generalized additive models, the smoothing parameter for each chromosome being chosen via cross-validation. The obtained smoothing parameter of each chromosome is used in permutations.

We assessed statistical significance by permuting the positions through the whole genome. For each gene the permutations of genes that are in an area with similar density (distance to tenth gene) were used to compute P-values (p-values were adjusted using the Benjamini and Hochberg method).

Alterations in the genome copy number in the highly bone metastatic BoM2 cells were analyzed. High-molecular DNA was isolated from in vitro cultured MCF7 and BoM2 cells using GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following manufacture's instructions. DNA quantity and quality was determined by NanoDrop ND-1000 UV-Vis Spectrophotometer and electrophoresis in 1% agarose gel. Genetic aberrations were detected using NimbleGen Human CGH 3x720K Whole-Genome Tiling v3.0 Array consisting of 72,000 probes. Samples were independently

labeled with indicated fluorochromes: Cy3 for BoM2 and Cy5 for MCF7 reference sample, and co-hybridized. The copy number analysis was performed using Bioconductor. Briefly, log2 fold changes were normalized by a mode normalization and outlier smoothing(Venkatraman and Olshen, 2007). Preprocessing, segmentation, post-segmentation and segment classification were performed as implemented in the Bioconductor'sCGHcall package. For all chromosomes, dots and blue horizontal lines represents normalized log2 intensity ratios and segments. Copy number gain probability is indicated by the length of the green downward bar, and loss probability is represented by red upward bar.

From The Cancer Genome Atlas (TCGA) breast cancer project, a total of 773 breast tumours were assayed using Affymetrix 6.0 SNP arrays. Segmentation analysis and GISTIC were used to identify focal amplifications/deletions and arm-level gains and losses. In particular, we evaluated DNA copy-number changes of each gene using Level 3 GISTIC data (high level amplification, low level amplification, neutral, low level deletion and high level deletion) that is publicly available at the TCGA portal (https://tcga-data.nci.nih.gov/tcga/).

Statistical Analysis

A) Cumulative incidence

For figures 1F, 1G, 2A and 4B and supplementary figures S2A, S2D and S6E, p-values were obtained after fitting Cox proportional hazard models and performing likelihood ratio tests.

Kaplan-Meier

For figures 1F, 2B and 2C and 2E p-values were obtained using log rank test

B) Statistical group comparison

Statistics were calculated by means of Wilcoxon two-sided test including figures 2D, 3A, 3E, 3F, 3G and supplemental figures unless indicated.

C) Survival analysis (mRNA, figureS3E)

A multivariate Cox proportional hazard model was fitted to test the correlation between MAF high versus the rest of the tumors and bone metastasis. Tumor size, lymph node status, tumor grade and proliferation were used as adjustment variables. R's function step was used to perform Backward elimination by AIC. P-values were obtained with Cox proportional hazards likelihood ratio tests.

D) Comparison of baseline characteristics (Table S1 and S3).

Differences in age are tested with Student's t test. All other variables are tested with

Fisher's exact test.

E) Measure of agreement (Figure S6B)

We assessed the agreement of both IHC and FISH methodological events in each sample. A Wilcoxon rank-sum test has been used.

F) Diagnostic performance FISH and IHC (Table 1, 2 and S1, S2, S3, S4 and supplemental figure S2B)

- A multivariate Cox cause-specific hazard model with competing events (death) was fitted to test the correlation between 16q23 or MAF and bone metastasis. Tumor size, lymph node status, tumor grade, Her2 and proliferation were used as adjustment variables. Diagnostic performance was evaluated by comparing the AUC of the ROC curves.
- Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) were computed for each of the classification categories based on the most predictive variables (16q23 FISH and MAF IHC). Bootstrapping of the PPV and NPV was done.

G) Prognostic role

- Cox regression modeling of the outcome time to bone metastasis was done with an "efron" management of ties. The maximum number of considered variables was 1 variables for each 5-10 events.

Protein extraction and Western Blot

Cells were lysed with a buffer containing 1% Triton in 50mM Tris/HCl (pH 7.4) for protein extracts and processed as in(Tarragona et al., 2012). The antibodies used were anti-MAF (AbCam or Inbiomotion) and α-Tubulin (Sigma).

Quantitative real-time PCR

Total RNA was isolated and processed as described(Tarragona et al., 2012). Human *MAF long, CND1, MYC* and *B2M* as endogenous control were amplified with commercially designed TaqMan gene expressions assay (Applied Biosystems). Expression levels of human *MAF short* were assessed and normalized to beta-actin levels using Syber Green real time PCR reaction (Applied Biosystem) with following primers: *MAF short-F*: 5'GATCCACAGCACTGGTCTTG3', *MAF short-R*: 5'GGGATGCTGCTGAGGTTAAA 3', *beta-ACTIN*-F: 5'

TCACCCACACTGTGCCCATCTACGA 3' and *beta-ACTIN*-R
5'CAGCGGAACCGCTCATTGCCAATGG 3'.

Fluorescence in situ hybridization FISH

Cells were processed as described(Arnal-Estape et al., 2010). The slides were incubated with and off the shelf 16q23 MAF and 14q32IGH probe mixture (Abbot). This SpectrumOrange probe flanks the MAF gene region and is composed of two segments that are each approximately 350 kb with an approximately 2.2 Mb gap. The centromeric segment is located at chr16:75729985-76079705 (March 2006 assembly, UCSC Genome Browser) and the telomeric segment is located at chr16:78290003-78635873 (March 2006 assembly, UCSC Genome Browser). This probe flanks five genes VAT1L, CLEC3A, WWOX, 5srRNA and MAF (ordered from centromer to telomer). Further, an independent BAC probe focused on MAF gene, RP11-1068n20 was used to further confirm the scorings. This probe covered the chr16: 79,460,645 - 79,657,297, 197 kb, region including the MAF gene in full (chr 16, 79,625,745 a 79,639,622, 14 kb). In parallel, a CEP16 (centromeric chr 16, 16q11.2)(Abbot) probe was used to score 16q23 CNA. DAPI counterstain was applied and images were acquired with a Leica TCS-SP5 confocal microscope.

Histopathology and immunohistochemistry

Hind limb bones were excised, fixed in 10% neutral-buffered formalin, decalcified, embedded inparaffin and subjected to staining with hematoxylin and eosin (H&E, Richard-Allan ScientificInc.) and anti-Ki67 antibody (Novocastra).

MAF immunostaining was performed using 3μm human breast cancer tumor tissue sections, placed on plus charged glass slides in a Dako Link platform. After deparafinization, heat antigen retrieval was performed in pH6.1, 0.01 mol/L citrate-based buffered solution (Dako). Endogenous peroxidase was quenched. A rabbit polyclonal anti-MAF antibody was used for 30 minutes at room temperature, 1:100 dilution, followed by incubation with an anti-rabbit Ig dextran polymer coupled with peroxidase (Flex+, Dako). Sections were then visualized with 3,3'-diaminobenzidine (DAB) and counterstained with Hematoxylin.

MAF antibody sensitivity (1:100) had been calculated in a range of crescent dilutions of primary antibody from 1:10 to 1:1000. Specificity was determined using parental and MAF-overexpressing (plusMAFlong/short) MCF7 and T47D human breast cancer cells. Formalin-fixed cell pellets were processed as described for IHC and results confirmed by western blot from whole lysates. Specificity was also shown in heterotopic MCF7

and MAF-overexpressing MCF7 xenoimplants in BALB-c nude mice. Sections from the same specimens incubated with normal rabbit IgG2 (IS600, Dako) instead primary antibodies were used as negative controls.

