



Involvement of microRNA-30 family in metastatic dissemination of breast cancer cells to bone

Agnieszka Frackowiak

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Involvement of microRNA-30 family in metastatic dissemination of breast cancer cells to bone

INSERM UMR1033

Pathophysiology, Diagnosis and Treatment of Bone Diseases

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Abstract

Bone metastasis is a common complication of advanced breast cancers and is clinically responsible of bone fractures, hypercalcemia and pain for which only palliative therapies are proposed. Breast tumor cells that preferentially invade bone express a set of deregulated genes that enhance bone tropism and facilitate bone marrow engraftment which may lead to the formation of overt osteolytic lesions. Molecular pathways underlining these steps are regulated through the tight control of genes expressed by cancer cells interacting with cells from the bone microenvironment. In this context, microRNAs act as regulators of gene expression and control multiple aspects of bone metastasis, including tumor cell escape from the primary site, dissemination, invasion of the bone marrow and secondary outgrowth.

MicroRNA transcriptomic profiling of osteotropic breast cancer cell lines identified drastic down-regulation of the miR-30 family (miRs-30). In the clinic, low expression of miRs-30 in breast primary tumors is associated with poor distant metastasis-free survival and hormone-insensitive status. In a model of human bone metastasis *in vivo*, the forced expression of miRs-30 in a breast cancer cell line that is highly and specifically metastatic to bone inhibited bone metastasis. We demonstrated that miRs-30 inhibit tumor cell invasiveness and stimulate osteoblastogenesis, *in vitro*, and reduces tumor burden and osteoclast activity, *in vivo*. Consistent with that, the expression of several genes that promote bone metastasis were inhibited by miRs-30. Among these, expression of connective tissue growth factor (CTGF) was up-regulated in human bone metastasis.

The early steps of bone metastasis were studied in a mouse model using spontaneously metastatic mouse breast cancer cell lines inoculated in the mammary gland. In this model, miRs-30 did not alter tumor growth or metastatic dissemination to bone. However, miRs-30 inhibited cell invasiveness and cancer stem cell-like phenotype of these metastatic cells.

We conclude that miRs-30, by interfering negatively with bone metastasis, represent a potential therapy to repress gene targets that promote bone metastasis.

Résumé

Les métastases osseuses sont des complications fréquentes du cancer du sein, responsables sur le plan clinique d'hypercalcémie, fractures osseuses et douleurs, pour lesquelles, il n'existe que des traitements palliatifs. Les cellules tumorales de carcinomes mammaires qui métastasent au site osseux expriment des gènes qui favorisent le tropisme osseux de ces cellules ainsi que leur ancrage et développement dans la moëlle osseuse. Les mécanismes moléculaires sous-jacents à ces processus sont contrôlés par l'expression génique des cellules tumorales qui interagissent avec le microenvironnement et les cellules osseuses. Dans ce contexte, les microARNs en tant que régulateur endogène de l'expression génique, interfèrent avec les différentes étapes de la formation des métastases osseuses, incluant l'échappement des cellules tumorales de la tumeur primaire, la dissémination et l'invasion du site osseux, ainsi que l'apparition de lésions ostéolytiques.

Les profils transcriptomiques des microARNs de cellules tumorales mammaires à caractère ostéotrope montrent que l'expression de la famille de microARNs-30s (miRs-30) est inhibée dans ces cellules. En clinique, la faible expression des miRs-30 est associée à un mauvais diagnostic de rechute et au statut hormono-résistant. Dans un modèle animal de métastases osseuses, l'expression forcée des miRs-30 dans des cellules tumorales qui métastasent fortement et spécifiquement à l'os, inhibe la formation des métastases osseuses. Nous montrons que les miRs-30 inhibent l'invasion et stimulent l'ostéoblastogenèse, *in vitro* et réduisent la charge tumorale et l'ostéoclastogenèse, *in vivo*. En accord avec ces résultats, l'expression de gènes qui stimulent les métastases osseuses est inhibée par les miRs-30. Parmi ces gènes, l'expression du CTGF (connective tissue growth factor) est augmentée dans les métastases osseuses humaines. Les étapes précoces des métastases osseuses sont étudiées par inoculation de cellules tumorales métastatiques murines dans la glande mammaire de souris. Dans ce modèle, les miRs-30 n'altèrent pas la croissance tumorale et la dissémination métastatique à l'os. Cependant les miRs-30 inhibent l'invasion et le caractère de cellules souches tumorales de ces cellules métastatiques.

Ces résultats suggèrent que les miRs-30, en régulant négativement les métastases osseuses, représentent une thérapie potentielle pour réprimer des gènes cibles qui stimulent les métastases osseuses.

Abbreviations

AGO2	Argonaute 2
ALP	Alkaline Phosphatase
Bcl2l11	BCL2-like 11 (apoptosis facilitator)
BCL9	B-cell CLL/lymphoma 9
bFGF	Basic Fibroblastic Growth Factor
BGLAP	Bone Gamma-carboxyglutamate Protein
BLI	Bioluminescence
BM	Bone Marrow
BMPs	Bone Morphogenetic Proteins
BMU	Basic Multicellular Units
BRC	Bone Remodeling Compartment
BRCA1	Breast Cancer 1, Early Onset
BSP	Bone Sialoprotein
BT-ICs	Breast Tumor Initiating Cells
CAMs	Cell Adhesion Molecules
CCD	Cleidocranial Dysplasia
CDH11	Cadherin 11
c-myc	Myelocytomatosis Oncogene
COL1A1	Type I Collagen
COX-2	Cytochrome c Oxidase Subunit II
Cre	Cre Recombinase Protein
CSC	Cancer Stem Cell
c-SRC	SRC proto-oncogene, Non-receptor Tyrosine Kinase
CT-1	Cardiotrophin-1
CTC	Circulating Tumor Cell
CTGF	Connective Tissue Growth Factor
CTHRC1	Collagen Triple Helix Repeat Containing 1
CXCL-12	Chemokine Ligand 12
CXCR4	Chemokine Receptor 4
DKK1	Dickkopf-related Protein 1

DLX5	Distal-less Homeobox 5
ECM	Extracellular matrix molecule
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-mesenchymal Transition
EphB4	EPH Receptor B4
ER	Oestrogen Receptor
ErbB-2	v-erb-b2 Erythroblastic Leukemia Viral Oncogene Homolog 2, Neuro/glioblastoma Derived Oncogene Homolog
FRZ	Frizzled
GEM	Genetically Engineered Mouse
GFP	Green Fluorescent Protein
GSK3 β	Glycogen Synthase Kinase 3 beta
HEF1 (NEDD9)	Human Enhancer of Filamentation
HER2	Human Epidermal Growth Factor Receptor 2
<i>HOXD10</i>	Homeobox D10
H-Ras	Harvey Rat Sarcoma Virus Oncogene
HSC	Hematopoietic Stem Cell
i.c.	Intracardiac
i.t.	Intra-tibial
i.v.	Intravenous
i.a	Intra-arterial
IGF-1	Insulin-like Growth Factor 1
IL-11	Interleukin 11
IL-6	Interleukin-6
IL-8	Interleukin 8
Itga5	Integrin Alpha 5
ITGAVB3	Integrin Alpha V Beta 3
Itgb3	Integrin Beta 3
Ki-67	Antigen Ki-67
Klf10	Krupper-like Factor 10
KO	Knock-out
Lox	Lysyl Oxidase
LRP5/6	Low Density Lipoprotein Receptor-related Protein

Luc	Luciferase
M-CSF	Macrophage Colony Stimulating Factor
miRs	microRNAs
MMO	Mammosphere
MMP	Matrix Metalloproteinase
mRNAs	messenger RNAs
MSC	Mesenchymal Stem Cell
mTOR	Mechanistic Target of Rapamycin
NF- κ B	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells
NFYC	Nuclear Transcription Factor Y gamma
NOV	Nephroblastoma Overexpressed
OB	Osteoblast
OC	Osteoclast
OCN	Osteocalcin
OCP	Osteoclast precursors
Oct4 (Pou5f1)	POU domain, class 5, Transcription Factor 1
OPG	Osteoprotegrin
OSM	Oncostatin M
OSX	Osterix
P53 (TP53)	Tumor Protein p53
PBS	Phosphate-Buffered Saline
PDGF	Platelet-derived Growth Factor
PR	Progesterone receptor
PSA	Prostate-specific Antigen
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone-related Protein
PyMT	Polyoma Middle T Antigen
RANKL (TNFSF11)	Rank Ligand, tumor necrosis factor (ligand) superfamily member 11
RISC	RNA-induced Silencing Complex
ROCK	Rho-associated Kinase
RUNX2	Runt-related Transcription Factor 2
s.c.	subcutaneously

S1P	Sphingosine-1-Phosphate
S1PR2	Sphingosine-1-Phosphate Receptor 2
sFRP	Secreted Frizzled-related Proteins
SOST	Sclerostin
Sox2	SRY (sex determining region Y)-Box 2
SPARC (ONC)	Secreted Protein, acidic, cysteine-rich (osteonectin)
SV40	Simian Virus 40
TGF	Transforming Growth Factor
TGF- α	Transforming Growth Factor Alpha
TGF β 1	Transforming Growth Factor, Beta 1
TGF β R2	Type-II TGF- β Receptor
TIC	Tumor Initiating Cell
TIMP	Tissue Inhibitor of Matrix Metalloproteinases
TNBC	Triple Negative Breast Cancer
TNF- α	Tumor Necrosis Factor Alpha
TRAPase	Tartate-resistant Acid Phosphatase
TWF	Twinfilin
TWIST	Twist Family bHLH Transcription Factor 1
Ubc9	Ubiquitin-conjugating Enzyme 9
UTR	Untranslated Region
VCAM1	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VIM	Vimentin
WNT10b	Wingless-type MMTV Integration Site Family, member 10B
WNT3A	Wingless-type MMTV Integration Site Family, member 3A

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INTRODUCTION

Solid primary tumors spread to distant organs with selective affinity, the skeleton being one of the most common organs to be affected by metastatic tumors. Breast cancer is one of the main osteotropic tumors leading to osteolytic lesions characterized by bone destruction that results from enhanced osteoclast activity. These skeletal lesions cause pathological fractures, hypercalcemia and nerve compression and can negatively impact on survival (Ell and Kang, 2012) (Weilbaecher et al., 2011). Therefore there is a need to understand underlying molecular mechanisms that are deleterious to the skeleton in order to improve existing treatments and develop new targeted therapies (Coleman et al., 2012). The metastatic cascade leading to bone metastasis is initiated in primary tumours where a subset of cancer cells undergoes epithelial-mesenchymal transition, acquires mobility to migrate into the surrounding stroma and seeds at distant sites to grow (Buijs and van der Pluijm, 2009). The specific development of bone metastasis requires the recruitment of circulating tumour cells in the bone marrow, their adaptation to survive in the surrounding microenvironment where they alter the functions of osteoclasts and osteoblasts, and hijack signals coming from the bone matrix. Bone metastasis, being a very inefficient process, is regulated by multiple factors and molecular pathways, mainly through the tight control of genes expressed by interacting cells. The small endogenous non-coding microRNAs (miRs) which have the capacity to control multiple genes are master regulators of gene expression and thus redirect or reprogram biological pathways. Consequently, in cancer, they can act either as promoters or suppressors of tumor development and metastatic progression. Large sets of miRs are underexpressed in tumors compared to normal tissue, whereas miR genes located in fragile chromosomal regions are susceptible to amplification or deletion, often promoting tumorigenesis and metastatic dissemination (Krol et al., 2010). Here, we asked whether miRs are involved in bone metastasis formation and progression in breast cancer, and whether modulation of their expression might regulate specific steps of bone metastasis.

In the first part of the work, the activity of miRs on bone metastasis has been study in an experimental mouse model of human bone metastasis, after inoculating the breast cancer cells in mice arterial blood. In this, we could investigate the regulation of specific steps of bone tropism,

homing, invasion and second outgrowth of breast tumor cells in bone. Analyzing transcriptomic profile of miRs in osteotropic human breast tumor cells identified a drastic down-regulation of the miR-30 family (miRs-30) in these cells. Restoring miRs-30 in an osteotropic breast cancer cell line decreased bone metastasis *in vivo* predominantly through the down-regulation of genes associated with osteomimicry.

This work is reported in the manuscript under the form of a paper (*in preparation of submission for publication*), entitled “The Tumor Suppressor miRs-30-5p Family Regulates Human Breast Cancer Cell Colonization in Bone” (pages 59 to 111).

In the second part of the work, the activity of miRs on breast cancer cell dissemination to bone has been studied in an orthotopic tumor xenograft mice model, in which spontaneously metastatic breast cancer cells were inoculated into mammary gland. These results are reported in “Experimental work” (pages 112 to 140).

Finally, we have shown that the transcription factor Twist1 facilitates breast cancer bone metastasis formation through a mechanism dependent of an oncomir, miR10b, which in turn promotes the development of osteolytic lesions. This paper entitled “TWIST1 expression in breast cancer cells facilitates bone metastasis formation” is included in the manuscript.

PART 1

LITERATURE REVIEW

1. Breast cancer and its complications

Breast cancer is the most common cancer affecting women with 1.67 million new cases in 2012. It is more common in less developed (883,000 cases) than more developed regions (794,000).

Regarding mortality, it ranks as the most frequent in women in less developed regions (324,000 deaths) followed by cancers of the lung (281,000 deaths). In more developed regions, breast cancer is the second leading cause of cancer death (198,000 deaths), after lung cancer (210,000 deaths) (Figure 1) (Ferlay et al., 2015).

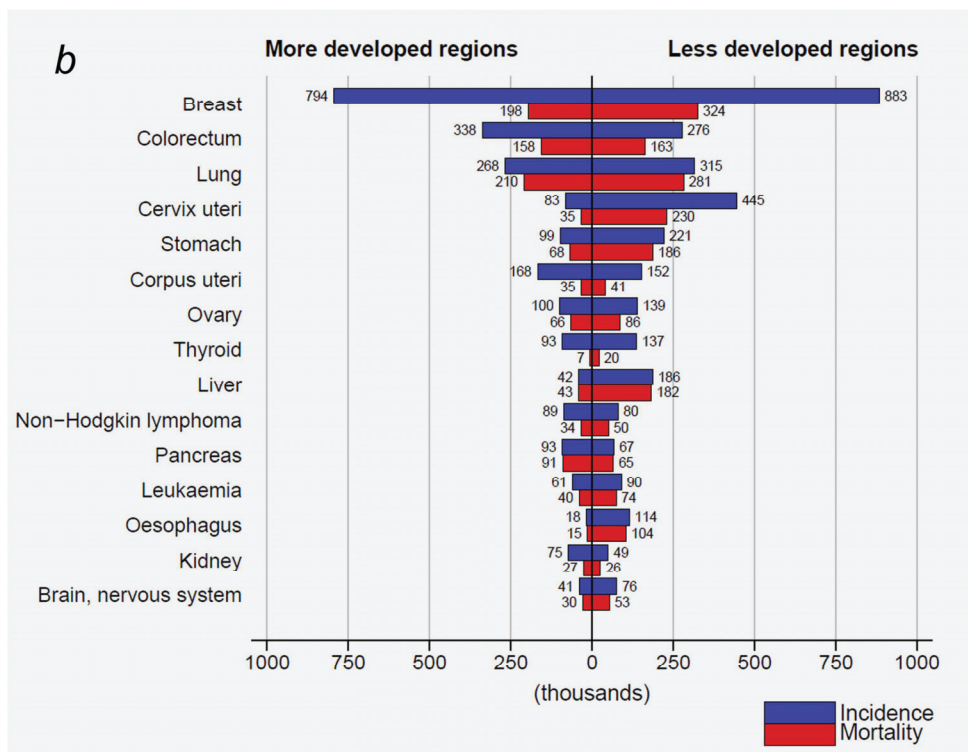


Figure 1. Incidence of new cancer cases and death in women in more developed and less developed regions of the world in 2012 (Ferlay et al., 2015).

Breast cancer is a complex and diverse disease and in order to stratify patient risk and outcomes, ongoing research strives for accurate classification of the disease. An important histopathological feature of breast cancer related to patient outcome is the expression of certain receptors, such as

oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Based on receptors subtypes and differences in gene expression, molecular subtypes of the cancer have been defined. These include: luminal A, luminal B, HER2-expressing and triple-negative breast cancer (TNBC).

Luminal A subtype accounts for 40-55 % of breast cancers, making it the largest tumor gene subtype. It demonstrates ER/PR positivity and HER2 negativity with a low cellular proliferation index ($Ki-67 < 14\%$). These carcinomas are well-differentiated and they have the lowest systemic relapse rates. The median duration of survival from time of first metastasis is approximately 2.2 years (Chikarmane et al., 2015), this is statistically higher than any other subtype (Kennecke et al., 2010).

Luminal B subtype tumors express ER/PR, can be HER2 positive or negative and often have high cellular proliferation index ($Ki-67 > 14\%$) (Chikarmane et al.). Patients with ER/PR positivity are more likely to be post-menopausal women with lower-grade tumors at presentation and with worse survival than luminal A patients (1.6 years) (Kennecke et al., 2010).

The HER2-positive tumors constitute 12-30 % of invasive breast cancers and can be ER/PR positive or negative. This subtype is associated with increased proliferation rate, angiogenesis, tumor invasiveness, high nuclear grade and these patients are more likely to have multifocal/multicentric cancers and nodal involvement. It is more often found in pre-menopausal women and was originally associated with poorer clinical outcome (Chikarmane et al.). In 1998, the clinical course for HER2-positive subtype tumors was significantly changed with the introduction of trastuzumab, the monoclonal antibody against extracellular portion of HER2 tyrosine kinase receptor (Slamon et al., 2001). However, without adjuvant trastuzumab treatment these patients have a higher risk of local recurrence than luminal A or B cancers, with a median survival from time of first metastasis of approximately 0.7 years (Kennecke et al., 2010).

TNBC refers to ER/PR and HER2 negativity in immunohistochemistry. It comprises around 13-25 % of breast cancers and presents with higher histological grade and proliferation rate than other subtypes. TNBC is more frequent in young African Americans, and in women with BRCA1 mutation. These tumors are more likely to be present at the physical examination rather than at mammographic screening because they have a rapid growth rate (Chikarmane et al.,

2015). Furthermore, patients with TNBC have a poorer prognosis (relapse-free survival and overall survival) when compared to luminal A and B subtypes (Lin et al., 2008).

Approximately 30 % of newly diagnosed breast cancer patients will have tumor spread to regional lymph and up to 5 % will already have metastases at presentation (Chikarmane et al.). Molecular phenotyping of breast cancer on the basis of three receptors mentioned above has become standard of care in the management of breast cancer patients due to the fact that the molecular phenotype of breast cancer determines the timing, pattern and outcome of metastatic disease (Kennecke et al., 2010; Perou et al., 2000). At 10 years from the diagnosis, 70 % of patients with luminal A tumors were alive compared with 62.6 % of nonbasal TN patients, 54.4 % of luminal B, 52.6 % of basal-like, 48.1 % of HER2-enriched and 46.1 % of luminal/HER2 patients. Virtually all relapses occurred within the first 5 years among basal-like, TN nonbasal and HER2 groups, whereas luminal subtypes experienced relapses between 5 and 15 years. Regarding the correlation of breast cancer subtype with site of metastases, it has been found that molecular subtype predicts the secondary events (Table 1).

Subtype	Metastatic organ [%]				
	bone	lung	liver	brain	distal nodal
Luminal A	66	23.8	28.6	7.6	15.9
Luminal B	71.4	30.4	32	10.8	23.3
HER2 positive, ER/PR positive	65	36.8	44.4	15.4	22.2
HER2 positive, ER/PR negative	59.6	47.1	45.6	28.7	25
Basal-like	39	42.8	21.4	25.2	39.6
TN nonbasal	43.1	35.8	32.1	22	35.8

Table 1. Frequency of site specific metastases according to molecular breast cancer subtype.

The predominant metastatic site for each subtype is marked in green. Numbers reflect the percentage [%] of patients from the specific subtype positive for metastasis in a given organ (Kennecke et al., 2010).

Almost all breast cancer subtype, except for basal-like, predominantly metastasize to bone. This study confirms the previous observation that bone is of particular clinical importance in breast

and prostate cancers because of the prevalence of these diseases. At postmortem examination, 70% of patients dying of these cancers have evidence of metastatic bone disease (Coleman, 2006). Due to such a high incidence rate of bone metastases in breast cancer patients, it is of urgent need to find effective treatment for bone metastasis. In the next session, the bone physiology, the process of bone metastasis formation and current treatment regimens will be discussed.

2. Physiology and pathophysiology of bone

Skeleton is responsible for the maintenance of the shape of the body, it shields vital organs from external insults, serves as a scaffold for the muscles allowing their contraction to be translated into bodily movements, resists mechanical load during locomotion and weight bearing, and provides a reservoir of calcium, magnesium, bicarbonate and phosphate. The skeleton is made up of mineralized and unmineralized connective tissue matrix, highly specialized cells, and spaces that include the bone marrow cavity, vascular canals, canaliculi and lacunae. There are two different designs in the architecture of the normal skeleton: cortical and cancellous. Cortical bone accounts for about 80 % of bone mass and forms the solid outer wall of all bones, whereas cancellous, or trabecular, bone rates for 20 % of the total bone mass and consists of three-dimensional network of trabeculae located mainly in the metaphysis of long bones such as the femur and inside of flat bones such as ileum or cranium. It is in intimate contact with the hematopoietic marrow (Body, 2000).

Like in other tissues, the bone undergoes a process of regeneration or remodeling throughout life. During development and growth, the skeleton is sculpted in order to achieve its shape and size by the removal of bone from one site and deposition at a different site. Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone by new bone at the same location. This process is called *remodeling* and it is carried out by osteoclasts and osteoblasts, termed *basic multicellular units (BMU)* (Body, 2000).

Osteoblasts and osteoclasts are derived from progenitors originating in the bone marrow and the process of bone remodeling is controlled locally by growth factors and cytokines (Capulli et al., 2014).

2.1. The cellular components of bone and their function

2.1.1. Osteoblasts-bone forming cells

Osteoblasts are cells commonly known for their bone building function. They compose approximately 4–6% of total resident cells in the bone (Capulli et al., 2014).



Figure 2. Histological section of a mouse tibia, stained with the Masson's trichrome.

Black arrows indicate a row of osteoblasts on a bone trabecula (blue staining), white arrow indicate bone-lining cells, bar = 10 μm (Capulli et al., 2014).

As many other cells of the connective tissues (fibroblasts, chondrocytes, myoblasts and adipocytes), osteoblasts arise from a common pluripotent mesenchymal stem cell (MSC).

The expression of Bone Morphogenetic Proteins (BMPs) and Wnt pathways is crucial for the early steps of osteogenesis, where they promote MSCs commitment towards an osteo/chondroprogenitor. The osteo/chondroprogenitor cell is defined when at least the following osteoblast-specific transcription factors are expressed: Runt-related transcription factor 2 (RUNX2), Distal-less homeobox 5 (DLX5) and Osterix (OSX) (Figure 3).

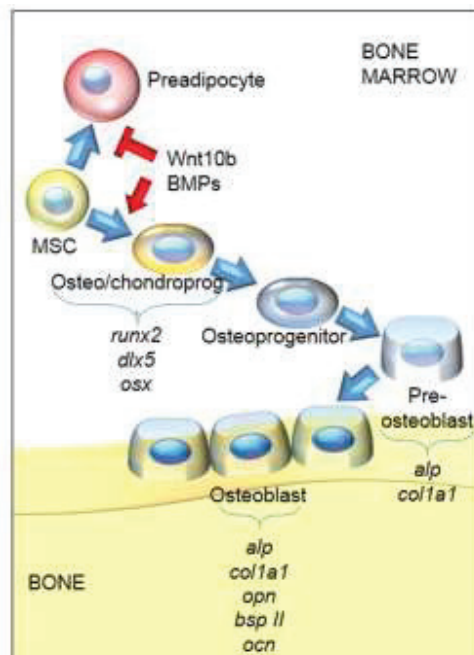


Figure 3. Osteoblast differentiation.

The expression of BMPs and Wnt pathway promotes MSCs commitment towards an osteo/chondroprogenitor. Next, RUNX2 and DLX5 direct MSCs into osteoprogenitor differentiation. A pre-osteoblast arising from an osteoprogenitor expresses high amount of ALP and Type I Collagen. A mature osteoblast additionally expresses bone matrix proteins, such as OCN, OPN and BSP II (Capulli et al., 2014).

The master gene of osteoblast differentiation is RUNX2 (Capulli et al., 2014). It has been demonstrated that Runx2-null mice lack of osteoblasts (Komori et al., 1997). In humans, mutations of RUNX2 cause Cleidocranial Dysplasia (CCD), an autosomal-dominant disease with dramatic abnormalities in bones (Lee et al., 1997). RUNX2 up regulates osteoblast-related genes (COL1a1, ALP, BSP, BGLAP) in osteoblasts. Moreover, the overexpression of RUNX2 in human MSCs isolated from adipose tissue triggers commitment towards osteoblasts, by increasing Alkaline Phosphatase (ALP) activity and osteocalcin (OCN) expression (Lee et al., 2011). At later stage, RUNX2 inhibits osteoblast maturation and causes osteopenia (Capulli et al., 2014), showing a dual role for RUNX2 in osteoblastogenesis.

Another transcription factor needed to direct MSC into osteoprogenitor differentiation is DLX5. It promotes osteogenesis under the control of BMPs, is highly expressed in the developing skeleton and is induced during fracture healing (Han et al., 2011).

The last mentioned transcription factor regulating osteoblast differentiation is OSX, also known as SP7. It is a downstream target of RUNX2, whose expression in MSC progenitors is stimulated by BMPs and Insulin-like Growth Factor (IGF) (Capulli et al., 2014).

Once these main transcription factors have been expressed and a pool of osteoblast progenitors has been established, there is a proliferation phase, during which osteoblast progenitors acquire ALP activity. These cells become now pre-osteoblasts which express relatively high amounts of ALP (Body, 2000). ALP has been long thought to play a role in bone mineralization, however, the precise mechanism of mineralization and the exact role of ALP in this process remain unclear (Sharma et al., 2014). Pre-osteoblasts also produce Type I Collagen which is a major product of the bone-forming osteoblast. It is initially secreted in the form of a precursor which is further processed extracellularly to mature three-chained Type I Collagen molecules. These then assemble themselves into a collagen fibril. Individual collagen molecules become interconnected by the formation of pyridinoline cross-links which are unique to bone (9).

The late stage of osteoblast differentiation is characterized by a higher expression of the bone matrix proteins OCN, BSP I and II, of Type I Collagen and of proteoglycans, such as decorin and biglycan. This newly formed, non-mineralized bone matrix is called osteoid. Osteoblasts producing osteoid are mature osteoblasts which appear in histological sections as a single row of cuboidal-shaped cells with a round basal nucleus (Figure 3). They also play a role in the early steps of mineralization, which relies on the synthesis of hydroxyapatite crystals within the matrix vesicles, derived from the membrane surface of osteoblasts. The aging osteoblasts have three possible destinies: (I) undergo apoptosis, (II) give way to the osteocytes or (III) become bone-lining cells (Figure 4).

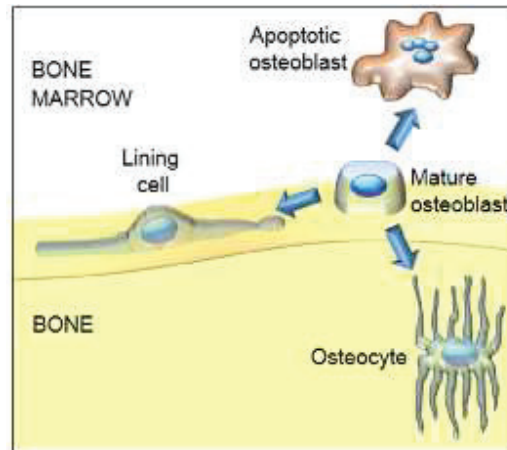


Figure 4. Possible fate of mature osteoblast (Capulli et al., 2014).

Osteoblast differentiation is mainly controlled by two signaling pathways, the Wnt pathway and Bone Morphogenetic Proteins pathways (BMPs).

Wnt proteins are a family of at least 19 secreted glycoproteins involved in many cell functions. They signal through a canonical (WNT3a, WNT10b) and a non-canonical pathway (WNT1, WNT5A). The former relies on β -catenin stabilization, and is crucial for a correct bone mass achievement. More precisely, in the canonical pathway, Wnt proteins bind to Frizzled (FRZ) and to LRP5/6 receptors, which in turn activate a downstream signaling that inhibits the activity of Glycogen Synthase Kinase 3 beta (GSK3 β), with a consequent prevention of β -catenin phosphorylation. When hypophosphorylated, β -catenin is more stable, it can translocate into the nucleus where it regulates the transcription of Wnt target genes. Whereas in absence of Wnt, GSK3 β is active and phosphorylates β -catenin, eventually leading to its ubiquitination, thus turning off Wnt signaling (Capulli et al., 2014). Moreover, knock-down of WNT5a and WNT10b show reduced in trabecular bone and bone mass (Takada et al., 2007). Some molecules specifically interfere with the Wnt pathway, such as the Dickkopf-related protein 1 (DKK1). Deficiency of DKK1 has been associated with increased bone formation (Capulli et al., 2014). Other inhibitors of Wnt pathway acting in a similar way is sclerostin (SOST), which is predominantly produced by osteocytes and the secreted frizzled-related proteins (sFRP), whose members inhibit Wnt pathway by sequestering Wnt from binding to FRZ receptors or by directly binding to FRZ (Capulli et al., 2014).

The second pathway crucial for osteoblastogenesis is related to Bone Morphogenetic Proteins (BMPs). These proteins (at least 20) belong to the Transforming Growth Factor (TGF) β

superfamily and they were first described as being involved in bone metabolism as components of the bone matrix able to cause ectopic bone formation when injected subcutaneously or intramuscularly in mice (Capulli et al., 2014). Mutations in the genes coding for BMPs in mice clarified the role of these proteins in bone metabolism (Kingsley et al., 1992). Among the different isoforms, BMP-2, 4, 5, 6, and 7 are clearly implicated in osteogenesis, likely due to their ability to stimulate the expression of the transcription factors RUNX2 and OSX. BMP-2 is especially expressed in areas surrounding cartilage condensation, where it has a prominent role for chondrocyte proliferation and maturation, thus enhancing endochondral ossification. Moreover, it is crucial for osteogenesis. It has been shown that a lack of BMP-2 and BMP-4 in osteoblast conditional KO mice led to a severe impairment of osteogenesis (Bandyopadhyay et al., 2006). Finally, BMP-7 has been shown to increase ALP activity in osteoblasts and to trigger bone mineralization (Capulli et al., 2014).

2.1.2. Osteocytes and bone lining cells

As mentioned in the previous section, mature osteoblast can end up as osteocytes being the most abundant cells in bone. They are characterized by a striking stellate morphology, reminiscent of the dendritic network of the nervous system. Their major feature is multiple extensions of their plasma through narrow canaliculi, connecting them to each other as well as with the bone-forming osteoblasts and the lining cells. The cell-cell communication is also achieved by an interstitial fluid that flows through the osteocytes canaliculi.

This network is essential in coordinating the response of bone to mechanical and biological signals. Currently, a general consensus exists that osteocytes are involved in translating mechanical stimuli into biochemical signals. Following that, osteocytes orchestrate bone formation and bone resorption acting on osteoblasts and osteoclast differentiation and function.

Regarding the role of osteocyte in osteoblast regulation, it has been shown that osteocyte apoptosis or osteocyte network disruption increased bone formation (Moriishi et al., 2012). Also, it is well known that SOST is a secreted glycoprotein specifically expressed in osteocytes which inhibits osteoblasts differentiation and function by antagonizing the canonical Wnt pathway. Consistent with this finding, bone formation is enhanced in Sost-deficient mice (Capulli et al., 2014). Osteocyte also regulates osteoclast formation. Tatsumi et al. showed that osteocyte

apoptosis results in an increase in RANKL (Rank Ligand) expression in bone, thus enhancing the osteoclast formation and function, leading to increase of the bone resorption (Tatsumi et al., 2007). These data suggest that osteocytes can act as mechanosensing cells but also are able to regulate bone homeostasis in a coordinated and complex manner.

Another possible destiny for mature osteoblast is becoming the so-called *bone lining cells*. They are quiescent and flat shaped osteoblasts which cover the bone surface, functionally representing the resting phase of the bone remodelling process. There is not much known about their function. A likely role of these cells is to prevent the direct interaction between osteoclasts and bone matrix when bone resorption should not occur (Capulli et al., 2014).

2.1.3. Osteoclasts-bone resorbing cells

Osteoclasts are multinucleated giant cells (50-100 μm in diameter) with abundant mitochondria, numerous lysosomes and free ribosomes. They differentiate from myeloid precursors under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF- κB ligand, being RANKL, supplied by osteoblasts and/or osteocytes (Figure 5) (Charles and Aliprantis, 2014).

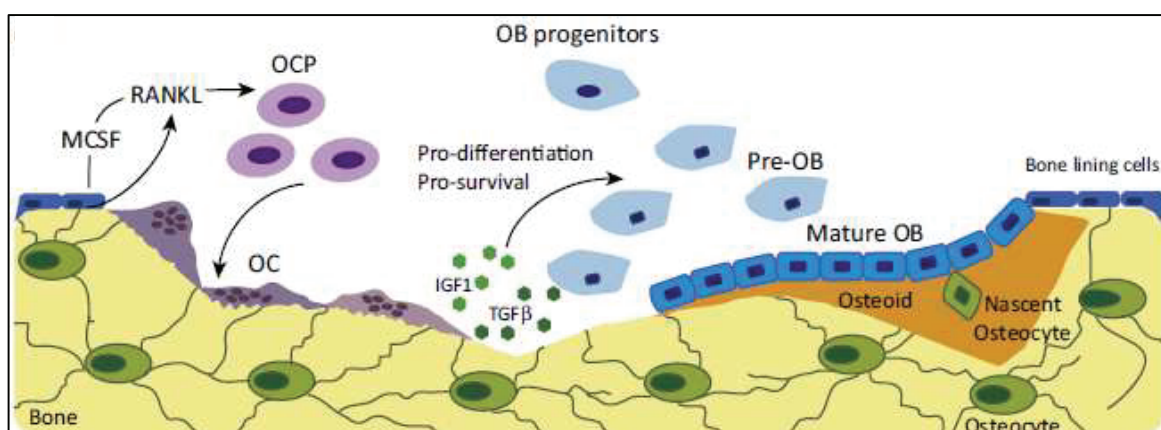


Figure 5. Osteoclasts–osteoblast interactions in the basic multicellular unit (BMU).

Osteoclasts (OCs) differentiate from OC precursors (OCP) under the influence of M-CSF and RANKL produced by osteoblast (OB) lineage cells including osteocytes. As OCs create a resorption pit, growth factors, including TGF β and IGF1, are released from the bone matrix. These growth factors may recruit mesenchymal osteoblast progenitors and promote their differentiation into mature cells that secrete osteoid to fill the area of resorbed bone. Some OBs differentiate further into matrix-embedded osteocytes.

A decoy receptor for RANKL, termed osteoprotegerin (OPG), which is synthesized by the osteoblast lineage, inhibits osteoclast differentiation.

The main function of osteoclasts is degradation of bone. They secrete proteolytic enzymes (e.g., cathepsin K, O, B, L) and acid, which hydrolyze and solubilize the organic and inorganic components of bone, respectively (Charles and Aliprantis, 2014). Enzyme and proton secretion is directed into the resorption lacunae where the degraded bone matrix components are endocytosed. Next, these degraded bone matrix components are transcytosed to the membrane area opposite the bone, where they are released (Body, 2000). Subsequent to the osteoclastic resorptive phase, coupling mechanisms promote the recruitment and differentiation of mesenchyme-derived osteoblast progenitors at the resorption lacunae. After these cells mature into osteoblasts, they line the eroded bone surface and secrete the organic component of bone-osteoid, which is mineralized over time by the incorporation of hydroxyapatite. As osteoblasts secrete osteoid, some cells are entrapped within the matrix where they eventually become osteocytes (Figure 5) (Charles and Aliprantis, 2014).

Osteoclasts are characterized by high amounts of the phosphohydrolase enzyme tartrate-resistant acid phosphatase (TRAPase). This feature is frequently used for the detection of osteoclasts in bone specimens. Osteoclasts bear on their surface several antigens that are also present on hematopoietic cells, such as: CD45, the common leukocyte antigen; ICAM-1, the cellular ligand for certain integrins; integrin α 2 and integrin β 1 that constitute the collagen receptor; integrin α v and integrin β 3 which constitute the vitronectin receptor responsible for mediating the attachment of osteoclasts to the bone surface, and finally CD9, a gene encoding for tetraspin family (Body, 2000).