MAF immunostaining was scored by a computerized measurement. Nine representative images from each specimen were acquired at 10-nm wavelength intervals between 420 and 700 nm, using a DM2000 Leica microscope equipped with the Nuance FX Multispectral Imaging System (CRI Inc). Before acquiring a spectral dataset of an image, an autoexposure routine was performed while imaging a blank area of slides to determine the exposure time necessary to approximately 90% fill the device wells at each wavelength to compensate for variations in source intensity, filter transmission efficiency and camera sensitivity. A library of pure DAB and Hematoxylin dye colors was created and used to separate the colors using the Nuance 1.6.4 software. A cube (stack of images taken at the different wavelengths) of reference was then acquired for each new case, followed by spectral imaging of three representative tissue fields using the same exposure times. After deconvolution of the images, the spectral data was flat fielded to compensate for unevenness in illumination and background was filtered. The positive signals were converted from transmission to optical density units by taking the negative log of the ratio of the sample divided by the reference cube using a Beer law conversion. A computer-aided threshold was set, which creates a pseudo-color image that highlights all of the positive signals. Analysis yielded quantitative data of MAF from the average intensity of regions of interest. Only the nuclei of epithelial cells (normal and malignant), but not stromal cells or lymphocytes, were automatically detected by setting distinct size threshold and confirmed by a pathologist. Each case was calculated for the mean value of the signal intensity of all regions of interest for statistical analysis. The output of the computerized measurement produced a continuous data ranging from 56 to 70,367 for MAF expression. The cut-off of 1000 OD was chosen based on receiving operating characteristic (ROC) curves.

X-RAY analysis

Development of bone metastasis was monitored by X-Ray imaging (CT-Scan). Visible metastatic lesions were measured using Image J software and osteolytic area and calculated in arbitrary units.

Reporter assays

Renilla and luciferase reporter assays were performed as previously described(Tarragona et al., 2012). C-MARE sequence was cloned in renilla expressing vector. A CMV-RFP plasmid (Promega) was included to control for transfection efficiency.

Osteoclast differentiation assay

In order to isolate bone marrow mononuclear cells, 4-6 week wild type C57BL/6 mice were sacrificed and their femora and tibias were flashed with cold PBS solution and bone marrow mesenchymal cells cultured in 100mm dishes overnight in α-MEM (Invitrogen) media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml of streptomycin and 0.29 mg/ml of glutamine. Non-adherent cells were collected and plated in the same media. After 2 days the adherent cells were scraped and counted in order to be plated in 24 well dishes and osteoclast differentiation was induced by adding 20ng/ml RANKL (peproTech), 30ng/ml M-CSF (R&D Systems) and conditional media from breast cancer cells. Medium was changed at day 3 and TRAP+ staining (Sigma-Aldridge) was performed at day 6. For PTHrP antagonist treatment, the 7-34 PTHrP peptide (Bachem) (5μg/ml) was added to the wells.

Chromatin Immunoprecipitation (ChIP)

Indicated cells were grown to 70% confluence and subsequently cross-linked with 1% formaldehyde at room temperature for 15 min. ChIP was performed as described previously(Arnal-Estape et al., 2010). The antibodies used were MAF (Santa Cruz), anti-acHis4 (Usptate). A 166-bp segment of the distal region of the PTHrP promoter (nucleotides -3406 to -3240) was amplified with the following primers (PR4 set): 5'-GGTGCTCTTGCTGTGTCTC-3' (sense) and 5'- CTTTCCGTAGAAATTCTCCTC-3' (antisense). In the proximal region of the PTHrP promoter (nucleotides -425 to -566) a two primer sets were used. A 181-bp was amplified with the following primers (PR1 set): 5'- GGCTAACCGCCTCCTAAAAG-3' (sense) and 5'-TTGTTCTCAGGGTGTGTGGA-3' (antisense). As a negative control, a 166-bp region of the □-actin promoter (nucleotides 29 to 195) was amplified with the following primers: 5'-TCGAGCCATAAAAGGCAACTT-3' (sense) and 5'-AAACTCTCCCTCCTCCTCTTCC-3' (antisense).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Description of the validation MSKCC/EMC data set

We used the EMC-344 and MSK-82 data sets, which are based on HG-U133A and were combined, and to EMC-189 data set, which is based on HG-U133plus2 and was processed separately (GSE2063, GSE5327, and GSE2034 available at the Gene Expression Omnibus (GEO) public database). In order to remove systematic biases, prior to merging the expression measurements were converted to z-scores for all genes. Patient clinical record of the 615 primary tumor samples has been extracted from the supplemental material described in Zhang, X.H., et al "Latent bone metastasis in breast cancer tied to Src-dependent survival signals" Cancer Cell. 2009, 6: 67-78. Following the indications of the Cancer Cell manuscript (table S1, page 33 of supplemental material), we retrieved the metastasis site annotation from table 8 of Bos, P., et al "Genes that mediate breast cancer metastasis to the brain" Nature. 2009, 459: 1005-9. The metastasis site annotation was reported for 560 of the 615. The median duration of follow-up was 7.667 years (range, 0 to 14.25) for the 268 patients without metastasis and 1.917 years (range, 0 to 9.583) for the 292 patients with metastasis. The median follow-up among all 560 patients was 4 years (range, 0 to 14.25). Those 55 patients with no time to metastasis reported were not included in any ulterior time to metastasis analysis.

To examine the prognostic value of MAF in different subsets of breast cancers, we divided the breast cancer samples based on their ER status. For ER status, we used the intensity of ESR1 on the Affymetrix chip as the pathological status was not available (for GSE12276). The distribution of ESR1 gene showed strong bimodality. We defined the ER+ and ER- tumors based on this bimodality. We defined the ER+ and ER- tumors based on this bimodality (ER+ BC n=349, Figures 1D, 2A and S2D, E).

Missing Values Report

Five hundred and sixty patients are represented in the cohort. Of which, the variables "ER, "HER2mod", "tumor size" have 15(2%), 20 (3%), and 63 (11%) missing values, respectively.

Description of the validation van de Vijver data set

As described in van de Vijver et al N Engl J Med 2002; 347:1999-2009, surgical resection specimens from a series of 295 consecutive women with breast cancer were selected from the fresh-frozen–tissue bank of the Netherlands Cancer Institute

according to the following criteria: the tumor was primary invasive breast carcinoma that was less than 5 cm in diameter at pathological examination (pT1 or pT2); the apical axillary lymph nodes were tumor-negative, as determined by a biopsy of the infraclavicular lymph nodes; the age at diagnosis was 52 years or younger; the calendar year of diagnosis was between 1984 and 1995; and there was no previous history of cancer, except nonmelanoma skin cancer. All patients had been treated by modified radical mastectomy or breast-conserving surgery, including dissection of the axillary lymph nodes, followed by radiotherapy if indicated. Among the 295 patients, 151 had lymph-node–negative disease (results on pathological examination, pN0) and 144 had lymph-node–positive disease (pN+). Ten of the 151 patients who had lymph-node–negative disease and 120 of the 144 who had lymph-node–positive disease had received adjuvant systemic therapy consisting of chemotherapy (90 patients), hormonal therapy (20), or both (20). Sixty-one of the patients with lymph-node–negative disease were also part of the previous study used to establish the prognosis profile. All patients were assessed at least annually for a period of at least five years. Follow-up information was extracted from the medical registry of the Netherlands Cancer Institute. The median duration of follow-up was 7.8 years (range, 0.05 to 18.3) for the 207 patients without metastasis as the first event and 2.7 years (range, 0.3 to 14.0) for the 88 patients with metastasis as the first event. The median follow-up among all 295 patients was 6.7 years (range, 0.05 to 18.3). There were no missing data.

Description of the GSE14020

Similarly, GSE14020 data set, which is based on HG-U133A and HG-U133plus2 was analyzed. In order to remove systematic biases, prior to merging the expression measurements were converted to z-scores for all genes. No missing annotations were reported.

Description of the validation Spanish data set

As described in Rojo F., et al Ann. Oncol. (2012) 23: 1156-1164, surgical resection specimens from primary breast tumors and mammoplasties obtained from Parc de Salut Mar Biobank (MARBiobanc, Barcelona, Spain), Fundación Jiménez Díaz Biobank (Madrid, Spain) and Valencia Clinic Hospital Biobank (Valencia, Spain). Tumor specimens from formalin-fixed paraffin-embedded (FFPE) blocks were retrospectively selected from consecutive breast cancer patients diagnosed between 1998 and 2000,

which had the following criteria: infiltrating carcinomas, operable, no neoadjuvant therapy, enough available tissue and clinical follow-up.