Osteoclast function however is not only restricted to bone degradation. Coupling of bone formation and resorption requires cooperation between BMU and other cells residing within bone matrix, such as osteocytes, macrophages and T cells. Each of these cells produces a variety of factors involved in bone remodeling in health, but also in disease. In the next section, the process of bone remodeling in healthy condition will be reviewed.

2.2. Bone remodeling

The balance between bone deposition and resorption is crucial for the proper development and maintenance of bone size, shape and integrity. To maintain this balance, molecular communication between osteoblasts and osteoclasts, and also between bone cells and other bone marrow cells is fundamental. Ideas to explain the coordinate regulation of bone resorption and formation center developed on the notion that osteogenic growth factors are released from the bone matrix during bone resorption, and mediate the subsequent recruitment of osteoblasts to the remodeling site (Body, 2000). Matching bone formation and resorption, is referred to as osteoclast-osteoblast “*coupling*”.

An essential step in the process of remodeling is that sites needing to be remodeled must be selected from among the millions possible at any one time in the skeleton. Bone is maintained in a healthy state because remodeling takes place only where it is needed to replace old or damaged bone. To initiate a remodeling cycle, the site of activity must be chosen, regardless of whether remodeling is initiated in response to damage, to change in loading or to remove old bone. Although the initiating event has usually been described as resorption by osteoclasts, other mechanisms also occur to ensure that this takes place.

Such an initiating event might come from osteocytes recognizing that a specific area of bone needs to be replaced, and signaling through their canaliculae to surface cells. Subsequent signals could arise from apoptosis of osteocytes, or even of lining cells themselves, resulting in the release of paracrine factors and chemokines that attract osteoblast and osteoclast precursors and vascular elements.

Hematopoietic precursors for osteoclast formation are provided from nearby marrow precursors, as well as through the capillary blood supply closely associated to bone. As it has been mentioned before, osteoclast formation requires mainly the signals of M-CSF and RANKL which come from osteoblast lineage. Studies on genetically manipulated mice suggested that it is not only early osteoblast precursors but also fully differentiated and matrix-embedded osteocytes that provide RANKL to the osteoclast precursors (Xiong et al., 2011). Moreover, beside osteoblastogenic lineage, lymphocytes T and lymphocytes B have been shown to be high producers of RANKL (Capulli et al., 2014).

The most completely described intercellular interaction within the BMU is the balance in production of RANKL and its decoy receptor OPG by the osteoblast lineage, and their interaction with RANK on the cell membrane of osteoclast precursors. A number of local factors, produced by osteoblasts that stimulate RANKL production, have been identified such as semaphorin 3B, oncostatin M (OSM), interleukin-6 (IL-6), interleukin-1b (IL-1b), TNF α , family coreceptor subunit, glycoprotein 130 (GP130) (Sims and Martin, 2014).

2.3. Coupling of bone formation to resorption

It is essential that the formation component of remodeling needs to replace the exact amount removed by resorption.

Although circulating hormones, including PTH and 1,25-dihydroxyvitamin-D₃, were considered to be the prime regulators of remodeling, it has been clear for some years that locally generated cytokines are the key influences, influencing bone cell communication and subsequent function in complex ways, and often themselves regulated by the hormones and the nervous system. One of the main examples of osteoclast regulation by osteoblasts is the osteoblast lineage-derived RANKL which signals through its receptor RANK. However, what about the reverse direction? Does the signaling come also from osteoclasts to osteoblasts? Arguments began to be presented in favor of this, and in the past few years a number of candidate ‘coupling factors’ of osteoclast origin have been proposed.

One of such factors is active TGF β 1, released from the bone matrix during bone resorption. It might couple bone formation to resorption by inducing the migration of bone MSCs to sites that have been resorbed, thus making them available for differentiation and bone formation in remodeling (Tang et al., 2009). Complementing that, osteoblast- and matrix-derived insulin growth factor-1 (IGF-1) was found to promote osteoblast differentiation by favoring recruitment of MSCs, in response to osteoclastic bone resorption.

As coupling mechanism, direct interaction between osteoblasts and osteoclasts had been described. Semaphorins and ephrins are axon guidance molecules involved in remodeling and coupling process. Semaphorins include both secreted and membrane-associated signaling molecules that use plexins and neuropilins as receptors. Sema4D that is expressed by osteoclast,

but not by osteoblast, is an osteoclast-derived inhibitor of osteoblast differentiation and bone formation, acting as an inhibitor of remodeling (Negishi-Koga et al., 2011). Ephrins and their receptors Eph are axon guidance molecules acting as local mediators of cell function through contact-dependent processes. EphrinB2 has been shown to be expressed by osteoclasts both *in vitro* and *in vivo*. Studies *in vivo* and *ex vivo* of genetically manipulated mice suggested that osteoclast-derived ephrin B2 can act through its receptor, EphB4, in osteoblasts, to promote osteoblast differentiation, and that reverse signaling by direct contact with osteoblast-derived EphB4 can suppress the formation of osteoclast precursors. This contact-dependent action of osteoclast-derived ephrin B2 on osteoblastic EphB4 is part of coupling mechanism (Mundy and Elefteriou, 2006).

2.4. Bone metastasis

Bone is one of the most common sites of cancer spread. Although metastatic cells could theoretically invade any organ, clinical experience demonstrates that they have preference for bone and lung (Cleazardin and Teti, 2007). Why only certain cancers preferentially spread to bone is not fully elucidated, however it is true that bone provides a fertile microenvironment which facilitates colonization of metastatic cancer cells. The outer part of bone is composed of hard calcified matrix which shows relatively low cellularity and metabolic activity. Osteocytes are likely the only cells that are metabolically active and show some biological function. Calcified matrix, however, stores varieties of growth factors which may serve as essential nutrients for cancer cells that begin to house in bone. These growth factors are continuously released into the bone marrow cavity as a consequence of osteoclastic bone resorption during bone remodeling (Body, 2000). This appears to be one of possible explanations for preferential bone colonization. In this regard, bone is a representative target organ, which is consistent with the “seed and soil” theory, proposed by Paget more than a century ago (Paget, 1889).

The inner part of bone is made up of multicellular and highly metabolic bone marrow. It is rich in HSCs, which give rise to all blood cell elements and osteoclasts, as well as MSCs which differentiate into osteoblasts, chondrocytes, adipocytes and stromal cells. Especially important is the presence of osteoclasts because it seems that without osteoclastic bone resorption, growth factors stored in calcified matrix are less available to metastatic cancer cells. Tumor cells do not destroy bone by themselves but rather dependent on osteoclasts for the progression of osteolysis.

Therefore, osteoclastic bone resorption is essential for cancer cells to obtain sufficient nutrients and space for growth in the hard calcified tissue. However, cancer cells colonizing the bone will not only interact with osteoclasts, but also with osteoblasts, stromal cells and immune cells found in the bone marrow cavity (Body, 2000).

These interactions may lead to development of bone metastases even many years after the surgical removal of the primary tumor, suggesting that the osteotropic cancer cells may have a long period of quiescence before growing as secondary lesions. Growth of tumor cells in bone leads to the dysregulation of normal bone homeostasis, causing bone destruction and, ultimately, severe pains and fractures for which only palliative therapies are proposed. Thus, there is a need to better understand specific stages of tumor cell dissemination and growth in the bone marrow in order to prevent bone metastasis formation.

2.4.1. Classification and cellular features of bone metastases

Bone metastases are classified as osteosclerotic (osteoblastic), osteolytic or mixed lesions (Figure 6) (Guise et al., 2006).

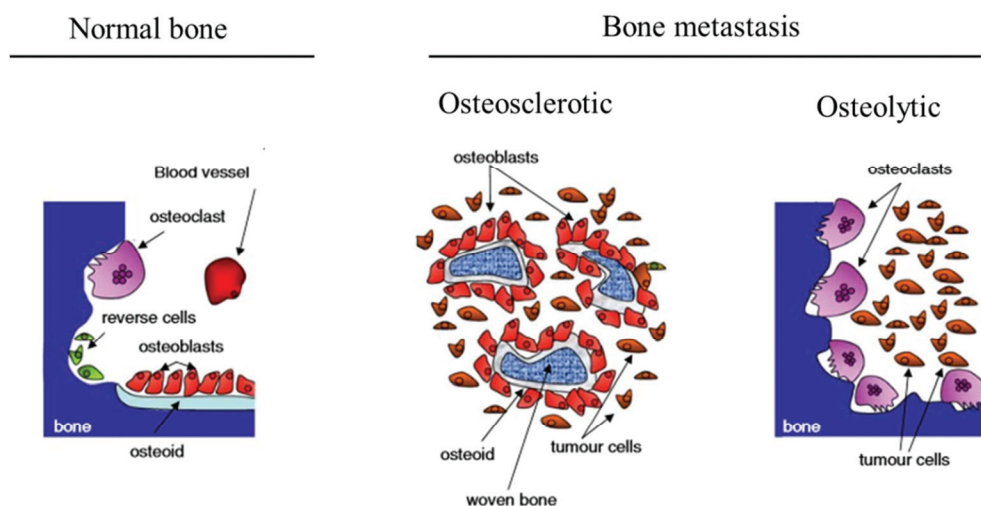


Figure 6. Classification of bone metastases.

Left panel depicts a normal bone in which osteoclasts and osteoblasts function in a coordinated manner. Osteoclasts remove the old bone matrix which, after reverse phase populated by mononuclear cells (reverse cells) participating to the coupling between osteoclasts and osteoblasts, is replaced by new osteoid released by osteoblasts. Middle panel: in osteosclerotic bone metastases, numerous osteoblasts appear forming new trabeculae that occlude bone marrow. This bone matrix has features of woven bone and is inordinately deposited in the medullary cavity. Right panel: in

osteolytic metastases, high number of osteoclasts is formed which resorb the mineralized matrix destroying the tissue (adapted from (Clezzardin and Teti, 2007)).

Osteosclerotic metastases are more typical of prostate cancer and are caused by cancer-derived factors that stimulate the differentiation and activity of bone forming cells, the osteoblasts, thus leading to increased bone formation. Osteosclerotic lesions appear in radiography as dense areas, often located to the axial skeleton and, particularly, in vertebral bodies and pelvis. Histologically, tumor cells residing in the bone marrow are surrounded by a high number of osteoblasts that form wide trabeculae of woven bone similar to that observed in primary ossification. Tumor-associated woven bone has a poorly organized microstructure, increasing the risk of pathological fractures. On the other hand, osteolytic metastases are common in breast cancer. They are caused by tumor-derived factors that stimulate the activity of bone-resorbing cells, the osteoclasts, leading to enhanced bone destruction. Radiographically, osteolytic lesions appear as radiolucent areas, frequently located in the skull and proximal ends of the long bones. Histologically, tumor cells reside in the bone marrow, and are surrounded by a number of osteoclasts, actively degrading bone (Chappard et al., 2011). These osteolytic areas frequently fracture.

As it has been noted in the previous section, bone resorption and bone formation are almost always coupled. The coupling of bone resorption and bone formation is altered in cancer, thereby leading to skeletal lesions that are predominantly osteolytic or osteoblastic. However, in many instances, bone metastases may consist of mixed lesions. It is believed that a bone metastasis may evolve from an osteoblastic to an osteolytic pattern through a continuous process of which we only have a static representation at the time of the radiographical or histological assessment (Clezzardin and Teti, 2007).

Bone metastasis formation consists of a series of inter-related steps. Molecular mechanisms involved in each of these steps are gradually being unraveled, and are potential therapeutic targets for the prevention and treatment of bone metastases. Further complexities are introduced by the fact that the bone microenvironment does not only include the cellular architecture of the bone tissue but also bone marrow-derived haematopoietic progenitors and cancer stem cells (CSCs) that altogether constitute a niche supporting the development of metastases. In the next section, these steps of so-called *metastatic cascade* and determinants for overt bone metastases formation will be debated.

2.4.2. Steps of cancer metastasis to bone

Cancer metastasis to bone consists of multiple sequential steps which include diverse and complex cellular and molecular events. The early steps of metastasis to bone are common to most solid tumors and start with local tissue invasion and tumor cell escape from primary tumors. After growing in the primary tumor, cancer cells invade surrounding tissue, intravasate, circulate in blood and entry into bone marrow sinus to migrate into bone marrow and colonize the tissue.

2.4.2.1. Growth at the primary sites, detachment from the primary site, local invasion and intravasation

First essential step for the successive colonization is cancer growth. Production of autocrine growth-promoting factors associated with the activation of receptor tyrosine kinase signaling pathways, oncogene expression and loss of tumor-suppressor gene expression are probably responsible for initiation and promotion of carcinogenesis. More than 30 % of human primary breast cancers exhibit increased expression of HER2/neu receptor tyrosine kinase which is a member of the epidermal growth factor receptor (EGFR) tyrosine kinase family. Targeted expression of neu proto-oncogene in the mammary gland of transgenic mice induces highly metastatic mammary tumors, suggesting that this growth factor influence invasive properties of cells. Apart from growth factors, mutations in tumor-suppressor genes also predispose to cancer. In aggressively growing tumors, cancer cells appear to be pushed further from existing vasculature due to unregulated cell proliferation and inadequate vascularization. These cancer cells are subject to hypoxia and nutrient depletion which induces angiogenesis (Body, 2000). In the 90s, Weidner et al. proved that there is a correlation between metastasis and angiogenesis. He found that the number and density of microvessels in primary breast tumors were significantly higher in patients with metastases than in patients without metastases (Weidner et al., 1991). Among the angiogenic molecules released by tumors or tumor stromal cells are: basic fibroblastic growth factor (bFGF), TGF- α , tumor necrosis factor alpha (TNF- α) and VEGF. Their expression correlated with aggressiveness of solid tumors. At certain time point, cancer cells start to detach from the primary site. Cell adhesion molecules (CAMs) play an important role in cancer invasion and metastases. Their downregulation is necessary for detachment from the primary site and initiation of invasion. One of CAMs which is critical in the detachment is E-cadherin. It is a cell-surface glycoprotein involved in calcium-dependent cell-cell adhesion. E-cadherin expression is decreased in breast and prostate cancer with high metastatic potential (Body, 2000). It has been

also shown that overexpression of E-cadherin in the human breast cancer cell line MDA-MB-231 profoundly suppresses bone metastases in a nude mouse model of experimental bone metastases (Mbalaviele et al., 1996). In parallel with the growth at the primary tumor site, cancer cells invade surrounding tissues to enlarge the tumor mass by secreting proteolytic enzymes. The matrix metalloproteinases (MMPs) are a family of Zn^{2+} -dependent endopeptidases. Increased plasma levels of MMPs have been correlated with invasion and metastasis in patients with breast cancer. Tissue inhibitors of matrix metalloproteinases (TIMPs) on the other hand, function as metastasis-suppressor proteins. The metastatic potential of cancer cells depends on the balance between MMP and TIMP production. For example, overexpression of TIMP-2 in MDA-MB-231 cells inhibits osteolytic bone metastases and prolongs survival of tumor-bearing animals (Yoneda et al., 1997). Subsequently to growth and invasion at the primary site, cancer cells enter the blood stream to start their journey to secondary sites. This process is initiated by the adhesion of cancer cells to the endothelium of interior of lymph vessels. Then, the endothelial cells retract, the basement membrane is exposed and cancer cells bind to extracellular matrix (ECM) molecules of the basement membrane. Two major components of the basement membrane ECM are type IV collagen and laminin (Body, 2000).

2.4.2.2. Migration in the circulation and arrival at the bone

Cancer cells that intravasate into the blood vessels are named CTCs. If they resist to anoikis or to killing by the immune system, they can extravasate from the sinusoids and reach the bone marrow. For this, they express specific gene signatures that would be predictive of bone metastasis. The preferential attraction and engraftment of CTC to bone is probably the result of several mechanisms. One of them, called *hemodynamic*, is based on random migration of cancer cells. In 1928, Ewing proposed that the development of metastases in a given organ is dependent on the blood volume flowing into that organ. This theory has been proven in some examples of colon cancer metastasis (Body, 2000). However, it does not explain the high frequency of cancer metastasis to bone, where blood flow is much lower compared with that in other organs which are also preferential sites of cancer spread, such as lung and liver (Table 2) (Weiss et al., 1980).

Organ	Blood flow (ml/min/g)
Lung	13.6
Adrenal gland	6.5
Thyroid	5.0
Kidney	3.2
Spleen	0.9
Liver/Brain/Prostate/ Bone marrow	0.4
Spinal cord	0.1

Table 2. Arterial blood supply in target organs of cancer metastasis (Weiss et al., 1980)

Therefore, it has been proposed that breast and prostate cancer cells possess distinct capacities that not only direct them to bone, but also enable them to be attracted to bone then to survive and proliferate and further colonize the tissue.

2.4.2.3. Osteotropism

This non-random bone seeking is called *bone tropism* (also *osteotropism*). Different molecular mechanisms are responsible for this propensity of cancer cells to metastasize to bone. One of them, the chemokine receptor CXCR4 controls the metastatic destination of breast cancer cells in organs where its ligand, the chemokine CXCL-12 is produced in high quantity. An organ which is eligible for these criteria is lung, liver and bone marrow. It has been shown that the blockade of CXCR4 reduces the formation of experimental lung and bone metastases caused by CXCR4-expressing breast cancer cells (Liang et al., 2004). However, the inhibition of chemokine receptors only partially blocks metastasis formation, *in vivo*, suggesting that there are additional factors involved in the bone tropism of cancer cells.

Bone-derived cytokine RANKL triggers the migration of RANK-expressing cancer cells *in vitro*, and OPG, a natural inhibitor of RANK–RANKL interaction, blocks the bone tropism of these cancer cells *in vivo* (Cleuzardin and Teti, 2007) (Cleuzardin, 2011).

There is also evidence from preclinical research showing that integrins mediate metastasis to specific organs. For instance, $\alpha\beta3$ integrin overexpression in breast cancer cells enhances bone metastasis incidence in animals, and a nonpeptide $\alpha\beta3$ integrin antagonist causes a profound and specific inhibition of bone colonization by $\alpha\beta3$ -expressing cancer cells, *in vivo* (Zhao et al., 2007). $\alpha2\beta1$ integrin has been also shown to mediate bone colonization by

prostate cancer cells (Cleardin and Teti, 2007). It is possible that these integrins act in concert with CXCR4 and RANKL to promote the bone colonization by cancer cells.

Another important chemoattractant for bone is the proto-oncogene c-SRC. In many tumors, c-SRC is upregulated or hyperactivated thus affecting cancer cell properties linked to proliferation, motility and responses to growth factors (Cleardin and Teti, 2007). Reduced c-SRC activity in breast cancer cells decreases their malignant phenotype and osteotropism in experimental metastases (Rucci et al., 2006).