TNM (tumor–node–metastasis) staging was classified using the American Joint Committee on Cancer (AJCC) staging system. Histological grade was defined according Scarff–Bloom–Richardson modified by Elston and Ellis, Histopathology (1991) 19: 403-410. ER and PR were determined by immunohistochemistry (IHC) (SP1 and PgR636 clones, respectively; Dako, Carpinteria, CA) establishing positivity criteria in ≥1% of nuclear tumor staining. HER2 amplification was assayed by FISH (Pathvysion; Abbott Laboratories, Abbott Park, IL). Ki-67 was studied by IHC (MIB1 clone; Dako). Patients referred to genetic counseling were studied for BRCA1 and BRCA2 gene status by direct sequencing. The Ethics Committees of the three hospitals approved the study.

Four hundred and fifty six infiltrating carcinomas including 380 IDC (Ductal), 53 ILC (lobular) and 23 of other types were studied. Tissue microarrays (TMA) were constructed as described (Rojo et al Ann. Oncol., 2011). This TMA is an expanded version of that previously described.

In the Spanish validation data set, 38 out of 456 in the TMA (8,3%) patients suffer bone metastasis, which is in the range described in the literature (Jensen et al BMC Cancer 2011). We clarified that in this series, there were a total of 92 relapses (20.1%), and of those, 38 (8.3%) had bone metastasis at any time of relapse (either as the only site, following other sites of relapse or followed by other sites of relapse). Therefore, the proportion of relapsing patients that had bone metastasis was in fact of 38 (41,3%) out of 92 cases, in line with the proportion of bone metastasis expected as first site of relapse in breast cancer.

i. 16q23 Copy variation analysis (FISH)

Missing Values Report

Three hundred and fifty six patients have information on 16q23 FISH in the database. The variables “ER, “PR”, “HER2mod”, “grade” and “age” have 7(2%), 7(2%), 8(2%), 3(1%), and 13(4%) missing values respectively. 9 (2.5%) lack time to bone metastasis event. As these missing values constitute less than 5% of the sample size, individuals with missing values in these variables are excluded from the analysis. The variable “Ki67 consenso” has 25 (7%) of missing values and these values are also excluded but reported. Effective sample size for the following analyses is 334 patients, of which 47

(14%) have equal or more than 1.5 copies of the 16q23 compared to reference. Effective sample size for the following analyses in ER+ BC is 250 patients.

ii. MAF protein expression (IHC)

Missing Values Report

The exposure of interest, MAF OD, has 63 missing values out of the 456. These cases are not included in the analysis; the remaining sample size is 393 patients. The variables "ER, "PR", "HER2mod", "grade" and "age" have 5(1%), 5(1%), 10(2.5%), 5(1%), and 17(4%) missing values respectively. 12 (3%) lack time to bone metastasis event. As these missing values constitute less than 5% of the sample size, individuals with missing values in these variables are excluded. After excluding individuals with missing values in the survival covariate, a total sample size of 372 individuals are used in the analysis, of which 70 (19%) have more than 1000OD signal intensity.

Description of the TCGA data set

As described in Cancer Genome Atlas Network Nature 2012; 490, 61-70 surgical resection specimens from 825 primary breast tumors were obtained from several institutions according to the following criteria: the tumor was primary invasive breast carcinoma including T1 to T4. It is not reported in the original publication how patients were surgically and systemically treated. Among the 825 patients, 384 had lymph-node–negative disease (results on pathological examination, pN0) and 405 had lymph-node–positive disease (pN+). Tumor and germline DNA samples were obtained from 825 patients. Different subsets of patients were assayed on each platform: 466 tumors from 463 patients had data available on five platforms including Agilent mRNA expression microarrays (n = 547), Illumina Infinium DNA methylation chips (n = 802), Affymetrix 6.0 single nucleotide polymorphism (SNP) arrays (n = 773), miRNA sequencing (n = 697), and whole-exome sequencing (n = 507). Owing to the short median overall follow up (17 months) and the small number of overall survival events (93 out of 818), survival analyses were not reported in the publication.

Missing Values Report

The variables "ER, "PR", "HER2Final Status" and "age" have 45(5%), 48(6%), 59(7%), and 7 (1%) missing values respectively. There is no time to bone metastasis event, thus bone metastasis was not analyzed. 773 patients have CNA information on

16q23.2 region (including MAF gene) in the database, of which 47 (6.1%) are annotated to have a gain of the 16q23.2 region compared to reference.

Statistical Methods (more details)

Cumulative incidence functions for recurrence were estimated. These functions estimate the actual percentage of patients who will experience the various competing events within the study cohorts as opposed to the overestimated percentages obtained with the Kaplan-Meier method based on the cause-specific hazards.

Differences between the cumulative incidence functions according to patient subgroups were tested for statistical significance. Analyses were conducted to determine whether the risk of recurrence in bone at any time increased according to baseline characteristics. A cumulative incidence function regression model, Cox regression of cause-specific hazards, was used for multiple regression analyses. Covariates included in the model were nodal status, tumor size and tumor grade. To assess statistical significance of each factor in the multivariate Cox regression model we used likelihood ratio tests, including all other factors in the model.

Categories of Sites of recurrence

Different estimates were used depending on the data set.

For the MSKCC/EMC, all recurring breast cancer events reported were classified according to their sites, as follows: bone metastasis, lung metastasis, brain metastasis if this event is reported irrespectively of other metastasis sites reported at the same time. Time to event was defined as time from surgical resection to occurrence of event.

For the Spanish Data set, all recurring breast cancer events reported were classified according to their sites, as follows: local recurrences, confined to the ipsilateral chest wall and including mastectomy scars; regional recurrences, including ipsilateral axillary, internal mammary lymph node metastasis; distant recurrences in soft tissue; bone metastases; and visceral metastasis, including all other organ involvement metastasis. Other events were also recorded and this event is reported. Time to event was defined as time from surgical resection to occurrence of event.

Because special emphasis was being placed on the incidence of recurrence in bone, occurrence of bone metastases with or without recurrence at any other site or subsequent bone metastasis after recurrence at any other site was classified as the event of interest. Time to recurrence in bone at any time was defined as the time from tumor ressection to the first or subsequent event in bone after a metastasis elsewhere,

whichever occurred first. Death before recurrence in bone was considered the only competing event in this analysis.

In a subset analysis, interest is placed on the incidence of visceral and soft tissue metastasis. Occurrence of bone metastases without recurrence at any other site or death before recurrence in soft tissue was considered the only competing event in this analysis. Otherwise, patients' data were censored at the time they were last known to be alive without recurrence in soft tissue metastasis.

Analyses of the association between 16q22-24 and bone metastasis in MSKCC/EMC data set

For every ER+ sample in the cohort (n=349) we select the genes in the cohort that belong to 16q22-16q24 (169 genes). We test if the average expression of these genes is different form 0 using a one-sided Wilcoxon test where the alternative hypothesis is that the average expression is bigger than 0.

The samples with a p-value for the test below 0.05 are considered CNA. The rest are considered no CNA. We fit a Cox proportional hazards model to see how well this grouping predicts overall metastasis. We obtained a hazard ratio for CNA vs. no CNA of 1.37 (1.01-1.88) with a p-value of 0.04849

Evaluation of the biomarker in the adjuvant setting

The efficacy of treatments in the adjuvant setting is usually analyzed in a "relapse event-free survival" manner in randomized clinical trials. This analysis considers both relapse (bone metastasis in our case) and death as events of same relevance (FDA guidelines). To mimic this analysis, we provide below an analysis of bone metastasis-free survival for both CNA and MAF IHC.

Multivariate analysis of bone metastasis-free survival (models are adjusted by tumor size, affected lymph nodes, grade and ki67 proliferation rate) based on the Spanish data set.

	HR (95% CI)	P value
16q23 CNA negative	1 (Ref.)	
16q23 CNA positive	4.17 (2.12-8.20)	3.5e-5
IHC MAF LOW	1 (Ref.)	
IHC MAF HIGH	2.81 (1.72-4.59)	3.7e-5

Evaluation of the 16q23 chromosomal gain frequency based on the TCGA consortium breast cancer data set

16q23 Chromosomal gains frequency estimation was based on genes within each segment using the “The Cancer Genome Atlas (TCGA) breast cancer project” data. DNA copy-number changes of each gene were evaluated using Level 3 GISTIC data (high level amplification, low level amplification, neutral, low level deletion and high level deletion) that is publicly available at the TCGA portal (https://tcga-data.nci.nih.gov/tcga/).

As described in "Comprehensive molecular portraits of human breast tumours" Nature 2012, DNA from each tumor or germline-derived sample was hybridized to the Affymetrix SNP 6.0 arrays using protocols at the Genome Analysis Platform of the Broad Institute. From raw .CEL files, Birdseed was used to infer a preliminary copy-number at each probe locus28. For each tumor, genome-wide copy number estimates were refined using tangent normalization, in which tumor signal intensities are divided by signal intensities from the linear combination of all normal samples that are most similar to the tumor. This linear combination of normal samples tends to match the noise profile of the tumor better than any set of individual normal samples, thereby reducing the contribution of noise to the final copy-number profile. Individual copy-number estimates then undergo segmentation using Circular Binary Segmentation. As part of this process of copy-number assessment and segmentation, regions corresponding to germline copy-number alterations were removed by applying filters generated from either the TCGA germline samples from the ovarian cancer analysis or from samples from this collection.

Segmented copy number profiles for tumor and matched control DNAs were analyzed using Ziggurat Deconstruction, an algorithm that parsimoniously assigns a length and amplitude to the set of inferred copy number changes underlying each segmented copy number profile. Analysis of broad copy number alterations was then conducted as previously described. Significant focal copy number alterations were identified from segmented data using GISTIC 2.0. NMF consensus clustering of copy number data was performed using the presence or absence of amplifications or deletions in regions identified by GISTIC 2.0 analysis.

Chromosomal region gain	Frequency (n=773)	Percentage
16q23.1	53	6.8
16q23.2 (MAF gene included)	47	6.1
16q23.3	47	6.1

Author Contributions

M.P. designed and performed experiments, analyzed experimental and patient data and participated in manuscript preparation. A.A.E. designed and established the model system, performed microarrays and FISH. F.R. did pathological analysis and contributed to Spanish data set. M.T. established the model system. E.P. adapted ACE-like algorithm and provide support to statistical studies of patient data. X.G.A. reviewed all statistical analyses. M.M., S.G., A.B. and J.U. performed experiments. M.G. performed experiments with animal models. A.R., and A.LL. contributed to Spanish patient sample collection. A.P. did the analysis using the TCGA breast cancer data. R.C., L.N., and J.J-M participated in text preparation. J.A. participated in Spanish patient sample collection, clinico-pathological correlation and text preparation. R.R.G conceived and designed the project, analyzed data, supervised the overall project and wrote the manuscript.

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3.2 Identification of NOG as a specific breast cancer bone metastasis-supporting gene.

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CHAPTER 4. DISCUSSION

DISCUSSION

Our work provides novel and clinically relevant insights into the mechanism of breast cancer metastasis to the bone. Herein, we describe the16q22-24 copy number gain that occurs in approximately 10 percent of breast cancers and predicts patients at high risk of bone relapse. Moreover, we identified *MAF* gene, within the 16q22-24 region, as a critical mediator of bone metastatic colonization. We show that *MAF* exhibits bone metastatic function trough transcriptional control of its downstream targets. We further demonstrate *MAF*-driven expression of *PTHLH* which controls osteoclast differentiation and contributes to the pronounced osteolytic phenotype of *MAF* overexpressing tumors. In addition, we identified *NOG as a* secreted factor that contributes to the bone metastatic process in both paracrine and autocrine manner.

4.1 Identification of bone metastasis genes in breast cancer

The current view of tumorigenesis implies that cancer cells within the tumor have different biological characteristics, including their metastatic potential. Such biological diversity results from different genetic transformations that occur during malignant progression. Therefore, metastatic cells that thrive in secondary organs encompass certain genetic and epigenetic alterations that support successful colonization of metastatic site (Fidler, 1990; Heppner, 1984; Nicolson, 1984). Indeed, only a small percentage of tumor disseminated cells, released from the primary site into circulation, is able to initiate metastatic growth. Accordingly, cells with different metastatic properties have been isolated from the same parental tumor, confirming that not all cells of primary tumors have same capacity to disseminate and colonize secondary site (Fidler, 1973; Poste, 1982). Moreover, tumor cell heterogeneity and metastatic speciation of cancer cells could very well explain the metastatic organ tropism and simultaneous metastases that occur in cancer patients (Table 3). As Steven Paget described in 1989, particular tumors display restrictive profile of metastatic dissemination. He defined the ‘seed and soil’ hypothesis based on

the clinical appearance of cancer metastasis, where non-random patterns of metastasis to visceral organs and bone were observed (Fidler, 2003). This hypothesis implies that certain tumor cells, 'seeds', have specific affinity for the milieu of certain organs, 'soil'. However, metastatic dissemination is also highly influenced by mechanistic factors such as venous circulation or lymphatic drainage. In colon cancer, metastatic cells are drained from the primary site directly to the liver, where colon cancer metastasis commonly occurs. Nevertheless, distant metastases of certain cancers are clearly site specific and these tumors types show a prominent capacity to form metastases in the particular secondary site. In the case of breast cancer, tumors show obvious preference to develop bone, lung, liver and brain metastasis (Kennecke et al., 2010). Molecular classification of breast tumors demonstrated that organ tropism of breast tumors can be further distinguished between different molecular subtypes. Thus, bone is the predominant site of metastasis for estrogen receptor expressing groups (luminal A, luminal B and luminal/Her2 groups) and the least common site in basal group. In contrast, high rates of brain metastasis are demonstrated among HER2-enriched, basal-like and triple negative non-basal group. Moreover, basal-like tumors have a higher rate of brain, lung, and distant nodal metastasis, similarly to triple negative non-basal group (Kennecke et al., 2010).

Tumor type	**Principal site of metastasis**
Breast	Bone, lungs, liver and brain
Lung adenocarcinoma	Brain, bones, adrenal gland and liver
Skin melanoma	Lungs, brain, skin and liver
Colorectal	Liver and lungs
Pancreatic	Liver and lungs
Prostate	Bones
Sarcoma	Lungs
Uveal melanoma	Liver

Table 3. Typical sites of metastatic relapse for solid tumors

In the last decades many genes that act as oncogenes or tumor suppressors in early steps of malignant transformation have been identified. These genes provide functions necessary for tumorigenesis, such as autonomous cell proliferation, resistance to cell death, angiogenesis, altered cell adhesion and motility (Hanahan and Weinberg, 2000; Nguyen and Massague, 2007). On the contrary, the knowledge of genetic determinants of metastasis is limited, despite of the fact that the conceptual framework of biological traits and steps of metastasis has been established (Eckhardt et al., 2012; Gupta and Massague, 2006; Nguyen et al., 2009; Nguyen and Massague, 2007). Thus, the identification of genetic determinants of metastasis remains a major issue in cancer field. *In vitro* systems had shown limited application in screenings for metastasis genes since they have been mostly used for analysis of pre-metastatic actions. Conversely, the transcriptional profiling of primary breast tissue provided important information on the biology of metastasis (Perou et al., 2000). Van't Veer et al. have shown that clinical outcome of patients with breast metastasis could be predicted based on the gene expression signature of their tumor (van 't Veer et al., 2002). Moreover, microarray platforms led to the identification of other transcriptional signatures, gene copy number abnormalities, single nucleotide polymorphisms (SNPs), epigenetic changes, microRNA expression levels, proteomic alterations and somatic mutations relevant in breast cancer progression (Ellsworth et al., 2005; Laird, 2003; Lu et al., 2005; Sebat et al., 2004; Sjoblom et al., 2006; Varambally et al., 2005). Next, murine models of metastasis are also emerging as indispensable in screenings for metastatic determinants, as well as in assessing a functional role of new targets and testing new therapeutics in preclinical settings (Eckhardt et al., 2012). Moreover, animal models were widely used to select different tumor clones with certain metastatic phenotypes (Bos et al., 2009; Fidler, 1973; Fidler and Kripke, 1977; Kang et al., 2003; Minn et al., 2005a; Minn et al., 2005b; Nicolson, 1988; Poste, 1982). Therefore, most efforts to identify metastasis genes currently rely on use of *in vivo* systems and human tissue based approach. (Fidler, 1990; Nguyen and Massague, 2007).