2.4.2.4. Osteomimicry

Osteomimicry is the acquisition of bone cell-like properties by tumor cells, which improve homing, adhesion, proliferation and survival in the bone microenvironment. Osteotropic malignant cells are able to express RUNX2 which is a master regulator of osteoblast differentiation and strong inducer of the expression of bone proteins. For example, bone matrix proteins, such as osteopontin, osteocalcin, osteonectin and bone sialoprotein II, are frequently highly expressed in breast and prostate cancers, which represent tumors with the highest propensity to colonize bone. They are also highly expressed in human breast cancer cell lines that form experimental bone metastases in mice. In our lab, a subclone variant from the human breast cancer cell line, MDA-MB-231 has been obtained by selection pressure in mice. This MDA-B02 cell line, highly and specifically metastasizes to bone and presents an osteoblast phenotype (Peyruchaud et al., 2001). A global transcriptome analysis performed on MDA-B02 cell line, has confirmed that several genes among those up- or down-regulated relative to the parental cell line, correspond to genes whose expression is associated with the osteoblast differentiation process. Also, the proteins such as: cadherin 11 (CDH11), cytochrome c oxidase subunit II (COX-2), connective tissue growth factor (CTGF), connexin 43 (CX43) and secreted protein, acidic, cysteine-rich (SPARC), which are overexpressed by MDA-B02 and known to be up-regulated during osteoblast differentiation, are selectively overexpressed in human breast cancer bone metastases compared to the primary tumor and liver metastases (Bellahcène et al., 2007).

These data suggest that malignant cells acquire osteomimetic properties, favoring the development of secondary lesion in bone microenvironment. The question remains if these

osteomimetic properties refer only to the osteoblast phenotype or if they can be extended to the osteoclast phenotype as well. For instance, it has been shown that cathepsin K, a highly specific osteoclast gene, is overexpressed in human breast cancer cells that metastasize to bone (Gall et al., 2007). Therefore, it seems that a multigenic mimicry program is crucial for a tumor cell to develop in bone, and that this program includes both osteoblastic and osteoclastic genes.

2.4.2.5. Entry into the bone marrow sinus and migration into the bone marrow cavity

There are four major arteries running into bone: the nutrient artery, the metaphyseal artery, the epiphyseal artery and the cortical capillaries communicating with the marrow arteries. Cancer cells arriving at bone enter the bone marrow cavity through one of these arterial routes. Cells penetrating to the bone marrow cavity must have certain capacities to make this step possible. It has been shown in vitro that cancer cells show preferential attachment to the endothelial cells of their in vivo target organs (McCarthy et al., 1991). The interactions between the endothelial and cancer cells are mediated by cell adhesion molecules (CAMs). One of them is vascular cell adhesion molecule 1 (VCAM1). When it is aberrantly expressed in breast cancer cells, VCAM-1 mediates distinct tumor stromal interactions facilitating metastasis to bone (Lu et al., 2011).

2.4.2.6. Colonization through interactions with bone cells-the vicious cycle

Among factors that regulate bone remodelling are systemic hormones and local factors such as interleukins, cytokines, colony-stimulating factors, eicosanoids, the RANKL/OPG axis and parathyroid hormone-related protein (PTHrP). In addition, many growth factors are synthesized by the osteoblasts and embedded into the bone matrix, mostly as inactive peptides, during the bone formation phase. These factors, which include TGF- β , platelet-derived growth factor (PDGF), BMPs, are then released from the matrix during bone resorption and activated both by the low pH created by the osteoclasts to remove the bone mineral, and by a set of proteases present in the microenvironment. Therefore, during bone resorption, the bone/bone marrow microenvironment is enriched in agents that regulate many cellular activities.

According to the “seed and soil” theory, these environmental factors provide a fertile ground in which tumor cells can grow. Moreover, during the formation of bone metastases, the perturbation of the microenvironment is initiated by the tumor cells that produce many factors

stimulating the osteoclasts, with the end point of an increased bone resorption. In addition, cancer cells secrete bone morphogenetic and Wnt protein antagonists (noggin, DKK-1) that inhibit osteoblast activity which, in turn, enhance the osteolytic pattern of bone metastases. It is a so called “*vicious cycle*” (Clezardin and Teti, 2007) (Figure 7).

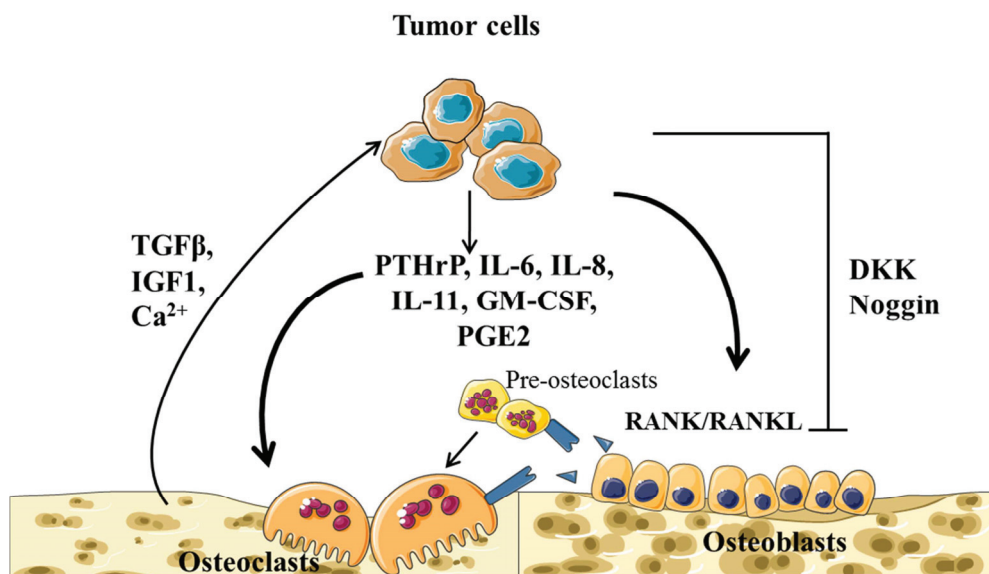


Figure 7. The vicious cycle.

The figure illustrates the many factors that stimulate on one hand osteoclast, osteoblast and vascular cells activity, and on the other hand tumor cell growth and survival in the bone microenvironment.

Bone metastases are likely to rely on multiple factors: the ability of cancer cells to exhibit specific receptor-ligand interactions that direct the cells to bone, the osteomimicry that leads to tumor development on the bone/bone marrow tissue and the microenvironmental factors which establish a vicious cycle. The stem cell niche maintaining cancer cell dormant until permissive conditions waken them up is also an important player in establishing bone metastases (Clezardin and Teti, 2007). Recently, it has been published that the perivascular niche regulates breast tumor dormancy. Specifically, work from Ghajar et al. reveals that stable microvasculature constitutes a dormant niche, whereas sprouting neovasculature sparks micrometastatic outgrowth (Ghajar et al., 2013).

3. In vivo models of breast cancer bone metastasis

3.1. Transplantable tumor model system

In this model, cancer cell lines or tissues can be grown in mice. There are various ways to mimic breast cancer growth and metastasis in tumor transplantation experiments. The primary and secondary metastatic growth is largely defined by the site of injection, together with the cell line specific tropism. Moreover, depending on the species or genetic background of donor and host, xenograft or syngeneic tumor transplantations can be distinguished (Fantozzi and Christofori, 2006).

3.1.1. Xenograft tumor transplantations

The majority of *in vivo* studies of bone metastases have used xenograft models, where human tumor cells are implanted in immunocompromised mice (Holen I. and Lawson M. A., 2014). The resultant tumors are a mosaic of human cancer cells and murine stromal cells. Cancer cell-stromal cell interactions are important in the biology of cancer progression and metastasis. For some pathways, it cannot occur across species boundaries. This limited tumor-stromal interaction is one of the major drawbacks in xenograft models. Secondly, in order for human tumors to grow in mice, the murine host must be immunocompromised to prevent immune rejection. Such an approach eliminates the ability to examine the role of the immune system in tumor progression in xenografts. Thirdly, each immunocompromised mouse strain is characterized by specific features that can influence the biology and study of metastasis. For instance, nude mice that are depleted in T cells and have impaired T and B cell function, have been shown to have impaired angiogenesis (Khanna and Hunter, 2005). Human tumor cells are now routinely engineered to express green fluorescent protein (GFP), luciferase or other tags, allowing non-invasive *in vivo* imaging of tumor development. Depending on multiple factors, such as: the cell line used, the age and strain of the animal, as well as the route of implantation, tumors will develop in bone between 3 and 5 weeks, providing the opportunity to study both the biological aspects of bone metastases and effects of therapy (Holen I. and Lawson M. A., 2014).

Human breast cancer cells for metastasis models can be injected intra-arterially, intracardially or intraosseously. First type of injection results mainly in lung metastasis, whereas intracardiac (i.c.) injection gives rise to a broader target organ spectrum, including bone (Khanna and Hunter, 2005). Intraosseous injection is generally used to investigate late-stage disease, as a large number of tumor cells are introduced directly into bone, bypassing the early steps of homing and colonization (Holen I. and Lawson M. A., 2014).

3.1.1.1. Intra-arterial injection

We set up a specific breast cancer cell lines with high propensity to metastasize to bone, by isolation of cells from bone metastases (originally introduced by i.c. injection), expanding the cells in culture and re-inoculating them into the left ventricle. Following six passages through bone marrow, a cell line that homes specifically to bone following intra arterial. injection has been generated.

With this approach, the MDA-B02 model has been developed. This is a bone-homing strain of MDA-MB-231 breast cancer cells (Peyruchaud et al., 2003), subsequently used to investigate whether therapeutic targeting of tumor $\alpha v \beta 3$ integrin prevents bone metastasis formation (Zhao et al., 2007).

3.1.1.2. Intracardiac injection

In this approach, human breast cancer cells are injected into the left ventricle of the heart. The cells will bypass the pulmonary vasculature resulting in widespread metastasis formation in several skeletal sites (mainly long bones) within 4–5 weeks (Holen I. and Lawson M. A., 2014).

The main disadvantage is that it does not represent the initial steps of the metastatic cascade, where tumor cells leave a primary site, enter the circulation and subsequently recolonize a distant site (Holen I. and Lawson M. A., 2014). Phadke et al. used the i.c. injection model to perform a detailed and informative study of the kinetics of breast cancer cell trafficking in bone. GFP-expressing MDA-MB-435 cells were injected into the left ventricle of female nude mice and animals were sacrificed at time points ranging from 1 h and up to 6 weeks. This allowed them to precisely localize breast cancer cells from the initial colonization of bone to advanced disease. One hour post-injection, tumor cells can be detected in the highly vascularized metaphysis of

the femur and they are gradually cleared over the next 72 h. One week after injection, small tumor cell foci were detected mainly in the distal metaphysis, and a few of these developed into larger tumors associated with loss of the majority of the trabecular bone during the following 2–3 weeks. Moreover, increasing numbers of tumor cells were associated with a substantial decrease in osteoblast number through induction of apoptosis. There was also a decrease in the number of osteoclasts and the osteoblast:osteoclast ratio was reduced from around 40:1 at baseline to 4:1 by 4 weeks, as bone turnover balance shifted towards increased resorption. This resulted in a rapid depletion of bone forming cells by an unknown mechanism, likely to prevent repair of tumor-induced osteolytic lesions. From the point of view of human disease these results suggest that therapeutic strategies to protect the osteoblast from the detrimental effects of tumor cells should be developed, for use in combination with anti-resorptive agents (Phadke et al., 2006).

3.1.1.3. Intraosseous implantation

Implanting cancer cells directly into bone can also be used to model bone metastasis, although, it is generally used to investigate late-stage disease. Most common site of intraosseous injection is intratibial (i.t.) injection. Holes are drilled in the tibiae of anesthetized animals, the bone marrow flushed out and replaced by a suspension of tumor cells. This will inevitably cause damage to the bone around the injection site, complicating analysis of tumor-induced changes of bone structure. Bone tumors are generated within 3–4 weeks, depending on cell lines.

3.1.2. Syngeneic models

Syngeneic models, unlike the xenograft models, provide the possibility to study the initial steps of metastasis by injecting murine tumor cells into the mammary fat pad of immunocompetent BALB/c mice.

4T1 is a commonly used murine breast cancer cell line that is inoculated into the mouse mammary gland to study tumor growth and cancer cell dissemination. This model allows to study growth of initial mammary tumor in an anatomically correct site, meaning mammary fat pad, followed by metastatic spread to a range of distant organs, thereby mimicking human breast

cancer. The main advantage is that the transplanted tissues, the tumor microenvironment and the host are from the same species, therefore, it can be used to study the role of the immune system in tumor progression in bone, as well as in response to anti-cancer therapy. However, syngeneic models do have some important limitations. The murine tumor cells differ from human breast cancer cells in the genetic makeup and potentially in their growth characteristics and responses to therapy. In addition, the primary mammary tumors grow quickly once established, leaving a limited window of opportunity for studies of the metastases unless the primary tumor is removed (Holen I. and Lawson M. A., 2014). Moreover, these model systems lack many of the important features of human tumors. For instance, they are usually derived from homozygously inbred mice and therefore lack the genetic complexity of human tumors (Khanna and Hunter, 2005). Most importantly, syngeneic models are not bone-metastasis specific, and a large number of animals are required in order to generate sufficiently large groups with skeletal involvement. There is also considerable variability in time for metastasis appearance in animals, presenting particular challenges in terms of when to initiate treatment and the timing of therapeutic interventions (Holen I. and Lawson M. A., 2014).

Historically, subcutaneous (s.c.) models were used due to rapid primary tumor growth. It was however, uncommon to observe spontaneous metastasis to distant organs. Application of “seed and soil” hypothesis resulted in the use of orthotopic transplantation of tumor cells into mice. Orthotopic inoculation refers to delivery of cancer cells to the anatomic location or tissue from which a tumor was derived. It is a model in which the primary tumor is formed followed by the development of metastasis (Khanna and Hunter, 2005). Orthotopic model in part resembles the multiple stages involved in malignant breast cancer development in patients. Therefore, it has the advantage over other models in which the direct introduction of cancer cells into the blood circulation should be considered rather as an assay of organ colonization and not a complete metastatic process.

Transplantable models have yielded many important insights into the potential molecular mechanisms of metastasis, however a number of important caveats remain. Neither injection of the tumor cells into the circulation, nor the orthotopic implantation is free of drawbacks. Tumorigenesis is not simply the result of proliferative activation of a mutated cell, but rather is a complex interaction between neoplastic tissue and the stromal and environment in which it arises. Injection of the tumor cells does not necessarily recapitulate all of the interactions and

microenvironmental components that may play important roles in tumor dissemination. Important issue is also mechanical disruption of the target tissue during the implantation process which may permit escape of tumor cells into the circulatory system at the time of implantation, thus seeding distant sites at the onset of the experiment rather than subsequent to tumor growth, as is the intent. A further common caveat of transplantable models is their reliance on cultured cells. During the selection process and/or subsequent cell culture, the resulting cell lines and clones have been passaged *in vitro*. Thus, the cells are adapted to growth on a two-dimensional rather than a normal three-dimensional matrix platform, in an artificial or foreign milieu. The adaptations that permit perpetual growth in tissue culture may have significant impact on the pathways and mechanisms by which spontaneous metastases arise. While these experimental systems may imply some of the mechanisms and biological processes that function in tumor dissemination, we have to be aware that they only partially represent those steps in the metastatic process (Khanna and Hunter, 2005).

3.2. Genetically engineered mouse models of metastasis

To complement transplantable models, investigators need access to autochthonously arising tumors capable of completing the entire metastatic process. Here, genetically engineered models have the application. They encompass a variety of tissue types, with differing degrees of penetrance and different latencies. While it is possible that the constitutive activation or loss of a gene in these models may not completely replicate native metastasis, the tumors do arise in their normal context, more closely replicating the clinical setting and in a system possessing a functional immune system. They also permit the investigation of important determinants of cancer that are difficult or impossible to address using cell culture and transplant systems including the influence of genetic heterogeneity on tumor phenotype (Khanna and Hunter, 2005).

3.3. Human-to-human models

These models have been developed in order to capture the species-specific interactions involved in homing of breast cancer cells to bone. Pieces of human bone are implanted

subcutaneously (s.c.) in immunocompromised animals and subsequently, human breast cancer cells are injected i.a., in the mammary fat pad or directly into the bone marrow cavity.

The main advantage of these models is that the tumor cells have an access to the human bone microenvironment, although the bone fragments used are either fetal or from patients undergoing hip replacements, not from patients with bone metastases. In order to generate consistent bone metastasis, cancer cells must be introduced directly into bone, whereas tumors implanted in the mammary fat pad generate low frequency of bone colonization. These results are in agreement with the observation that not all breast cancer cells home to bone even when the right microenvironment is available. It might be due to a requirement that the human bone actually contains the appropriate niches. Also, there may be problems with a graft versus graft immune response, as the human bone and the human tumor cells are of different origin (Holen I. and Lawson M. A., 2014). Nevertheless, human-to-human models are becoming important as we seek to understand the pivotal role of the bone microenvironment in the development of bone metastases.

4. MicroRNAs

MiRs, a class of small noncoding RNAs are cell endogenous regulators of gene expression. Within 10 years of research, we have gone from discovering the existence of miRs in mammals to exploring their therapeutic applications in numerous diseases, including cancer.

4.1. Biosynthesis of microRNAs

MiRs are evolutionary conserved, small non-coding, single-stranded RNA molecules of around 19-23 nucleotides in length. These miRs are predicated to regulate more than 50% of the human protein coding genes. Mature miRs are generated via a two-step processing pathway (Figure 8) (Lee et al., 2002).

The miR genes are first transcribed by RNA polymerase II into primary transcripts (pri-miRs) in the nucleus. Initial cleavage is catalyzed by Drosha, a RNase III nuclease existing in a multiprotein complex called the *microprocessor*, (Drosha-DGCR8 (Pasha)), which produces pri-miRs of around 60 nucleotides in length (Denli et al., 2004; Gregory et al., 2004). The

resulting precursor hairpin, the pre-miR, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase III enzyme, Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miR hairpin to its mature length (Bernstein et al., 2001). The functional strand of the mature miR is loaded together with Argonaute 2 (AGO2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target messenger RNAs (mRNAs) through either mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is often degraded (Winter et al., 2009).