There are various animal models of metastasis and each model carries its own advantages and disadvantages. The choice of model used depends on the question being addressed. Direct inoculation of tumor cells into circulation is commonly employed for simulation of metastatic seeding to the secondary site. In such model, tough, the gradual dissemination of tumor cells from the growing primary site and establishment of pre-metastatic niche are absent. However, having in mind that efficient metastatic seeding is provided, this experimental model of metastasis is advantageous in case of cells populations that that cannot form metastasis spontaneously from the orthotropic to secondary site (Eckhardt et al., 2012). Moreover, Ding et al have shown that human xenografts retained all primary tumor mutation and moreover display a mutation enrichment patterns that resembled the metastasis from the same patient (Ding et al., 2010).

The mouse model of breast cancer carries limitations since the transgenic animals that spontaneously generate bone metastasis have not been described to date. Therefore, we relayed on human cancer cells lines that have been extensively used to model cancer (Burdall et al., 2003; Vargo-Gogola and Rosen, 2007). Transcriptional profiling of breast cancer cell lines showed that they can reflect molecular profiles present in patients (Burdall et al., 2003; Holliday and Speirs, 2011; Poste, 1982). Such notion is important when having in mind that few mutations acquired in mice models of tumorigenesis do not truly reflect multiple mutations and general genomic instability present in human cancers. Furthermore, cancer cell lines were also widely used for profiling of gene amplification and fragile sites in cancers (Bignell et al., 2010). These cell populations are derived from cancer patients that have undergone the treatment and therefore are suitable for modeling metastasis that occurs in patients despite the treatment. Cancer cell lines carry drawbacks such as already being aggressive tumor cells susceptible to genetic alteration *in vitro* and without a human stroma component. Nevertheless, they reflect certain features of human tumors and are a useful tool for studying human cancers.

In order to screen for genetic determinants of breast cancers metastasis to the bone, we took an advantage of an experimental model of metastasis and human breast cancer cell lines. Since ER positive tumors show clear preference to relapse to the bone, when compared to ER negative tumors, we performed our screening using MCF7 ER+ human breast cancer cells. MCF7 cells were previously described to have mild bone metastatic capacity when injected intracardiscly into immunodeficient mice (Lu et al., 2011; Yin et al., 2003). Our data have confirmed that MCF7 (parental) cells are able to colonize hind limbs, after being inoculated via left ventricle into Balb/c nude mice. Moreover, these cells were able to develop adrenal, soft tissue, lung and in some cases brain metastasis. The detected bone metastatic incidence (60%) in our experimental setting was higher than in previously described studies (Yin et al., 2003). Possibly, because of differences in number of cells injected, differences in estrogen supply method and differences in genomic profile of those cells during culturing conditions *in vitro.* Indeed, these cells retained estrogen dependency to grow and similar phenotype was observed with two independent ATCC purchased cell populations. Nevertheless, bone metastatic derivative (BoM2) caused significantly higher incidence of bone metastasis (90%), comparing to parental cells. Metastatic incidence to non-bone sites in BoM2 cells was in the same range as for parental cells. Additionally, bone metastatic derivative and parental cells showed similar growth rates when implanted into primary site (mammary fat pads of mice) or subcutaneously. Therefore, we could conclude that we selected for a cellular population that was more competent to metastasize to the bone of immunodefficent mice, but otherwise retained similar growth capacities as parental cells. Isolation of specific metastatic cell populations was in agreement with previous reports of metastatic speciation trough rounds of *in vivo* selection (Kang et al., 2003; Lu et al., 2011).

CGH and transcriptional array analysis show differences between BoM2 and parental cell population both at the level of gene alterations (genomics gains and losses) and gene expression. However, epigenetic analyses of these cell lines did not reveal significant differences (data now shown). Almost 200 genes

were differentially expressed, more than 2 fold, between BoM2 and parental cells. Moreover, in principal component analysis (PCA), these two cell populations were clearly separated on both first and second component axis. Such results supported that the bone-tropic population encompasses particular phenotypic associated genetic profiles compared to parental cells. Bone metastatic derivative specific genes are involved in proliferation, migration, tumor stroma interaction, possibly underlining particular biological processes that occur during bone metastatic speciation.

Only a small subset of BoM2 differentially expressed genes were significantly associated with bone metastasis in primary tumors (MSKC/EMC) according to their expression levels in these samples. Such result is concordant with the percentage of genes that were significantly correlating with organ-specific relapse in primary tumor cohorts in previously reported screenings for metastatic genes (Kang et al., 2003; Minn et al., 2005a). As already emphasized, using an experimental model can carry certain drawbacks. With direct injection of tumor cells into circulation we were able to model seeding at secondary site but not the whole metastatic cascade. Therefore, such screening would identify mostly metastatic virulence and progression genes that mediate late steps of metastasis progression (Nguyen and Massague, 2007). Genes expressed in primary tumors are mainly metastatic initiation and progression genes that couple function important for both primary tumor progression and metastasis (Minn et al., 2005a; Nguyen et al., 2009). Next, our system was restricted to the MCF7 cell line that only partially reflects the heterogeneity of breast cancer tumors. Therefore, some of the genes selected in this cancer cell line may be present only in small subset of primary tumors and do not reach significance in the whole cohort. These limitations of the experimental system could explain that small percentage of BoM2 differentially expressed genes correlated with bone metastasis in primary breast tumor expression cohorts.

4.2 Breast tumors harbor 16q22-24 copy number gain

The results of CGH analysis revealed significant genomic alterations in bone metastatic derivatives. Namely, we were able to detect the gain of 16q region amplification and significant losses in chromosomes 6, 12, 19, 20, and 21. Thus, bone metastatic speciation was coupled with acquisition of genomic alterations in highly bone metastatic derivative.

Such result was in agreement with the fact that the scrutiny of breast cancers identified a number of recurrent copy number alterations displayed in breast tumors (Chin et al., 2006; Neve et al., 2006). Examples of tumor associated genes affected by copy number alteration include *HER2*, *EGFR*, *MYC*, *CCND1*, *MDM2*, *AIB1*, *FGFR1*, *S6K*, *TOPO2A*, *EMS1*, *FGF3*, *AKT2*, and *PIP4K2* genes. Importance of copy number gain/lost in breast cancer is clearly demonstrated by tyrosine kinase HER2 gene amplification. HER2 amplification leads to overexpression of HER2 tyrosine kinase in 15-20% of breast tumors, and defines a particular group of breast cancer patients who receive biological treatment with trastuzumab (Al-Kuraya et al., 2004).

We have found that the genomic gain of the 16q22-24 region, observed in bone metastatic derivative, was clinically relevant and associated with metastasis in primary breast tumors expression data sets. Analysis of breast cancer expression cohorts by means of ACE algorithm was previously used to identify clinically important genomic alterations, with example of 8q22 genomic gain in breast cancer (Hu et al., 2009). 6,12,19, 20 and 21 chromosome losses were discarded for further inquiry due to clinical irrelevance in patient samples.