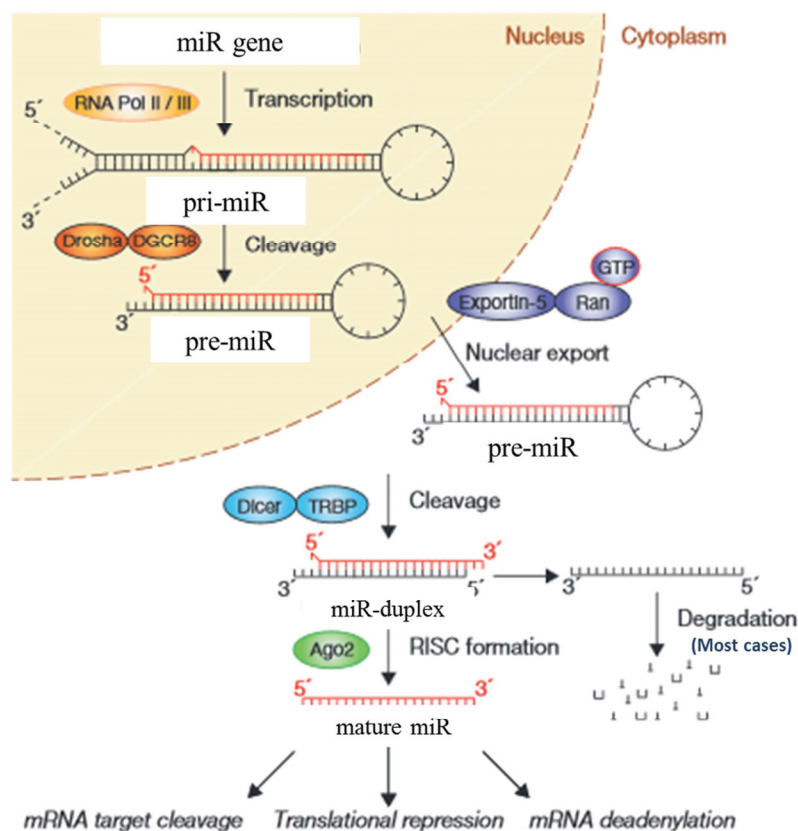


Figure 8. MicroRNA biogenesis.

The primary miR transcript (pri-miR) is produced by RNA polymerase II or III and is cleaved by the microprocessor complex Drosha–DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miR, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miR hairpin to its mature form. The functional strand of the mature miR is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation. The passenger strand is often degraded (adapted from (Winter et al., 2009)).

Numerous studies have uncovered highly specific miR profiles during development or tumorigenesis. Their functions as important regulators of differentiation, proliferation, apoptosis or metabolism are extensively studied.

4.2. MiRs discovery and first experiments

The discovery by Lee, Feinbaum and Victor Ambros, on the role of the *lin-4* and *lin-14* genes in temporal control of development in the model organism *C. elegans* has busted an era of intense miR studies (Lee et al., 1993).

They showed that *lin-4* gene does not encode a protein product, but instead gives rise to a 61-nucleotide precursor gene that matured to a more abundant 22-nucleotide. Also, it has been found that *Lin-14* levels are inversely proportional to those of *lin-4* RNA and that it is complementary to the 3' untranslated region (3'UTR) of the *lin-14* gene. This led to the hypothesis that *lin-4* regulated *LIN-14*, in part, through Watson–Crick base pairing, revealing the first miR and mRNA target interaction (van Rooij, 2011). For 7 years, *lin-4* was considered an anomaly, until the discovery of a second *C. elegans* miR, called *let-7*, which repressed *lin-41*, *lin-14*, *lin-28*, *lin-42*, and *daf-12* expression during development (Reinhart et al., 2000). *Let-7* homologs were found in many vertebrate species including humans, which lead to the finding that miRs are evolutionarily conserved across many species and are often ubiquitously expressed (van Rooij, 2011).

In 2002, shortly after the expression of mammalian miRs was recognized, Calin et al showed a correlation between the loss of miR-15 and -16 and the occurrence of B-cell leukemia (Calin et al., 2002). In 2006, the first cardiac miR-profiling study appeared, linking dysregulation of many different miRs to cardiac remodeling in both mice and humans. Today, microarray analysis and deep-sequencing approaches enable researchers to correlate the dysregulation of miRs to the progression of many different diseases in both animals and humans. When such a disease-specific miR expression signature was first characterized, researchers started investigating whether these expressional changes were causally related to disease. Although many *in vitro* studies indicated prominent and defined functions for miRs in different aspects of cell biology, the first genetic evidence for the importance of miRs in mammals came when it was

shown that homozygous deletion of Dicer disrupted prenatal development of the murine embryo through its role in miR biogenesis (Yang et al., 2005).

4.3. MiR-mediated regulation of bone metastasis formation

Bone metastasis is a multi-step process involving a series of molecular events that might already be genetically determined in primary tumours. The misexpression of miRNAs in tumour-initiating cells might up- or down-regulate master genes that drive invasion, extravasation and finally bone seeding of cancer cells. Besides these regulatory mechanisms within tumour cells themselves, miRs interfere with tumour-stroma cross-talks within the bone (Ell et al., 2013). In this way, stroma-tumour interactions may be mediated by miRNA transfer through cell-to-cell interactions or exosomes to influence osteoclastogenesis and metastasis-related bone destruction.

4.3.1. Involvement of miRs in the early stage of metastatic dissemination

Molecular pathways underlining bone metastasis formation are regulated by multiple factors, through the tight control of genes expressed by cancer cells interacting stroma and with cells from the bone microenvironment. In this context, miRNAs can act as master regulators of gene expression to control multiple aspects of bone metastasis formation, including cancer cell escape from the primary tumour site, cancer cell dissemination to bone, invasion of the bone marrow, as well as secondary outgrowth and tumour-stroma cell interactions (Croset et al., 2015). A small subset of cancer cells in breast primary tumours express specific gene signatures that would be predictive of bone metastasis (Smid et al., 2006) (Kang et al., 2003). In this respect, the identification of miRNA and gene signatures expressed in primary carcinomas and their matching bone metastases provide information on molecular mechanisms driving bone metastasis. It is very likely that before patients are diagnosed with cancer, dormant disseminated tumour cells (DTC) are already present in the bone marrow (Aguirre-Ghiso, 2007). Given this information, dysregulated miRNAs that are associated with the early onset of bone metastasis might be a useful clinical biomarker of the minimal residual disease in patients with cancer. Tavazoie et al. identified human miRs that affect breast cancer metastasis to lung and bone. MiR profiling of the MDA-MB-231 cell line was compared to its derivatives that are highly metastatic to bone and lung. Hierarchical clustering defined a set of miRs (miR-335, -126, -206, -122a, -199*a, and -489) whose expression was decreased in the two metastatic derivatives. Restoration of miR-335,

miR-126, or miR-206 expression in LM2 and BoM2 cells reduced their colonizing activity in both lung and bone when they were inoculated to mice by the intravenous route or orthotopically (Tavazoie et al., 2008). MiR-126 suppresses tumorigenesis and metastasis, in part, through an inhibition of tumor cell proliferation whereas miR-335 and miR-206 do not affect primary tumor outgrowth, but reduce migratory and invasive capacities of these tumor cells. MiR-335 suppresses breast cancer cell invasion and metastasis by targeting the transcription factor SOX4 and tenascin C, proteins involved in cell adhesion. Additionally, expression levels of miR-335 and miR-126 are low in breast tumors from patients who relapse, further indicating that these miRs act as metastasis suppressors in breast cancer.

Studies performed on human primary prostate tumors and matched bone metastasis showed that expression levels of miR-145, -143, -33a, -100, and -508-5p were the most highly downregulated in bone metastasis specimens. The expression levels of several miRs previously reported as metastasis suppressors (miR-125b, 30e, c, -99a, -20b,c, let7) were also deregulated to a lesser extent. Ectopic expression of miR-143 and miR-145 in the aggressive and androgen-insensitive PC-3 prostate cancer cell line reduced the migratory and invasive capacities of these tumor cells *in vitro* and their propensity to metastasize to bone *in vivo*. Neither miR-143 nor miR-145 interfere with the tumorigenic and metastatic properties of the hormone-responsive and weakly-metastatic LNCaP prostate cancer cell line. Upon upregulation of these two miRs, the expression of the mesenchymal markers fibronectin and vimentin (VIM) decreased, whereas E-cadherin expression increased, in PC-3, but not in LNCaP cells. Further, oncogenic protein HEF1 (human enhancer of filamentation, also known as NEDD9) was shown to partially mediate miR-145 suppression of epithelial-mesenchymal transition (EMT) and invasion (Peng et al., 2011). In a retrospective investigation of 12 patients without bone metastasis and 10 patients with bone metastasis the low expression levels of these two miRs were negatively correlated to bone metastasis, the level of free prostate-specific antigen (PSA) and the Gleason score. Therefore, miR-143 and miR-145 can be potential candidates for biomarkers in discriminating different stages of prostate carcinomas and predicting bone metastasis.

Regarding metastasis-promoting miRs, Huang et al. found that miR-373 and miR-520c, two miRs that share the same seed sequence, promoted cancer cell migration and invasion *in vitro* and *in vivo* and that several cell lines required miR-373 expression in order to be invasive. *In vivo* studies on SCID mice were conducted by tail-vein injection with either MCF-7 cells

expressing miR-373 or miR-520c (miR-MCF-7) or a mutant of miR-373 (Mut-MCF-7) or a control vector (Ctrl-MCF-7). Metastatic nodules appeared in the skull, spine and lungs in the mice inoculated with miR-MCF-7 but not in the mice inoculated with Ctrl-MCF-7 or Mut-MCF-7. Histological analysis showed osteolytic metastases infiltrating into the medullary bone spaces and skull (Huang et al., 2008). Evidence of metastasis-inducing potential of miRs was reported by Weinberg's laboratory when they identified miR-10b as being highly expressed in metastatic breast tumors and promoting tumor cell migration and invasion. 50 % of the metastasis-positive patients had elevated miR-10b levels in their primary tumors when compared to metastasis-free patients. This was in agreement with the observation that miR-10b is highly expressed in metastatic breast cancer cell lines. When miR-10b was overexpressed in otherwise non-metastatic breast tumor cell lines, it initiated strong invasion of orthotopically implanted mammary tumors in animals and promoted dissemination of tumor cells in the lungs. The transcription factor TWIST directly induced miR-10b expression which, in turn, inhibited the expression of the transcription factor homeobox D10 (*HOXD10*) (Ma et al., 2007). Interestingly, Twist1 is expressed in disseminated breast cancer cells that persist in the bone marrow after chemotherapy (Watson et al., 2007). In an experimental model of human breast cancer bone metastasis, Twist1 facilitates bone metastasis formation through a miR-10b-dependent mechanism (Croset et al., 2014). Moreover, miR-10b could be a circulating marker of bone metastasis in regard of its high level in serum of breast cancer patients with bone metastasis (Zhao et al., 2012). The potential of miR-10b as target for anti-metastatic therapy has been therefore studied in an animal model of breast cancer metastasis. MiR-10b antagomir was delivered to rapidly growing tumor cells preventing metastasis dissemination to secondary organs without marks of cytotoxicity (Ma et al., 2010).

4.3.2. Involvement of miRs in tumor cell homing and colonization of bone marrow

MiR-203 have an anti-metastatic role in prostate cancer progression and bone metastasis (Saini et al., 2011). Its level in human prostate adenocarcinoma is lower than in normal adjacent tissue. This low miR-203 expression correlates with advanced clinical-pathological stage and high Gleason score in patients with prostate cancer. Moreover, miR-203 expression is downregulated in prostate cancer cell lines derived from bone metastasis (PC-3, MDA-PCa-2b, and VCaP cells). Ectopic expression of miR-203 significantly decreases the metastatic capacity

of PC-3 cells in a mouse model of bone metastasis. The phenotype of miR-203-transfected cells (marked morphological changes, from fibroblasts- to epithelial-like phenotype, and reduced migration/invasion) is reminiscent of a reverse transition from an EMT to a mesenchymal-to-epithelial state. Moreover miR-203 decreases proliferation and increases apoptosis of PC-3 cells. MiR-203 repressed a cohort of metastatic genes including a master regulator of bone metastasis, the bone-specific transcription factor RUNX2. Additionally, expression levels of two key osteoblastic genes, OPN and OCN, were decreased, suggesting that miR-203 may also inhibit osteomimetic properties of PC-3 cells. Collectively, this work identified a miR-203 regulatory pathway that acts at multiple steps of prostate cancer progression in bone, inhibiting the colonization of the bone marrow by prostate cancer cells. Iorio et al. performed a genome-wide miR expression analysis in a set of normal and malignant breast tissues. They reported that the miR-30s were expressed at higher levels in hormone-dependent (ER+/PR+) and well differentiated breast tumors, compared with that observed in hormone-independent tumors (Iorio et al., 2005). Moreover, they have been shown to act as tumor suppressors in anaplastic thyroid carcinoma (Visone et al., 2007), lung (Kumarswamy et al., 2012a), prostate (Kao et al., 2013) and breast (Zhang et al., 2013) cancers, mostly by regulating EMT. MiR-30s might play a role during metastatic dissemination of breast carcinomas, as their expression levels was lower in lymph node metastases versus primary tumors (Baffa et al., 2009). TGF- β is released from bone in the microenvironment during bone resorption, and it stimulates cancer cells to produce pro-osteolytic factors such as IL-11. Pollari et al. identified three miRs, miR-204, miR-211, and miR-379, as potent inhibitors of TGF- β -induced IL-11 secretion. In addition, gene expression analysis showed that miR-204 and miR-379 downregulated a set of genes involved in TGF- β signaling (Pollari et al., 2012). MiR-33a is a tumor suppressor that target PTHrP, a potent stimulator of osteoclastic bone resorption, in lung cancer. MiR-33a levels are inversely correlated with PTHrP expression between human normal bronchial cell lines and lung cancer cell lines. When miR-33a expression was restored in lung cancer cells, the production of PTHrP, IL-8 (another stimulator of osteoclast-mediated bone resorption) and OPG decreased. Thus, low miR-33a expression contributes to cancer-mediated bone destruction and may even predict a poor prognosis for lung cancer patients (Kuo et al., 2013). Rho-associated kinase (ROCK) signaling plays an important role in regulating cell morphology, adhesion, and motility. ROCK is involved in the enhanced ability of cancer cells to detach and become motile and is highly expressed in primary breast

tumors that give rise to metastasis. It has been shown that inhibition of ROCK activity reduces cell migration and metastasis to bone *in vivo*. A downstream target of ROCK is c-myc which facilitates the dissemination of MDA-MB-231 breast cancer cells to bone. Coupled to increased c-myc, miR-17-92 cluster expression is increased in metastatic MDA-MB-231 breast cancer cells *in vitro* and *in vivo*. The intratumoral administration of anti-miR-17 molecules into mice bearing MDA-MB-231 tumor xenografts greatly repressed bone metastasis without significant effect on primary tumor growth (Liu et al., 2009). Therefore, inhibition of ROCK/c-myc by anti-miR-17 strategy might prevent breast cancer bone metastasis. Another miR with reported oncogene role is miR-218. It appears as an osteo-miR by controlling bone formation through the upregulation of RUNX2 and the downregulation of Wnt signaling inhibitors (sclerostin, dickkopf-2, and secreted frizzled-related protein2) in osteoblasts. Additionally, miR-218 stimulates the expression of Wnt-related proteins (bone sialoprotein and osteopontin) in metastatic breast cancer cells. Moreover, miR-218 may facilitate MDA-MB-231 breast cancer bone metastasis by upregulating CXCR4, a chemokine receptor supporting breast cancer cell migration to bone and mediating tumor growth in bone (Hassan et al., 2012). Although miR-218 regulates positively the molecular mechanisms underlying bone metastasis formation it appears as tumor suppressor in other types of cancers (Tie et al., 2010), suggesting that its biological activity depends on the cellular microenvironment.

Evidence that the bone is able to produce miRs that modulate the growth of DTCs has been reported (Ono et al., 2014). There are also reports that cancer cells can modify the microenvironment to become more favorable for metastatic tumor initiation. Conditioned media from highly metastatic cell lines are able to induce osteoclast differentiation (Ell et al., 2013). Indeed, exposure of differentiating osteoclasts to tumor-conditioned media activates a specific miRNA signature in osteoclasts that is partly induced by the secretion of sICAM1 by bone-metastatic cells. This suggests that miRNAs from the tumor cells may also alter the bone microenvironment, disrupting bone turnover.

MiRs activity in interfering with bone colonization relies on mRNA targets whose expression is altered in bone metastasis. Uncovering the genes under the control of these miRs and how they integrate into the bone metastatic gene interaction network will further help us understand the disease and hopefully lead to new targeted therapies. Identifying these targets and transferring their bone metastasis-suppressive activity from bench into clinical settings for

predictive biomarkers and/or treatment of bone lesions is essential. The already existing miR-based therapeutic approach to interfere cancer is an attractive one. However, it still remains puzzling, mainly due to the large numbers of transcripts sometimes with opposing functional consequences that could be targeted by a single miR. Therefore, the efficiency and safe delivery of agents such as miR mimics or antagomirs to a growing metastatic tumor or to already-established metastasis need to be further studied.

4.4. MicroRNA-30 family

4.4.1. Genomic arrangement of miR-30 family

In most multi-cellular organisms there are hundreds of miR genes. MiRs can be located within exons, or introns of coding or non-coding transcripts. Some miR genes are intergenic and not subject to splicing. MiRs can also be co-transcribed as part of one primary transcript in cluster, which can be intronic or exonic (Figure 9) (Finnegan and Pasquinelli, 2013).

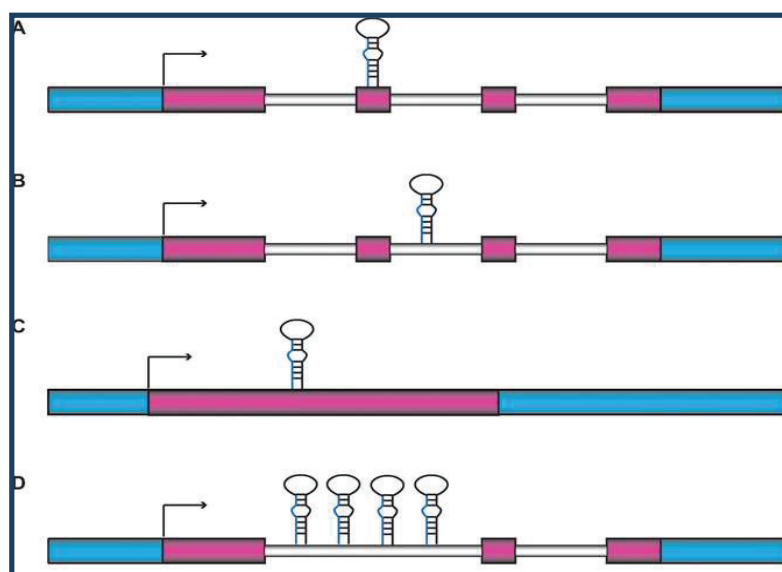


Figure 9. MiR genomic locations.

MiR can be located within exons (A) or introns (B) of coding or non-coding transcripts. Some miR genes are intergenic and not subject to splicing (C). Multiple miRNAs constitute miR clusters co-transcribed as part of one primary transcript, which can be intronic (D) or exonic (not shown) as part of a coding RNA or a non-coding RNA. Blue represents non-transcribed DNA, pink represents exons, and white represents introns. Adapted from (Finnegan et al, 2013).

The miR-30 family is made up of five members in human, encoded by six genes: *a*, *b*, *c-1*, *c-2*, *d* and *e* which share a conserved seed sequence. They are encoded in the genome in three clusters: miR-30 *e* and *c-1* cluster together on chromosome 1 which is found within a nuclear transcription factor Y gamma (NFYC) gene; miR-30*d* and *b* on chromosome 8 and miR-30*a* and *c-2* cluster on chromosome 6.

In mouse, there are six members of the family, encoded by seven genes: *a*, *b*, *c-1*, *c-2*, *d*, *e* and *f*. MiR-30*e*, *f* and *c-1* cluster together, like in human, in the exon of *Nfyc* gene, but on chromosome 4. Mir-30*d* and *b* cluster on chromosome 15 and miR-30*a* and *c-2* cluster on chromosome 1.

Regarding the similarity between the members of the family, they are conserved between human and mouse. Not a single base is changed between these two organisms (Table 3).

	Mouse sequence	Human sequence
30a	UGUAAACAUCCUCGACUGGAAG	UGUAAACAUCCUCGACUGGAAG
30b	UGUAAACAUCCUACACUCAGCU	UGUAAACAUCCUACACUCAGCU
30c-1	UGUAAACAUCCUACACUCUCAGC	UGUAAACAUCCUACACUCUCAGC
30c-2	UGUAAACAUCCUACACUCUCAGC	UGUAAACAUCCUACACUCUCAGC
30d	UGUAAACAUCCCGACUGGAAG	UGUAAACAUCCCGACUGGAAG
30e	UGUAAACAUCCUUGACUGGAAG	UGUAAACAUCCUUGACUGGAAG

Table 3. Comparison of genomic sequences of miR-30 family members between mouse and human.

(based on <http://www.mirbase.org/>)

There is one base difference between the member *a* and *e* or *d*. Thus, between *d* and *e* this difference is of 2 nucleotides. On the other hand, members *b* and *c* differ in 7 or 6 nucleotides respectively when compared to member *a*. The member *b* is of the same length, even though, there are two deletions in its sequence. Member *c* is one base longer (Table 4).

MiR-30 member	Sequence	Length
30a	UGUAAACAUCCUCGACUGGAAG	22
30d	UGUAAACAUCC <u>C</u> CGACUGGAAG	22
30e	UGUAAACAUCCU <u>U</u> GACUGGAAG	22
30b	UGUAAACAUCCUACACUC - - AGCU	22
30c	UGUAAACAUCCUACACUCUCAGC	23

Table 4. Differences between miR-30 family members in the nucleotide sequence

Nucleotides that are different are marked in red. Deletions are marked with a hyphen.

Based on <http://www.mirbase.org/>

Typically, a family of miRs includes all miRs, regardless of genomic location, that share 2–7 nucleotides, called the *seed sequence* (Bartel, 2009). In miR-30 family, all members share the same seed sequence 5' **UGUAAAC** 3'. By this sequence, miR-30 members bind to target 3'UTR region of the mRNA based on the antisense complementarity. If the complementarity is perfect, the target mRNA is cleaved and the miR acts as a siRNA. If the complementarity is partial, the translation of the target mRNA is repressed (Nelson et al., 2003).

4.4.2. The biological role of miR-30 family

MiR-30 has been reported to stimulate adipogenesis (Zaragosi et al., 2011), B-cells differentiation (Lin et al., 2011), pronephros development (Agrawal et al., 2009), endothelial cell behavior (Bridge et al., 2012), structural changes in myocardium (Duisters et al., 2009) and cellular senescence (Martinez et al., 2011). MiR-30s can suppress apoptosis in the heart (Li et al., 2010), osteoblast differentiation (Wu et al., 2012) and EMT (Braun et al., 2010; Joglekar et al., 2009; Zhang et al., 2012), a process which correlates with more invasive potential of cancer cells.

Regarding cancer disease, in the majority of reports miR-30s are described as tumor suppressors. Reduction of miR-30s has been correlated with more invasive properties of cancer cells. Downregulation of the family in aggressive BT-ICs (breast tumor initiating cells) results in the maintenance of self-renewal and apoptosis inhibition in these cells (Yu et al., 2010). In this view, miR-30a reduction may correlate with the expansion of putative BT-ICs. In addition, miR-30d overexpression suppresses cell proliferation and induces apoptosis in renal carcinoma cells (Wu et al., 2013). By targeting Wnt/ β -catenin/BCL9 (B-cell CLL/lymphoma 9) pathway, miR-

30s reduce tumor burden and metastatic potential *in vivo* in murine models of human multiple myeloma (Zhao et al., 2014). It has been also shown that miR-30c influences the chemotherapy resistance of breast cancer cells. In fact, by targeting twinfilin 1 (TWF1) and IL-11, a secondary target of TWF1, miR-30c reduces chemotherapy resistance of these cells (Bockhorn et al., 2013). MiR-30c expression correlates with endocrine therapy resistance in advanced ER-positive breast cancer (Rodríguez-González et al., 2011). In this study, miR-30c has been suggested as a predictor of endocrine therapy in ER+ breast cancer. Interestingly, it has been reported that miR-30 family members are all downregulated in both ER and PR negative tumors, suggesting that expression of these miRs is regulated by these hormones (Iorio et al., 2005). Indeed, miR-30 have been recently shown to be downregulated by progestins (Cochrane et al., 2012).

Despite a well-recognized role of tumor suppressor in epithelial cancer and activity to oppose EMT and to maintain epithelial phenotype, miRs-30 has been reported as oncomir. A miR analysis of human melanoma, a highly invasive cancer, found that miR-30b/30d upregulation correlates positively with stage, metastatic potential, shorter time to recurrence, and reduced overall survival. Ectopic expression of miR-30b/30d promoted the metastatic behavior of melanoma cells by directly targeting the GalNAc transferase GALNT7, resulted in increased synthesis of the immunosuppressive cytokine IL-10, and reduced immune cell activation and recruitment (Gaziel-Sovran et al., 2011). MiR-30c actually promotes the invasive phenotype of MDA-MB-231 by a mechanism dependent on NOV gene (Dobson et al., 2014). Taking into account the high expression of miRs-30, it is important to investigate the impact of their dysregulation in specific tissue, pathology and cell lines. Depending of gene expression in the considered tissues, miRs-30 would have different regulating activity. As a consequence of their different location in genome, they may be differently regulated, especially in cancer, and this will impact on their own regulative property.

PART 2

EXPERIMENTAL WORK

1. Objective of the project

The early steps of breast cancer metastasis to bone are common to most solid tumors and start with local tissue invasion and tumor cell escape from primary tumors. Following tumor cell dissemination into the blood and/or lymphatic system, cancer cells that express a specific gene signature disseminate to bone and invade the bone marrow. If they adapt to the local microenvironment and exit the dormancy step, they may further colonize the tissue. Each of the molecular pathways underlining these steps is regulated by multiple factors, through the tight control of genes and their master cell endogenous regulators, the miRs.

The objective of this work is to find out if tumour-derived miRs act as endogenous regulators of bone metastasis, in breast cancer. We have first studied miR activity in the steps of bone tropism, bone homing and engraftment of cancer cells and subsequent osteolytic lesions of bone. We used a model of human bone metastasis *in vivo*, in which a tumor cell line that highly and specifically metastasizes to bone is injected in caudal artery. We have also studied the potential activity of miRs-30 in breast tumorigenesis and dissemination of cancer cells, the early steps of metastasis in a syngeneic orthotopic mouse model, based on mammary gland injection of breast cancer murine cells.

Overall, we looked for miRs that could be prognosis and/or diagnosis markers of bone metastasis and might represent potential therapeutic targets of the disease.

1.1. The tumor suppressor miRs-30-5p family regulates human breast cancer cell colonization in bone.

“Manuscript in preparation for publication”

The Tumor Suppressor miRs-30-5p Family Regulates Human Breast Cancer Cell Colonization in Bone

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Running title: The tumor suppressor miRs-30-5p family in the control of metastatic bone disease

Key words: Bone metastasis, MicroRNA, MicroRNA-30, Breast cancer, osteomimicry

Abstract

Breast cancer cells that escape primary tumors disseminate to bone with a high affinity at late-stage disease. Breast tumor cells that preferentially invade bone, express a set of deregulated genes that enhance bone tropism and facilitate bone marrow engraftment which may lead to the formation of overt osteolytic lesions. MicroRNAs are endogenous regulators of gene expression that target bone turn-over and pro-metastatic genes. To identify genes involved in bone metastasis, we established microRNA transcriptomic profiling of osteotropic cell lines and identified drastic down-regulation of the miR-30 family (miRs-30), composed of miR-30a, miR-30b, miR30c, miR30d and miR30e. Induced over-expression of miRs-30 in a breast cancer cell line that is highly and specifically metastatic to bone inhibited bone metastasis in an experimental model of human bone metastasis, *in vivo*. Moreover, specific to bone, miRs-30 inhibited osteoclasts and stimulated osteoblasts through regulation of IL-11 and WNT signaling DKK1, respectively. Consistent with this, miRs-30 reduced expression of osteomimetic genes, *ITGβ3*, *ITGα5*, *CDH11*, *CTGF* and *RunX2*, and of oncogenic genes, *BCL9*, *MTDH*, *NT5E*, *TNC* and *NEDD4* and impacted negatively on tumoral invasion. In the clinic, the low expression of miR-30a-5p, miR-30d-5p and miR-30e-5p in primary tumors from patients with breast cancer was associated with poor distant metastasis-free survival.

In conclusion, we identified for the first time, miRs-30 as inhibitors of bone metastasis from breast cancer which may constitute a potential therapeutic in breast cancer-induced bone lesions.

Introduction

Breast primary carcinoma is one of the most common osteotropic tumors that spread to skeleton with high affinity at late-stage disease (1),(2). In breast cancer, osteolytic bone metastasis lead to occurrence of skeletal-related events, such as fractures, spinal cord compression, bone pain and disability and for which only palliative treatments are proposed (3),(4). Tumor-induced alteration in bone homeostasis disrupts the physiological balance between bone resorption and bone formation to promote bone destruction (5). Once in bone marrow, tumor cells may stay in the dormancy stage for decades as disseminated tumor cells to be reactivated, to proliferate, altering the functions of osteoclasts and osteoblasts and hijack signals coming from the bone matrix (6). Following escape from the primary tumor, they must express a specific gene signature to be attracted to bone, invade the bone marrow, adapt to the local microenvironment and finally exit the dormancy step to colonize bone (7). A classical view argues that cancer cells cumulate genetic alterations and express a specific set of genes that act cooperatively to render them competent for bone marrow engraftment (8),(9),(10). Indeed, transcriptional profiling of primary tumors has revealed gene signatures that correlate with metastatic relapse, enhanced invasive phenotype, increased angiogenesis and ability to modify the bone microenvironment (9),(11),(12).

How cancer cells are acquired into the regulatory network to lead such deregulation of gene expression is not fully understood. In that, the endogenous small non-coding RNAs, microRNAs (miRs) are able to post-transcriptionally regulate the expression of numerous genes and are attractive candidates to regulate metastatic dissemination (13),(14),(15). Indeed, abnormalities in their expression confer their properties as tumor suppressors or oncomirs, depending on the silenced cognate genes (16). Large sets of miRs are underexpressed in tumors compared to normal tissue, whereas miR genes located in fragile chromosomal regions are susceptible to amplification or deletion, often promoting tumorigenesis and metastatic dissemination (17). Insight into the molecular mechanisms of cancer cell dissemination to tissues has been obtained through associations between miR expression in primary tumor and metastasis with the involvement of specifically cognate genes during metastasis formation (18),(19).

Here we asked whether miRs are involved in bone metastasis formation and progression in breast cancer, and whether modulation of their expression might regulate specific steps of bone metastasis. Comparative expression of miRs in human breast cancer cell lines, MDA-MB-231 (non-osteotropic), MDA-B02 (an osteotropic subpopulation of MDA-MB-231) and micrometastases from bone marrow aspirate (BC-M1) identified the miR-30 family (miRs-30) as being down-regulated in osteotropic cell lines,

MDA-B02 and BC-M1. Screening for miRs-30 gene targets in predictive databases identified gene sets that are associated to tumor cell invasiveness, bone homing and colonization as well as bone physiology. Therefore, we have studied the role of miRs-30 in bone metastasis using a murine pre-clinical model of these metastasis.

Materials and Methods

Cell lines and cell culture

The human MDA-B02 cell line (MDA-B02) is a subpopulation of the MDA-MB-231 breast cancer cell line that was selected for the high efficiency with which it metastasizes to bone in animals (20). These cell lines were authenticated using short tandem repeat analysis. Human breast cancer lines, MDA-MB-231, Hs578T, T47-D, MCF-7, BT-474, ZR-751 and SK-BR were obtained from the American Type Culture Collection. MDA-MB-231 and MDA-B02 were cultured in 1 g/L glucose DMEM supplemented with 10% FBS and Hs578T in the same medium supplemented with 0.2 Units/mL of bovine insulin. ZR-751 was cultured in RPMI 1640, 10% FBS, MCF-7 and T47-D in the same medium supplemented with 0.2 Units/mL insulin. SK-BR3 was cultured in ATCC-formulated McCoy's medium modified, 10% FBS. BT-474 was cultured in ATCC Hybri-Care medium supplemented with 1 g/L sodium bicarbonate and 10% FBS. BC-M1, a cell line immortalized with DNA encoding for SV40 large T antigen, is a G0 state dormant DTC, obtained from bone marrow aspirate of a breast cancer patient with no signs of metastasis at time ((21). This cell line was cultured in RPMI 1640, supplemented with 1X IST (Insulin, Selenium, Transferin, Gibco), 50 ng/mL epidermal growth factor, 10 ng/mL human basic fibroblast growth factor (Mylenyi Biotec) and 10% FBS.

Cell transduction and transfection

MDA-B02 was transduced using the amphotropic retroviral packaging system (ClonTech Laboratories, Takara Bio Company, CA, USA). Briefly, GP2-293 cell line which has the viral gag and pol genes incorporated in its genome were transfected with the pantropic VSV-G envelope vector and the retroviral pmiR-Vec vector (kindly provided by Dr R. Agami, The Netherlands Cancer Institute, Amsterdam). Virus particles were collected at 72h after transfection, filtered and used to transduce the recipient cells in presence of 8 µg/mL polybrene. Vector transduced cells were selected with blasticidine.

Transient overexpression or downregulation of miRs-30 in MDA-B02 and MDA-MB-231 was achieved by transfecting the cells with chemically modified double-stranded RNAs that mimic endogenous miRs

(miRNA-mimics, Applied BioSystems) or chemically modified single-stranded RNA molecules designed to specifically inhibit endogenous miRs (miRNA-inhibitors, Applied BioSystems). Both cell lines were transfected with 10nM of each miR-30s mimics or antagonist sequences or with 50 nM of negative scramble controls (Applied BioSystems). These oligonucleotides were incubated with 10^6 cells in appropriated medium, in the presence of HiPerFect transfection reagent (Qiagen, Courtabeuf, France) for 30 min at 37°C in a 5% CO₂ incubator, prior to cell plating. Transfected cells were then grown in culture for 72 hours before cell assays. The efficiency of oligonucleotide delivery into these tumor cells was assessed using cell transfection with Alexa-fluor488-conjugated oligonucleotides followed by flow cytometry analysis of fluorescently-labelled transfected cells. MDA-B02 and MDA-MB-231 were transfected with Psicheck2 reporter vector (Promega) using Calcium Phosphate transfection Kit (Sigma).

Plasmid construct

pLentiVectors containing genomic sequence of miR-30b, miR-30d, miR-30b-d, miR-30c-b and miR-30e-b-c-d-a were constructed by inserting the PCR amplified miR-30 genomic DNA into the HindIII/EcoRI multiple cloning site (MCS) from PmiRVec. Genomic DNA was extracted from MDA-MB-231 with NucleoSpin® Tissue kit (Macherey-Nagel, Duren, Germany) and used as template PCR in presence of appropriate primers to generate PCR products (Supplementary methods S1). The 364-base pair of CDH11 ORF containing mir-30 sequence was amplified from genomic MDA-MB-231 by PCR, using appropriate primers and cloned into the psicheck-2 dual luciferase reporter Vector (Supplementary methods S2).

Real-time RT-PCR

Total RNA from human breast tumor cells was extracted using the Nucleospin RNA kit based on RNA adsorption on silica membranes, further treated by rDNase (Macherey Nagel, Duren). cDNA was produced from 1 µg total RNA using the iScript cDNA Synthesis Kit (Bio Rad). Real-time quantitative PCR reactions were performed using a SYBR Green qPCR kit (Qiagen, Courtabeuf, France) on Mastercycler RealPlex (Eppendorf GmbH, Wesseling, Germany). Relative gene expression levels were normalized according to the Ct value of the gene encoding the ribosomal protein L32 and results were expressed as fold differences equal to $2^{-\Delta\Delta Ct}$.

Analysis of miRNA expression

Taqman microRNA assays (Applied Biosystems, Life Technologies) were used to quantify miR levels and RNU48 levels after extraction by cell homogenization in Qiazol reagent followed by extraction on silica

column (miRNeasy Micro Kit, Qiagen) (22). Ten (10) ng of total RNA were reverse-transcribed using the MultiScribe reverse transcriptase and 50 nM stem-loop RT primers specific for miR and the endogenous control RNU48. Mature miRNAs were amplified using the TaqMan 2X universal PCR Master Mix and levels were expressed as fold differences equal to $2^{-\Delta\Delta C_t}$. Analysis of miR expression in primary breast tumors was performed on TaqMan 96 wells custom plates after multiplexed reverse transcription (RT) (Supplementary methods S3A). MiR profiling of breast cancer cell lines was performed by real-time PCR on TaqMan® Low Density Array Human MicroRNA Panels A and B (Applied BioSystems) (Supplementary methods S3B).