Interestingly, chromosome 16q was described as a site where structural abnormalities frequently occur in breast cancer (Dutrillaux et al., 1990; Pandis et al., 1992). Particularly, 16q was shown to participate in non-random translocation with chromosome 1 and to be frequently deleted in primary breast cancer (Chen et al., 1996; Devilee and Cornelisse, 1994). A high frequency of LOH on 16q (25-50%) has been reported by several groups (Curtis et al., 2012;

Larsson et al., 1990; Natrajan et al., 2009; Sato et al., 1990). Moreover, allelic loss of 16q23.2-24.2 was significantly correlated with metastasis free survival in primary breast cancer patients. LOH is a frequent event in small tumors, without lymph node metastasis, and with noninvasive histological phenotype (Iida et al., 1997; Tsuda et al., 1994). This data supports the observation that allelic loss of 16q is an early event in tumor development/progression. Therefore, the fact that tumors that harbor 16q loss actually display prolonged survival and freedom from metastasis could be explained by the existence of tumor suppressor/s genes in this region and metastasis-inducing gene in its vicinity. Tumors suppressor/s would be inactivated by allelic loss in early stage of cancer. However, the nearby presence of metastasis-inducing gene or, alternatively, a house keeping gene that in low dose could reduce metastatic spread would further explain better clinical aspect of tumors that harbor 16q23-24 loss (Hansen et al., 1998). In agreement with frequent chromosomal abnormalities on 16q, is the fact that 16q23.2 represents the second most active common fragile site, FRA16D (Mangelsdorf et al., 2000). Fragile sites appear as breaks, gaps or decondensation on metaphase chromosomes. Evidence suggests that common fragile sites are preferred sites of sister chromatid exchange, chromosomal rearrangements, deletion and amplification (Sutherland et al., 1998). Therefore, the fragile sites are potentially highly affected in cancers (Sutherland et al., 1998). In multiple myeloma, 25% of tumors contain a balanced reciprocal translocation between 14q32 and 16q 23.2. This rearrangement juxtaposes the IgH switch region and its very powerful enhancer element to genes in the 16q23.2 region (Krummel et al., 2000).

Interestingly, 16q22-24 genomic gain was detected in both MCF7 and BoM2 cells but with different frequencies, as shown by FISH analysis. Such result points out towards clonal evolution of metastasis. While only around 30 % of parental cells harbor 16q22-24 genomic gain, the majority of BoM2 cells (more than 80 %) encompass this genomic alteration. Enrichment of a cell population carrying 16q22-24 genetic gain demonstrates the selection of advantageous clones during tumorigenesis.

Importantly, the 16q22-24 genetic gain was confirmed in an independent cohort of paraffin embedded primary breast cancer samples (Spanish data set) by means of FISH analysis (using 16q23 and centromeric 16q11.2 (CEP16) probes). Our scrutiny has shown that around 16.2% of primary breast tumors encompass 16q22-24 genomic gain. Tumors were defined as 16q23 chromosome copy number gain positive when an average of at least 1.5 copies of the 16q23 normalized to the CEP16 was present in tumors. Interestingly, when looked at association of 16q22-24 gain and metastasis to particular secondary site, 16q22-24 copy number gain specifically predicted relapse to the bone, but not other (soft tissue or visceral) site (Spanish data set). Such result supported our hypothesis that genetic changes present in bone metastatic derivative should associate with bone metastatic recurrence in patient data.

The discovery of 16q22-24 copy number gain was initially made in ER positive breast cancer cell line. Concordantly, 16q22-24 predicted bone relapse in ER positive patents (Spanish data set). However, this observation was extended to ER negative and HER2 positive patients. Consistently, 16q22-24 genetic gain is an independent prognostic marker for bone metastasis regardless of the hormonal status of tumors. Of note,16q22-24 copy number gain is characterized by low copy number gain, less than 8. Previous reports have shown that selection of low copy number changes can occur during cancer formation because they increase basal metabolism and provide survival/proliferative advantages to cell carrying them (Chin et al., 2006). Interestingly, 16q22-24 copy number gain is associated with high Ki67 proliferative index in primary tumors. Moreover, the *MAF* gene located within this region is involved in cell cycle progression. Therefore, 16q22-24 genomic gain could partially contribute to proliferative capacity of primary tumors (see discussion chapter 4).

Hence, although complete or partial 16q deletion of 16q chromosome is frequently displayed in luminal breast tumors, herein we describe an independent genomic alteration affecting 16q22-24 region and present in all breast cancer subtypes. We report a novel copy number gain in breast cancer

that occurs in roughly 15% of tumors, depending of the cohort being analyzed, and predicts bone metastatic relapse.

4.3 *MAF* gene, located within 16q22-24 genomic region, predicts bone metastasis

Among the different genes located within 16q22-24 region we identified transcriptional factor *MAF* to specifically predict bone metastasis in breast cancer. *MAF* was the only gene within the 16q22-24 region that reached the criteria of being differentially expressed between bone metastatic derivative and parental cells and whose expression significantly correlated with bone metastasis in patient samples. In addition, 16q23 FISH probe that we used to analyze the 16q22-24copy number gain encompassed *MAF* gene together with *VAT1L*, *CLEC3A*, *WWOX* and *5srRNA* genes. When using the FISH probe that narrowed down *MAF* genetic region to scrutinize the Spanish data set we obtained results that were consistent with initial FISH analysis of a broader 16q22-24 region. When MAF copy number gain was analyzed in yet an independent breast cancer cohort, we could confirm that 6% of tumors harbor *MAF* gains (TCGA data set) (2012). Furthermore, Moreover, *MAF* was already described to be an oncogene that in multiple myeloma is being over expressed due to translocation and contributes to the aggressive bone metastatic phenotype of these tumors. Therefore, we hypothesized that *MAF* gene within the 16q22-24 locus drives bone metastasis in tumors that harbor 16q22-24 copy number gain.

Among other genes flanked by 16q22-24 FISH gene probe *WWOX* was described to be related with tumor progression. However, *WWOX* is considered a potential tumor suppressor in breast cancer (Lewandowska et al., 2009; Ramos and Aldaz, 2006). Namely, expression of the *WWOX* gene was previously analyzed in breast cohorts and it has been associated with lower grade, ER positive and better prognosis tumors (Nunez et al., 2005). However, *WWOX* gene was not proven to be the dominant tumor suppressor since

deleting *WWOX* in mouse mammary glands did not cause tumor formation (Ferguson et al., 2012). We and others speculate that *WWOX* is a tumor suppressor and its expression is lost during the course of progression of some breast cancers, such as tumors with 16q LOH. Thus, in our model context, *WWOX* tumor suppressor deletion would be selected during the progression of certain tumorigenic clones while *MAF* copy number gain would occur in others. The presence of a common fragile site, FRAD16, where these genes are located, allows for different chromosomal rearrangements in this region. Depending on other genomic events, both *WWOX* deletion and *MAF* copy number gain can provide a selective advantage. In case of tumors with 16q22-24 copy gain, it seems probable that metastatic phenotype driven by MAF transcriptional factor overcomes any effect of such a mild tumor suppressor gene as *WWOX*. Based on literature and our data, the other genes comprised within 16q22-24 FISH probe were not expected to be involved in tumorogenesis or metastasis. However, we cannot exclude that some of the genes from this locus can have a contribution to bone metastatic phenotype in addition to *MAF*.

4.4 *MAF* as breast cancer bone metastatic gene

Our data has shown that *MAF* gene expression is elevated in bone metastatic derivative that harbors 16q22-24 genomic gain. Importantly, high levels of *MAF* mRNA expression levels in primary tumors were significantly associated with bone metastasis. *MAF* expression association with bone metastasis was retained in metastatic breast samples comparing to metastasis elsewhere. An interesting observation is that the linear ratio between *MAF* log expression and bone metastasis hazard ratio is observed only when a given level of *MAF* expression is achieved. This observation is concordant with the fact that *MAF* is located within 16q22-24 amplification and that *MAF* oncogenic transformation in mouse model of angioimmunoblastic T-cell lymphomas (AITL) and multiple myeloma (MM) requires multiple copies of *MAF* (Morito et al., 2006; Murakami et al., 2007). Moreover, in multiple myeloma *MAF* is highly expressed due to the 14q to chromosome 16q translocation. In our functional studies, *MAF* induced a

bone metastatic phenotype in MCF7 and T47D breast cancer cell lines, thus increasing bone metastasis incidence upon ectopic expression in these cells. In addition, downregulaltion of *MAF* in bone metastatic derivatives substantially reduced bone metastasis in our experimental mouse model.

We confirmed by immunohistochemical staining that MAF protein levels predict bone metastasis in the tissue microarray of breast cancer samples (Spanish data set). Importantly, MAF protein levels significantly correlated with 16q22-24 amplification in this data set. Therefore, we concluded that 16q22-24 amplification drives MAF overexpression fit breast tumor cells for bone metastatic colonization.

Our results demonstrate that *MAF* acts as a bone metastatic mediator whose expression is already selected in primary tumors. Hence, *MAF* could be classified as a metastasis progression gene (Nguyen and Massague, 2007). Importantly, the metastasis promoting role of *MAF* is confined specifically to the bone, since *MAF* expression did not correlate to metastasis event elsewhere (lung, brain, liver).

MAF is a member of AP-1 family of transcriptional factors. Thus, it was plausible to speculate that its role in bone metastasis is mediated through the transcriptional control of different downstream targets. MAF gene targets are context dependent and differ from one cellular system to another (Eychene et al., 2008). We defined the *MAF* transcriptional program upon ectopic expression of *MAF* in the MCF7 cell line. Interestingly, *MAF* upregulated genes were significantly associated with bone relapse in the MSKC/EMC expression data set. This observation suggested that *MAF* oncogenic function relays on its ability to regulate metastasis promoting genes, rather than to repress metastasis suppressors.

In multiple myeloma *MAF* was previously described to promote bone metastasis trough cell proliferation, invasion and adhesion to bone marrow cells (Hurt et al., 2004). This effect was accomplished through transcriptional targets such as cyclin D2, as mediator of proliferation, integrin beta 7 that promotes adhesion to

bone marrow cells and *ARK5* gene, involved in migration and invasion of myeloma cells. In breast cancer cells we identified the *MAF* transcriptional program that included genes important for cancer cell proliferation/survival (*CTGF*, *FGF18*, *EFEMP1*, *IGF1*, *GAS1*, *S1PR1*, *CCND2, CAV1*, *RERG*), cell migration and adhesion (*NAV3*, *CTGF*, *WIPF1*, *PODXL*, *S1PR1*, *CHL1*), cell signaling (*GEM*, *IRAK3*, *DOK5*, *LMCD1*, *S1PR1*), cell differentiation (*MAFB*, *FAT1*, *DAB2*) and tumor-stroma interaction (*CTGF*, *PTHLH*).

Similarly to myeloma cells, cyclin D2 expression is also regulated by *MAF* in breast cancer cells. Moreover, *MAF* regulates other genes important for proliferation such as caveolin1 (*CAV1*), as well as expression of breast specific gene *RERG*. Data from our lab has previously shown the metastasis suppressor role of *RERG* in breast cancer, where *RERG* expression impaired the growth of bone metastatic derivative cells in experimental metastasis model (Arnal-Estape, 2011). By controlling these proliferation genes *MAF* may promote tumor growth, a phenotype that we observed in our experimental model system. We speculate that *MAF* contribution to proliferation in context of already highly proliferating cells is probably limited. This could not exclude that the proliferative advantage is a reason for *MAF* selection in early breast cancers. However, our data from orthotropic tumor growth assay where already metastatic cells were used (mammary fat pad injections) could not address this question.

MAF expression was associated with osteolytic bone metastasis in our experimental model system. Such phenotype is caused by enhanced osteoclast activity, as shown by TRAP staining. Activation of osteoclasts is commonly occurring during the progression of bone metastasis in breast cancer (Kozlow and Guise, 2005). Exploration of well know molecules involved in osteolytic bone metastasis such as *PTHLH*, *RANK*, *RANKL*, *IL11* or *IL6* show that only *PTHLH* expression levels were changed upon *MAF* overexpression or downregulation in breast cancer cell lines we tested. Parathyroid hormone-related protein (PTHLH) was purified as a factor responsible for the hypercalcemia associated with malignancy (Strewler, 2000). However, PTHLH

also participates in malignancy as a mediator of the bone destruction associated with osteolytic metastasis. In addition, high percentage of breast carcinoma bone metastasis expresses *PTHLH*, which is not common for breast cancer metastasis to non-bone sites (Kozlow and Guise, 2005). Moreover, PTHLH was proven to be important for bone metastatic formation in MDA 231 breast cancer cell line (Zheng et al., 2013). Our data demonstrate that *PTHLH* is being transcriptionally regulated by direct binding of MAF to its promoter region. Such interaction promotes osteoclast differentiation both *in vitro* and *in vivo*. Consequently, *MAF* expressing bone lesions were more osteolytic compared to control ones, a phenotype which was reversed by anti-PTHLH treatment. Importantly, analysis of metastasis samples show that 77% of breast cancer metastases that expressed *MAF* and *PTHLH* above the average were in bone (Zhang et al., 2009). Of note, blocking of PTHLH action did not impair the high competence of MCF7 MAF expressing cells to initiate bone metastasis. This observation suggests the involvement of other *MAF* gene targets in initiation of *MAF* mediated bone metastasis.

Consistently, the role of *MAF* in bone metastasis initiation was proven in MCF7, T47D and bone metastatic derivative breast cancer cell line, since *MAF* expression had significant impact on bone metastatic incidence in these cell lines. Preliminary data from our lab indicates that *MAF* does not increase self-initiating capacity of breast cancer cells. Namely, *MAF* overexpression in breast cancer cells did not contribute to increased oncosphere formation in an *in vitro* assay (data not shown). Interestingly, parental and bone metastatic derivative cells showed *MAF* dependent capacity to adhere to bone marrow stromal cells *in vitro*. We speculate that this phenotype can contribute to the metastasis initiation without a direct effect on the process of self-renwal, since *MAF* could affect the ability of breast cancer cells to home to the bone. In addition, we envision other mechanisms to be involved in bone metastatic initiation. For instance, it is shown that survival of breast cancer cells in initial steps of micrometastasis formation is a key event in the initiation of bone lesions (Zhang et al., 2009). Therefore, we hypothesize that *MAF* could potentially confer

survival/proliferation capabilities of tumor cells at these initial steps. Different models of experimental metastasis can be used to address these questions. The role of *MAF* in homing of breast cancer cells to the bone could be assess by an intracardiac assay of experimental metastasis. In this setting the number of cells lodged into the bone could be quantified before the appearance of a bone lesion with detectable bioluminescence signal. Alternatively, the step of bone homing is bypassed and tumor cells are placed directly into bone environment, by mean of intratibial injection. In such scenario, the role of *MAF* oncogene in survival/proliferation of tumor cells at initial steps of micrometastasis formation could be investigated.

We further speculate that it would be of potential interest to decipher transcription factors that interact with *MAF* in breast cancer cells. Disclosure of these proteins could explain how the oncogenic effect of MAF is being more or less pronounced in the different cellular contexts. Additionally, posttranslational modifications are important for MAF transcriptional activity (Eychene et al., 2008). Identification of these modifications in breast cancer could further clarify *MAF* oncogenic activity in breast cancer context.