Cell invasion assay

Experiments were conducted in 24-well cell culture plates with 8- μ m diameter pore-size inserts, previously coated with 100 μ L basement membrane Matrigel (0.3 mg/mL), as previously described (22). MDA-B02, MDA-MB-231 transfectants and control (1.6×10^5 cells/mL) were re-suspended in culture medium containing 0.1% (w/v) bovine serum albumin and 300 μ L of this cell suspension were loaded into each insert (upper chamber). The chemoattractant (10% (v/v) FBS) was placed in the lower chamber (750 μ L/well). After a 24-h incubation at 37°C in a 5% CO₂ incubator, inserts were collected, the non-invading cells were removed and the invading cells on the under surface of the inserts were fixed and stained with crystal violet. Cells were then counted under microscope.

Cell enumeration assay

Cell growth curves were done after seeding the cells (10^4 per well) into 12-well plates and cultured them up to 96h. Cells were harvested at various time points following trypsinization and single-cell suspensions were then counted by the True Volumetric Absolute Counting technique of flow cytometry (Partec Instruments).

Flow-cytofluorimetric analysis

Cells (10^6 cells in 500 μ L PBS supplemented with 1% BSA) were stained with a phycoerythrin-conjugated human anti-CD24 or a fluorescein-isothiocyanate-conjugated human anti-CD44 monoclonal antibody (BD Biosciences). Stained cells were then immediately analyzed by flow cytometry (Canto II, Becton Dickinson). Aldehyde Dehydrogenase (ALDH) activity was performed with ALDEFLUOR™ reagent kit (STEMCELL Technologies) in which specific ALDH activity was obtained by monitoring fluorescence of an ALDH substrate retained in cells.

Osteoclastogenesis assay

Experiments were conducted as described previously (23),(22), with minor modifications. Briefly, murine bone marrow cells were cultured in α -MEM medium containing 10% (v/v) fetal calf serum supplemented with M-CSF (20 ng/mL) and RANK-L (30 ng/mL), in the presence of conditioned medium from transfectants (20 μ g/mL). A low concentration of RANKL was chosen in this study in order to maximize potential differences between conditioned media from MDA-B02 cell transfectants. Conditioned media were prepared from transfected cells cultured for 72h prior to replacement of complete medium by medium supplemented by 0.1% FBS. 72h later, media were harvested, centrifuged, filtered on 0.2 μ m filter and aliquoted for conservation at -80°C. After 7 days, mature osteoclasts were enumerated under a microscope on the basis of the number of nuclei (three nuclei) and TRAP activity. Results were expressed as the number of osteoclasts per well. The expression of genes involved in osteoclast differentiation and activity was analysed by RT-qPCR, as previously described, after RNA extraction with RNA easy microKit (Qiagen) (22).

Osteoblastogenesis assay

MC3T3-E1 were seeded in 12 well plates (10^5 cells/well) and cultured in α MEM medium, 10% FBS, supplemented with 50 μ g/mL ascorbic acid from day 1 and 10 mM β -glycero-phosphate from day 8 to induce differentiation, during 21 days (24). Conditioned media, prepared as above, were incubated (25 μ g/mL protein) with the cells two days after plating and changing medium every two days, until the end of experiment. Wells were washed with PBS and either further stained by modified Von Kossa to visualize mineralization, either harvested in lysis buffer for RNA extraction. MC3T3-E1 was also transiently transduced by equimolar concentrations of each miR-30 mimic (10 nM each) or corresponding control (50 nM) (Applied BioSystems) by incubating the cells in suspension with oligonucleotides in the presence of HiPerfect (Qiagen) for 30 min at 37°C prior to plating.

Western blotting

Total protein cell extracts were lysed in RIPA buffer (Sigma) containing a protease cocktail inhibitor (Roche). Protein cell extracts were electrophoresed on a 4-12% gradient SDS-polyacrylamide gel (Life technologies), then transferred onto nitrocellulose membranes (Millipore, Billerica, MA) and proteins were probed with a primary antibody against *CDH11* (1/2,000 dilution) (Human Cadherin-11 Polyclonal Ab, Goat IgG, R&D Systems, MN, USA) or β -tubulin (Cl B-512, 1/5,000 dilution; Sigma), according to

manufacturers' instructions. After incubation with primary antibodies, membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and anti-mouse secondary antibodies (Amersham; 1/2,000 dilution), and immunostaining was performed with enhanced chemiluminescence (ECL) detection system (Perkin Elmer).

Animals

Four-week-old female BALB/c nude mice were purchased from Janvier Laboratories. All procedures involving animals, including housing and care, the method by which they were sacrificed, and experimental protocols, were conducted in accordance with a code of practice established by the local ethical committee (CREEA: comite Regionale d'Ethique pour l'Expérimentation Animale).

Animal studies

Tumor xenograft experiments were conducted, as previously described (25),(22). Four-week-old female Balbc nude mice were subcutaneously inoculated on both flanks with MDA-B02 transfectants (10^6 cells in 50- μ L PBS), respectively. Tumor size was calculated by external measurement of the width (m_1) and length (m_2) of tumor xenografts using a Vernier caliper. Tumor volume (TV) was calculated using the equation $TV = (m_1^2 \times m_2) / 2$. At the end of the protocols, mice were sacrificed and tumors collected, then prepared for immunohistochemistry.

Bone metastasis experiments were conducted in female Balb/c nude mice, as described previously (22). Doxycycline-treated or untreated transfectants (5×10^5 cells in 100- μ L PBS) were injected into the tail artery of anesthetized nude mice. Radiographs of anesthetized animals were taken weekly with the use of MIN-R2000 films (Kodak, Rochester, NY) in an MX-20 cabinet X-ray system (Faxitron X-ray Corporation, Wheeling, IL). Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area of osteolytic lesions was measured using a computerized image analysis system, and the extent of bone destruction per leg was expressed in square millimeters. For measurement of skeletal tumor burden, bioluminescence imaging of animals was performed weekly using the Nightowl imaging system (Berthold, Germany), as previously described (26). On day 32 after tumor cell inoculation, anesthetized mice were sacrificed by cervical dislocation after radiography and bioluminescence imaging.

Bone histology, histomorphometry and immunohistochemistry

Bone histology and histomorphometric analysis of bone tissue sections were performed as previously described (22). Histomorphometric measurements [bone volume (BV)/tissue volume (TV) and tumor volume (TuV)/soft tissue volume (STV) ratios] were performed in a standard zone of the tibial metaphysis, situated at 0.5 mm from the growth plate, including cortical and trabecular bone. The BV/TV ratio represents the percentage of bone tissue. The TuV/STV ratio represents the percentage of tumor tissue. The *in situ* detection of osteoclasts was performed on tartrate-resistant acid phosphatase (TRAP)-stained longitudinal paraffin-embedded medial sections of tibial metaphysis with the use of a commercial kit (Sigma). Osteoclast resorption surface was calculated as the ratio of TRAP-positive trabecular bone surface to the total trabecular bone surface at the tumor-bone interface.

For immunohistochemistry, tumor sections were incubated with a mouse anti-human Ki-67 monoclonal antibody (DakoCytomation) or a rabbit polyclonal anti-CD31 antibody (AnaSpec) that specifically recognize proliferative cells and blood vessels, respectively. The mitotic index and tumor microvessel density were quantified, as previously described (22).

Breast cancer cohort of patients

In the cohort, patients ($n = 120$) were selected according to the following criteria: primary breast tumor without inflammatory features and no previous treatment (27). Breast cancer tissue biopsies were obtained from surgery, selected by the pathologist, and immediately stored in liquid nitrogen until processing. The biopsies were pulverized using a MikroDismembrator (B.Braun Biotech International), and total RNA was extracted using TRI Reagent (Sigma). RNA quality was verified using an Agilent Bioanalyser 2100 (Agilent Technologies) (27).

Transcriptomic profiling of breast cancer metastasis

The transcriptomic profiles of 80 breast cancer metastases including 21 bones, 24 lung, 23 brain, 10 liver and 2 skin metastases were compared *in silico* using two publically available data sets : the Gene Expression Omnibus, under IDs GSE11078 (MSK) and GSE14020 (Institut Curie). A class comparison was conducted based on univariate t test, with a stringent p value of 10^{-4} to identify genes differentially expressed by the bone and non-bone metastatic lesions. A filtering criteria ($p < 10^{-4}$, fold change > 1.5) was applied to remove poorly expressed genes in normal bones as compared to normal breast.

Statistical Analysis

All data were analyzed using GraphPad Prism 5 software (version 5.2; GraphPad Software, San Diego). Pairwise comparisons were carried out by performing nonparametric Mann-Whitney U test, one-way ANOVA, *post-hoc Bonferroni* test and Student t-test, as indicated in figures and tables. P-values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

Results and discussion

Identification of microRNAs that are deregulated in osteotropic breast cancer cell lines

To identify human miRs that may interfere with breast cancer metastasis to bone, we first performed miR profiling of human breast cancer cell lines with different affinities for bone. MDA-B02, a sub-variant of the parental MDA-MB-231 breast cancer cell line is highly and specifically metastatic to bone (20). We also analyzed miR expression in BC-M1, a human breast cancer cell line obtained from bone marrow aspirates of a breast cancer patient with no signs of metastasis at time of surgery (21). The expression of 152 miRs was identified to be significantly differentially expressed between the three cell lines (Fig S1). Hierarchical clustering of normalized miR expression levels for the 27 miRs that displayed the highest coefficient of variation between cell lines show that a large set of miRs was under-expressed in the two osteotropic cell lines (Fig 1A). The expression of miR-222, miR-125a-3p, miR-145, miR-34a, miR-146a, miR-146b, miR-10b was down-regulated in MDA-B02 but was up-regulated in BC-M1. Such an inverse miR regulation is probably related to a difference in BC-M1 phenotype since these cells are disseminated tumor cells in dormant G0-phase with a low proliferative index. A set of 20 miRs, previously reported as suppressors of breast cancer metastatic dissemination to organs including bone (such as miR-335, miR-206) (14), were down-regulated in both osteotropic cell lines. The tumor suppressor and EMT repressor, miR-200a, b, c and miR-429 cluster, were strongly down-regulated in MDA-B02 and BC-M1 (28). These data were associated with (1) positive and negative expression of cancer stem cells markers CD44 and CD24, respectively, as well as ALDH activity; (2) up-regulation of EMT-transcription factors Zeb1, 2 and snail1, 2; and (3) modulation of EMT-associated genes in the two osteotropic cell lines (Fig. S2A, C,D,E).

Both osteotropic cell lines expressed low levels of miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p, in accord with the property of miRs-30 which is involved in EMT switching in several solid tumors and metastatic dissemination (Fig. 1A, 1B) (29),(30). Although the genomic locus of the five miR-30s are located on three different chromosomes, they share a similar decreased expression pattern, suggesting a regulatory mechanism at the transcriptional level independent of chromosomal copy number alterations. RT-qPCR analysis confirmed differential expression of the miRs-30 expression and revealed that the complementary sequence excised from the opposite strand of the hairpin precursors, miR-30a-3p, miR-30d-3p and miR-30e-3p (but not miR-30b-3p and miR-30c-3p; currently named miR-30*) were also down-regulated in MDA-B02 and BC-M1 (Fig 1B). The low expression of miR-200 cluster and miRs-30 in osteotropic cell lines was explained with the down-regulation of the tumor suppressor

Dicer, a miRNA biogenesis enzyme, in agreement with the fact that these cells have undergone EMT to escape the primary tumor before reaching and invading the bone marrow, prior to isolation (Fig S2B) (31) (32). To gain insight into the functional consequences of miRs-30 expression, we investigated the expression levels of the five miR-30s-5p in breast cancer cell lines isolated from primary tumor sub-types that have different tumorigenic and metastatic properties in mice models (Fig S3). Basal tumors are aggressive in terms of metastatic dissemination, difficult to treat and of a general poor prognosis. The basal claudin-low subtypes (MDA-MB-231, MDA-B02 and BC-M1 and Hs578T) expressed all five miR-30s-5p at low levels, in contrast they were highly abundant in hormone responsive luminal A (T47D, MCF-7) and luminal B (BT-474, ZR-75) subtypes, and in non-hormone responsive but HER⁺ subtype (SK-BR3).

MicroRNA-30 family targets genes involved in metastatic dissemination and bone physiopathology, supporting its activity *in vivo* to reduce experimental bone metastasis in mice

To find out if miRs-30 may be involved in bone metastasis regulation, we performed an *in-silico* analysis of miRs-30 target genes by searching databases MirWalk, Microcosm, TargetScan and Pictar. We highlighted numerous genes that are either only predicted *in-silico* but also genes that are already experimentally validated (Table S1). Briefly, miRs-30 target several genes involved in tumorigenesis, cell survival, adhesion, invasion, ECM and finally bone physiology, making this miR family a good candidate to interfere with bone metastasis. Therefore, to experimentally validate the activity of miRs-30-targeted genes in bone metastasis, MDA-B02 was transduced by the lentiviral vector pmiRVec containing different combinations of the five miR-30 genomic sequences down-stream of CMV promoter. The genomic sequences of miR-30d-5p and miR-30b-5p were individually inserted in pmiRVec and also as a tandem sequence since they are located in a chromosome cluster. A third construct was obtained by the tandem insertion of genomic sequences of the 5 miR-30 in pmiRVec. The genomic sequences of miR-30c-5p and miR-30b-5p, the complementary sequences -3p of which is not expressed in MDA-B02, were similarly inserted into pmiRVec. Transduction by miR-30d and miR-30b genomic sequences induced a 17- and 6.2-fold increase of miR-30d and miR-30b, respectively when compared to endogenous levels. The lower enrichment of miR-30b compared to miR-30d reflects the higher endogenous level of miR-30b versus miR-30d. MDA-B02 transduction by pmiRVec-30d-b, pmiRVec-30c-b and/or pmiRVec-30e-c-d-b-a resulted in significantly enriched levels of each miR present in the construct (Fig 2). However, miR expression in tandem or as the complete family reduced the enrichment of each miR-30, compared to the level obtained after transduction with a single miR genomic sequence, probably due to the saturation of the RISC complex. A lower enrichment level obtained with miR-30b-5p reflects the higher

endogenous level of miR-30b versus miR-30d (Fig 2). Endogenous levels of the poorly expressed miR-335, the moderately expressed miR-152 and the highly expressed miR-21 were not modified by the transduction (not shown).

The activity of miR-30s-5p on bone metastasis development was investigated in a mice model of bone metastasis, in which bone seeking MDA-B02 cells were injected into the arterial circulation to mimic circulating breast tumor cells that invade the bone marrow and subsequently form overt osteolytic lesions. Restoring the expression of miRs-30 in MDA-B02 wild type (MDA-B02-wt) decreased the bone metastasis formation in mice irrespective of the miR-30 genomic sequence used to transduce the cells (Table 1). X-ray and histomorphometric analyses showed that mice bearing MDA-B02-wt tumors developed osteolytic lesions that were not significantly different than those of mice bearing MDA-B02-pmiRVec, whereas mice inoculated with MDA-B02-pmiR30d-b developed smaller osteolytic lesions (Fig 3A-E). Specifically, skeletal tumor outgrowth and bone destruction in metastatic animals were both markedly decreased by the increase of miR-30d and miR-30b levels in cells (Fig 3D,E,G). The inhibitory effect of miRs-30 on tumor-induced bone destruction was confirmed by the significant increase of BV/TV ratio in tibias from mice injected with MDA-B02-pmiRs-30e-c-d-b-a ($11.9\% \pm 2.4$ vs $26.6\% \pm 2.7$ $P < 0.01$). This was associated with a decrease in TB/TV ratio ($45.2\% \pm 5.4$ vs $21.7\% \pm 6.1$ $P < 0.01$) and a decrease of TRAP-positive osteoclast surface at the bone/tumor cell interface ($77.4\% \pm 8.1$ vs $41.7\% \pm 16.5$ $P < 0.05$) (Table 1). The miR-5p sequences and not the complementary sequences excised from the opposite strand of the hairpin precursors appear to specifically inhibit bone metastasis, since MDA-B02-pmiR30c-b (in which only 5p strands are expressed) also repressed bone destruction and tumor burden in mice tibias (Table 1). Bioluminescence quantification of bone metastasis revealed that cell enrichment with miR-30d-b repressed the tumor burden in legs, suggesting that miR-30s-5p negatively interfere with circulating tumor cell homing and/or extravasation and bone marrow engraftment (Fig 3C,F). We showed here that miRs-30 negatively affects the vicious cycle by repressing the tumor burden which in turn decreases osteoclast activity and bone destruction.

MiR-30s-5p regulate osteoclastogenesis and osteoblastogenesis in MDA-B02

Tumor-induced stimulatory effects on bone destruction were attenuated in hind limbs from mice inoculated with MDA-B02-miRs-30 (Table 1). The overall TRAP staining and the ratio of TRAP staining surfaces over bone surfaces at the tumor-bone interface were significantly decreased in decalcified bone tissue sections from these animals. This indicates a decrease in global bone destruction associated with a reduced tumor burden and with a lower osteoclast activity in mice bearing MDA-B02-miRs-30. Activity

of the five miR-30-5p on osteoclastogenesis were assessed after transfection of MDA-B02 by a mix of the five miR-30s-5p at equimolar concentrations (MDA-B02-miR-30a,b,c,d,e) and by equivalent concentrations of a miR scramble control sequence (MDA-B02-miR-Ctrl). The treatment of primary mouse bone marrow cell cultures in presence of RANKL and M-CSF together with conditional media from MDA-B02-miR-30a,b,c,d,e decreased the formation of TRAP⁺ multinucleated osteoclasts compared with that observed with the conditional medium of MDA-B02-miR-Ctrl (Fig 4A). The ability of miR-30s-5p to decrease osteoclast number was accompanied with lower expression of *Trap* (a marker associated with osteoclast differentiation) and inhibition of *Ctsk* (a gene encoding for cathepsin K, an osteoclast-derived cysteine protease enabling degradation of the collagenous matrix) (Fig 4B).