4.5 Identification of NOG as a specific breast cancer bone metastasis supporting gene

Besides metastatic progression genes that are expressed in primary tumors our screening led to identification of metastatic virulence genes whose expression was selected only in bone metastatic site (Nguyen and Massague, 2007). To this end, we focused on the soluble factor *NOG* that was upregulated in bone metastatic breast cancer samples, but did not associate with bone relapse in primary tumors. In the experimental model of metastasis, *NOG* expression had a significant impact on bone colonization in different breast cancer cell lines. Our data has shown that NOG contributes to successful bone colonization both in early and late time points, both during establishment and growth of the lesion. Therefore, we proved that *NOG* supports different steps in the process of bone metastasis. By increasing the self-renewing capacity of tumor cells, *NOG*

contributes to initiation of bone lesions. Thus, *NOG* has a cell autonomous effect on tumor cells. This observation was in agreement with the fact that *NOG* is an antagonist of the BMP pathway, a pathway that has been shown to induce the differentiation of cells (Gazzerro et al., 1998; Smith and Harland, 1992). Moreover, *NOG* has been previously related with the increase of the colon stem epithelial compartment (Hardwick et al., 2004). The hypothesis of tumor stem cells states that heterogeneity in cancer is reflecting by existence of pool of cell with higher tumor initiating capacity. Therefore, such cells would be much efficient in initiating metastatic lesions. The basic markers for stemness in breast are CD44 and CD24 (Clarke and Fuller, 2006). Also, an *in vitro* assay of oncosphere formation is used to assess stem cell capacity of breast tumors cells. As shown, *NOG* increased the capacity of oncosphere formation and number of $CD44^{high}/CD24^{low}$ cells. The effect of *NOG* was mediated through elevating levels of ID2 and consequently RANKL that was previously reported to be a main factor maintaining the pool of mammary epithelial stem cells (Asselin-Labat et al., 2010). Moreover, *NOG* expressing cells had a growth advantage in the bone environment by stimulating osteoclast differentiation and osteolysis of bone lesions. Osteolytic phenotype of bone lesion is one of the most devastating features of aggressive bone metastasis. This provokes bone loss followed with pain and causes skeletal complications. Under these circumstances *NOG* blocked BMP signaling, important for osteoblast differentiation, switching the balance between osteoclasts and osteoblasts toward osteoclast differentiation. There is emerging evidence that the increase in osteoclast number is associated with the impairment in osteoblasts number and activity in prostate cancer bone metastasis (Secondini et al., 2011). Moreover, previous studies have shown that conditioned media of MDA-231 breast cancer cells induce osteoblast apoptosis *in vitro,* which is in agreement with the osteolytic feature of these cell line and its derivate 1833 (Mastro et al., 2004; Mercer et al., 2004).

4.6 Possible clinical implications of 16q22-24 copy number gain and MAF protein expression in breast cancer

Despite the advances in the standard care of breast cancer patients, 20%-30% of patients with early breast cancers will relapse at distant metastatic site (Kennecke et al., 2010). Among different metastatic sites, bone is the most common place of relapse in breast cancer, accounting for 30%-50% of distant metastasis. Thus, approximately 10%-20 % of breast cancer patients will develop bone metastasis, in 15 years follow up time (Coleman and Rubens, 1987; Elder et al., 2006; Solomayer et al., 2000).

Herein, we identified 16q22-24 copy number gain and showed that subsequent *MAF* overexpression, occur in 6%-15% of breast cancer patients, and specifically predict relapse to the bone in breast cancer primary tumors. This is in contrast with *NOG* gene which expression has a predictive value only in metastatic sample and not in primary tumors; therefore *NOG* cannot be used as a biomarker. The percentage of 16q22-24 and *MAF* positive tumors depends on the cohort analyzed and technique used for their identification. Nevertheless, among the breast cancers that will relapse to the bone, 16q22-24 and MAF positive tumors are included. Both 16q22-24 copy number gain and MAF expression levels are shown to be independent markers of bone relapse with 97% negative predictive value (NPV) for cumulative bone metastasis (very low risk of false negative readouts). To date, no other clinical or pathological parameter is able to predict bone metastasis.

Existence of a bone metastasis marker that can predict bone disease and stratify patients according to this has direct clinical implications. Current standard of care of breast cancer patients implies surgical removal of primary tumor and subsequent systemic treatment with cytotoxic chemotherapy and/or biological therapy for ER receptor and HER2 positive tumors (Senkus et al., 2013). However, at time of diagnosis of primary tumors the patients that will relapse to the bone are not identified since clinically standardized bone metastasis markers are not available. Accordingly, tailored systematic treatment

that can target development of bone metastasis is not approved to date. Importantly, drugs that can possibly be used in the adjuvant treatment of bone metastatic recurrence are available. Namely, bone modifying agents like bisphosphonates (zoledronic acid) and RANKL neutralizing antibody (denosumab), are used for treatment of skeletal related events (SRE) associated with breast cancer bone metastasis in order to reduce hypercalcemia and cancer treatment induced bone loss (CTIBL) (Fizazi et al., 2009; Guise et al., 2010). Interestingly, preclinical data showed that these agents also have anti-tumorigenic potential (Coleman et al., 2010; Guise et al., 2010; Steger and Bartsch, 2011). In addition, several trials have shown that zoledronic acid leads to a reduction in the number of disseminated tumor cells in bone marrow aspirates (Coleman et al., 2010). However, despite the potential of bone modifying agents in treating and/or preventing bone metastasis, the outcome of some already finished clinical trials that scrutinized bisphosphonates in an adjuvant setting for reducing disease free survival is inconclusive (Coleman, 2012; Gnant et al., 2011). Namely, when randomized trials were conducted zoledronic acid did not show significant influence on disease free or overall survival. However, in prespecified subgroup analysis, zoledronic acid had an effect on disease free survival in women with low reproductive hormone levels. Therefore, in order to assess the effect of bone modifying agents in adjuvant treatment a careful design of clinical studies would be required. A bone metastatic marker would stratify those patients that are at high risk of bone relapse. Combining this knowledge with what is shown from previous clinical trials, bone modifying agents could be assessed in the adjuvant treatment of a more specified group of breast cancer patients where their effect on disease free and overall survival would be magnified. Thus, 16q22-24 copy number gain and MAF expression can be directly used for such purpose. Notably, FISH technique used for the identification of 16q22-24 gain and immunohistochemistry technique used for identification of MAF overexpression are gold standard techniques used in clinical practice. In case of favorable results, this bone metastasis marker could influence routine clinical practice.

Importantly, our data show that *MAF* performs its bone metastatic promoting role in part through tumor-stoma interaction and osteoclast activation. Since bone modifying targets are specifically targeting osteoclasts in the bone environment, we speculate that 16q22-24 and MAF positive breast cancer patients can indeed benefit from bone modifying agents. Our data further demonstrate that anti-PTHLH treatment reduces bone metastatic burden of *MAF* overexpressing cancer cells in experimental model of metastasis. Thus, we hypothesize that PTHLH antagonist treatment could be also beneficial for 16q22-24 and MAF positive breast cancer patients as well.

In the era of personalized medicine, stratification of patients at high risk of bone metastasis using a standardized biomarker could be a valuable tool in designing tailored patient therapies. Additional investigation of mechanisms underlining *MAF* mediated bone metastasis, could be further applied in the treatment of these patients. Moreover, we envision *MAF* itself to be a potential therapeutical target (Curtis et al., 2012).

CHAPTER 5. CONCLUSIONS

CONCLUSIONS

1. We have isolated a highly bone metastatic cell population of human ER-positive MCF7 breast cancer cells, named BoM2, through rounds of selection in experimental mouse model of metastasis.
2. We have identified the 16q22-24 genomic copy number gain in bone metastatic derivative comparing to parental MCF7 cells.
3. 16q22-24 copy number gain specifically predicts bone metastasis in primary breast tumors.
4. 16q22-24 copy number gain drives *MAF* gene overexpression, which in turn is also significantly associated with bone recurrence in breast cancer patient samples.
5. *MAF* genes acts as bone metastatic mediator in experimental model of metastasis, since its overexpression supports bone colonization and its downregualtion impairs it.
6. *MAF* contributes to bone metastasis, in part, though its transcriptional target *PTHrP*, which promotes osteoclast differentiation in the bone stoma and causes tumor induced osteolysis.
7. We have identified *NOG* as a gene that plays a pivotal role in bone metastasis of breast cancer.

CHAPTER 6. BIBLIOGRAPHY

BIBLIOGRAPHY

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