We next investigated the molecular inhibitory effect of miRs-30-5p on osteoclastogenesis. We first hypothesized that miRs-30 released in conditional media did not have a major impact on genes expressed by mouse bone marrow cells, in regard of very low amounts detected in conditional media (not shown). Rather, miR-30s might directly and/or indirectly modulate gene expression in MDA-B02. MiR-30c-5p has been reported as a human breast tumor prognosis marker that plays a role in chemoresistance by a direct targeting of *TWF1*, a gene encoding for an actin-binding protein that stimulates IL-11 as a secondary target of miR-30c signaling pathway (33). ELISA measurement of Interleukin levels in conditional medium showed a significant decrease of IL-11 and IL-8 in MDA-B02-miR-30a,b,c,d,e conditional medium whereas IL-6 and IL-18 levels were not affected (not shown). This was consistent with the significant decrease in levels of *IL-11*, *IL-8* and *TWF1* in MDA-B02 at the transcriptional level (Fig 4C,D,E). Thus, miR-30s-5p interfere negatively in the vicious cycle by decreasing IL-11 and IL-8 that in turn decrease osteoclast formation and contribute to attenuate the osteolytic lesions induced by breast tumor cells in mice bone marrow (5).

MiR-30s-5p have been described as negative regulators of osteoblast differentiation (34). Therefore, we next investigated whether tumoral miRs-30 may affect osteoblastogenesis. Incubation of conditional medium from MDA-B02-miR-30s with MC3T3-E1 osteoblast cells shown that miRs-30 reversed the inhibition of osteoblast differentiation and mineralization induced by MDA-B02 conditional media from MDA-B02 (Fig 4F) and increased *ALP* and *OCN* expression in MC3T3-E1 (Fig 4I). In accordance with this observation, conditional medium from MDA-MB-231 cells transfected with a mix of equimolar concentrations of the five miR-30 antagomirs did not reverse osteoblast differentiation and mineralization induced by conditional medium from MDA-MB-231, but increased osteoblast inhibition (Fig 4G). Previous data showed that miRs-30 negatively regulates BMP-2-induced osteogenic

differentiation of MC3T3-E1, by direct targeting of *smad1* and *RunX2* (35). Further, miR-30a and miR-30d are down-regulated during BMP-2-induced osteogenesis of C2C12 mesenchymal cells (34). Consistent with these previous results, we observed a strong inhibition of osteoblastogenesis in MC3T3-E1 transfected with miRs-30 (Fig 4H). Thus, we deduced that miRs-30-induced osteoblastogenesis stimulation resulted from modulation of MDA-B02 target genes. This tumor-induced stimulation of osteoblastogenesis was efficient enough to uncover direct miR-30s inhibition of MC3T3-E1 differentiation. Indeed inhibition of *DKK-1*, but not of other Wnt inhibitors such as *SOST* and *NOGGIN*, in MDA-B02, explains the pro-osteoblastic effect of tumoral miRs-30 (Fig 4J-L). This was observed at high as well as lower miRs-30 levels in MDA-B02. Overall, we conclude that miRs-30 interfere negatively with the vicious cycle induced by MDA-B02 in the mice bone microenvironment by regulating positively pro-osteoblastic genes and inhibiting pro-osteoclastogenic genes.

Stable over-expression of miRs-30 reduces tumorigenesis and invasion of MDA-B02

In order to decipher bone metastasis regulation by miRs-30, we used a xenograft mice model in which mice were implanted subcutaneously with MDA-B02-pmiRVec and with MDA-B02-pmiR30d-b to determine whether miR-30 would affect tumor take, tumor growth and angiogenesis. Tumor progression did not differ between groups until 35 days after inoculation, indicating that miR-30d and b did not interfere with tumor take. However, MDA-B02-pmiR30d-b grew more slowly after this time until sacrifice, resulting in a significantly lower tumor burden (Fig 5A-B). In regards to large necrotic areas in tumors, the number of Ki-67⁺ cells and of microvessels labeled by endothelial cell marker CD31, was measured at the edge of tumors (Fig 4C-F). Significant decrease of CD31 and of proliferative Ki-67 index in MDA-B02-pmiR30d-b tumors suggested that miR-30 decreased tumor neovascularization, a necessary event for growth and invasiveness of cancer cells, and impact negatively on tumor growth (Fig 5A-F). Indeed, previous works have reported that CTGF, a pro-angiogenic factor mediates local angiogenesis induced by breast cancer cells and stimulates bone osteolytic lesions in mice inoculated with osteotropic breast cancer cells (36). CTGF expression is down-regulated by miR-30c at the 3'UTR level, in a myocardial matrix remodeling (37). Similarly, some members of miRs-30 were reported as negative regulators of invasion and metastatic dissemination in several solid cancers by targeting genes involved in EMT, such as *SNAI1* (38) and *ERG* (39), and in breast cancer cell invasiveness, such as *MTDH* (40), *VIM* (30), and *TWF1* (33). Therefore, we analyzed proliferation and invasion of MDA-B02-pmiR30d-b *in vitro*. Growth curves were monitored after MDA-B02 low seeding. Mir-30d and miR-30b had no effect on MDA-B02 growth rates but markedly decreased cell invasion in plates coated with basement membrane

Matrigel (Fig 6A,B). The growth rates of MDA-B02pmiR-30e,c,d,b,a in the same seeding conditions were not altered (not shown). The absence of miRs-30 modulation on proliferation is consistent with our finding in xenograft tumors in which the decrease in angiogenesis and invasiveness would account for decrease in proliferative index. MiR-30a has been reported as suppressing breast tumor growth and breast cancer cell lines *in vitro* (40). This discrepancy might be attributed to a difference in targeted genes. According to most of predictive algorithms, the five miRNAs sharing the same seed sequence, they supposedly target the same genes, although differences in nucleotide sequences on 5', 3' sides may also modulated miR activity. Eguchi et al (41) have reported that among osteomiRs, miRs-30 could be classified into miR-30b-c and miR-30a-d-e on the basis of their mature structures, of their targets and also expression pattern during osteogenesis. In our work, by extending the invasiveness study to MDA-B02-miR-30e-d-c-b-a and MDA-B02-30b-c, we showed that the inhibition of invasion was quite comparable within cell lines (Fig 6C-D).

MiR-30s-5p mimics reduce invasion of MDA-B02 and MDA-MB-231 cells

To exclude the possibility that the modification observed in cell invasiveness may be due to nonspecific assay of the forced expression by transduction, such as saturation of tmiR machinery, cells were transfected with equimolar amounts of chemically-stabilized mature sequences of the five miR-30s (miR-30 mimics, or miR-30 antagomirs). The use of miRNA mimics also rule out the possible involvement of miR-30s-3p in regulation of MDA-B02 invasiveness, due to antisense strands (-3p). It also permits a roughly similar, since randomized cell content of each miR-30-5p. Treatment with miRNA mimics inhibited the invasion of MDA-B02 and MDA-MB-231 cells through Matrigel compared to cells transfected with a scramble miR sequence as control (Fig 6E-F). Surprisingly, antagonizing the miRs-30 activity did not affect invasion in either cell lines. qRT-PCR analysis revealed profound elevated expression of each miR-30 after mimic transfection (Fig S4A), whereas miR-30 expression was moderately repressed by antagonists that might explain lack of effect on invasion (Fig S4B). We next studied the expression of genes down-regulated by miR-30-5p which may interfere with breast cancer cell angiogenesis and invasiveness and in turn act as negative regulators of bone metastasis.

MiR-30s-5p modulate bone metastasis by targeting osteomimicry

We (20) and others (42),(43) have established bone seeking clones by the selection of human breast cancer cell lines from the parental MDA-MB-231 cells that spread and thrive in organs without selectivity. MDA-B02 express sets of deregulated genes, involved in bone homing, adhesion, invasion and

osteogenesis, that act cooperatively to form osteolytic bone lesions. Transcriptomic analysis has shown that MDA-B02 acquired selective properties to adhere to the bone surface, as well as to adapt and grow in the bone marrow by expressing a pseudo-bone cell phenotype through a process called osteomimicry (44). Here, we report that the expression of genes encoding for bone homing proteins, *ITGβ3*, *ITGα5* and *CDH11* was down-regulated by miRs-30 (Fig 7A). *ITGβ3* has been experimentally validated as a miRs-30 target in breast-tumor-initiating cells contributing to an anti-tumorigenic and anti-metastatic effect in these cells (see table S1). *ITGβ3* is part of the $\alpha v\beta 3$ integrin, an adhesion receptor expressed by breast cancer cells and osteoclasts that increases tumor cell invasion and osteoclast-mediated bone resorption. Inoculation of $\alpha v\beta 3$ -overexpressing MDA-MB-231 breast cancer cells in mice increases bone metastasis incidence and promotes both skeletal tumor burden and bone destruction (45). Expression of *ITGα5* was inhibited in MDA-B02 transduced by miRs-30 genomic sequences (Fig 7A), presumably through the 8-mer target sequence at position 206 of 3'UTR. Binding of integrin $\alpha 5\beta 1$ to its ligand fibronectin promotes the survival of dormant breast cancer cells in the bone marrow (46). MiRs-30 strongly decreased transcriptional and protein expression of cadherin11 in MDA-B02 that highly express this adhesion molecule compared to parental cells (Fig 7A, Fig S5A,E) and (Fig S5C,E). We described the presence of a conserved 8-mer site at position 16 of *CDH11* ORF that matches the seed region of each miR-30-5p, and was experimentally validated by luciferase reporter assay (Fig S5F). This site is well conserved through evolution and presents the highest free energy of -17.1 Kcal/mol for miR-30d and *CDH11* coding sequence (Fig S5B,C). *CDH11* is known to stimulate breast cancer bone metastasis. Indeed, overexpression of *CDH11* in MDA-MB-231 enhances bone, but not lung metastasis by promoting cell homing and early colonization of the bone marrow as well as osteoclastic bone resorption induced by PTH-rP (43). In our model of bone metastasis, miRs-30 that negatively regulated the adhesion molecule cadherin11 appears as a metastatic suppressor in bone.

Moreover, we found that CTGF and RUNX2, two proteins involved in osteogenesis and bone physiopathology, and that are already experimentally validated miRs-30 targets (see table S1), were down-regulated in MDA-B02-miR-30s (Fig 7). CTGF, a cysteine-rich secretory protein belonging to the CCN family and a mediator of local angiogenesis induced by breast cancers, is strongly expressed in MDA-B02 and BC-M1 (not shown). This follows from the pioneer work of Kang and al. (9), which reported that CTGF contributes to bone metastasis and converts low-metastatic breast cancer cells to become highly metastatic, while Shimo et al. have shown that osteolytic metastasis of breast cancer bone-seeking variant was inhibited by an anti-CTGF antibody (36). The miRs-30s induced decrease in

CD31 tumors from mice xenograft model may be sustained by the negative regulation of CTGF that in turn modulate negatively invasion and tumor growth. In this, miR-30s may reduce tumor burden in metastatic bone.

Based on immunoblot measurements and luciferase reporter binding assays, the master regulator of osteogenesis RUNX2 has been previously shown to be a direct target of miRs-30 in osteoblasts and chondrocytes (see Table S1). RUNX2 expression by breast cancer cells through osteomimicry is known to enhance interactions between tumor cells and bone microenvironment to promote osteolytic lesions in mice (47). Therefore, miR-30 targeting of this gene might contribute to decrease progression of osteolytic lesions. The gap junction protein CX43 that is highly expressed in osteocytes and osteoblasts, was also negatively regulated in MDA-B02-miR-30S, presumably through the 8-mer target sequences highlighted in its 3'UTR (see Table S1). Immunohistochemistry performed on human breast primary tumors and matching liver and bone metastases have shown that CX43 is highly expressed in bone metastasis compared to liver metastasis and primary tumors (44).

Tumor cells produce the soluble protein DKK1, a WNT inhibitor that inhibits osteoblastogenesis and promotes osteolytic lesions (48). MiRs-30-induced downregulation of *DKK1* levels might contribute to decrease bone metastasis. *DKK1* might be a direct target of miR-30s by a putative sequence in its ORF reported by MirWalk and/or an indirect target of these miRNAs. Kang et al. (9) have reported a gene specific signature (CXC4, OPN, IL-11 and CTGF) that drives metastasis to bone, these genes acting cooperatively to promote these metastasis. Expressed individually, these genes did not cause a significant increase in osteolytic lesions while these lesions were dramatically increased following the combined transfection of these genes in MDA-MB-231. We propose that miRs-30 act through the combined negative regulation of osteomimetic genes, *ITGβ3*, *ITGα5*, *CTGF* and *CX43* to decrease bone metastasis in our mice model of breast cancer bone metastasis.

Besides osteomimicry, we analyzed the expression of genes highly expressed in MDA-B02 and BC-M1 that have been involved in tumor cell dissemination and have also been reported as experimentally validated targets of miRs-30. For instance, MTDH (metadherin), a cell surface protein, is highly expressed in breast cancer and mediates lung metastasis (40). Direct targeting of metadherin by miR-30a on two putative binding sites in 3'UTR has been validated by dual luciferase assay. Consistently, overexpression of miR-30a in breast cancer cell lines inhibits cell invasion, tumorigenesis and lung metastasis in xenograft mouse models (40). MiR-30s-5p have been reported as tumor suppressors in multiple myeloma, in which their down-regulation enhances expression of BCL9, a transcriptional coactivator of

the WNT signaling pathway that promotes multiple myeloma cell proliferation, survival and migration (49). In this, miR-30s-5p are potential therapeutic in murine xenograft models of human multiple myeloma, reducing tumor burden and metastasis. We found that expression of *MTDH* and *BCL9* were downregulated by miRs-30 (Fig 7B) and this might enhance invasiveness of MDA-B02, a property that can be antagonized by miR-30s to inhibit tumor cell invasion in mice bone marrow. High expression of NTSE (5'ectopic nucleotidase, CD73), an enzyme that generates adenosine is associated with a poor prognosis in triple negative breast cancers (50). This protein enhances breast tumor cell migration, invasion and specific adhesion to ECM protein, tenascin C (51). *5NTE* was down-regulated by miR-30s in MDA-B02 (Fig 7B), consistent with the two 8-mer match to position 2-8 of miR-30s present at position 328-335 and 1442-1449 of 3'UTR (Table S1). We hypothesized that 5NTE could enhance bone metastasis formation by stimulating the adhesion of breast tumor cells to ECM, especially tenascin C which is highly abundant in MDA-B02 and BC-M1 (not shown). In MDA-B02-miR-30s, *TNC* was down-regulated at the transcriptomic level, despite the absence of reported miR-30s-5p targeted sequence in *TNC* (miRWalk, Microcosm), suggesting that this gene is indirectly regulated by miRs-30 by an unknown mechanism. *TNC* expression is associated with breast cancer aggressiveness through enhancing the expression of WNT target, *LGR5* (52). Tumor cell-derived *TNC* initiates metastasis in the niche until the tumor stroma can act as a source of *TNC* to support metastasis outgrowth. Both *5NTE* and *TNC* were up-regulated in bone metastasis compared to other tissue metastases from GEO datasets (Fig 7B). Thus, the direct down-regulation of *5NTE* and indirect inhibition of *TNC* expression by miR-30s in MDA-B02 that highly express these genes might interfere negatively with bone metastasis formation. *NEDD4* (E3 ubiquitin-protein ligase) is an oncoprotein and a metastasis-associated gene that prevents apoptosis in hepatocellular carcinoma and is a poor prognosis factor for relapse in gastric and colon carcinoma (53). *Nedd4* regulates ubiquitin-mediated trafficking, lysosomal or proteasomal degradation, and nuclear translocation of multiple proteins. It induces the ubiquitination and degradation of the tumor suppressor phosphatase and tensin homolog protein in colon cancer (54). Consistent with the presence of two 8-mer sites in position 942-049 and 1116-1122 in the 3'UTR, we found that *NEDD4* that is highly expressed in MDA-B02, but not the poorly expressed *NEDD9*, was down-regulated in MDA-B02 at the transcriptomic level.

Finally, we performed *in-silico* analysis of transcriptomic profiles of bone metastasis and other metastatic sites from breast cancer patients, in two data sets from Gene Expression Omnibus (GEO) (GSE11078 and GSE14020) (Fig 7C). Among genes that were down-regulated by miRs-30 in MDA-B02, we found that *CTGF* was up-regulated in bone metastasis from breast cancer patients compared to other metastatic sites. Genes associated to various solid cancer metastases which were down-regulated in MDA-B02-miR-

30s by direct miRs-30 targeting (*NEDD4*, *5NTE*) or indirect regulation (*TNC*) were also up-regulated in bone metastasis at the transcriptomic level (Fig 7C). We also found that the oncogene *Nid1* and *Vimentin*, that are *in-silico* predicted direct targets of miR-30s (see table S1), are up-regulated in bone metastasis at transcriptomic levels and could promote bone metastasis (Fig 7C).

Low miR-30 expression in breast cancer primary tumors is associated with short distant disease-free survival

We analysed the expression of miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p and miR-30e-3p by Taqman RT-qPCR in primary tumors from 120 breast cancer patients. 81 patients were metastasis relapse-free over a period of 5 years and 39 patients suffered metastatic relapse during this time period. Mir-30 levels were stratified in low and high expression groups, according to the median of each miR-30 expression. These levels were compared to the metastatic relapse by Cox regression analysis on continuous variable obtained in 120 patients. Low level of miR-30a-5p, miR-30d-5p and miR-30e-5p was significantly associated with metastatic relapse while association with miR-30b-5p level approached significance only for pN+ patients (Table 2A). In contrast to miR-30e-5p, its counterpart miR-30e-3p was not associated to metastatic relapse (Table 2A). This suggests that the down-regulation of miR-30-3p, as for miR-5p, occurs during EMT and/or by altered miR biogenesis in primary breast tumors. However, differences in the associations with patient outcomes may be explained through different target genes of miR-30-3p which may not be involved in metastatic dissemination.

In accordance with our data, miR-30a-5p was found to be significantly lower in primary breast carcinomas than in the paired normal tissues and negatively correlated with the extent of lymph node and lung metastases (30). Reduced tumor expression of miR-30a in breast cancer patients was associated with an unfavorable outcome, including late tumor stage, lymph node metastasis and worse out mortality and recurrence. In this study, the targeting of VIM by miR-30a inhibits cell migration and invasion. We found that VIM was highly expressed in human bone metastasis that suggests a role for this protein in bone metastasis. Surprisingly miR-30c-5p levels, despite a tendency to be expressed at low levels in metastatic patients, were not associated to metastatic relapse. Meta-analysis conducted in the GSE22216 breast cancer dataset by Bockhorm et al (33) have shown that low levels of miR-30c, but not miR-30b was significantly associated with distant relapse-free survival during a 10 year follow-up. This discrepancy with our results may be explained by the shorter time of follow-up in our study and/or by the fact that miR-30c is significantly decreased in basal tumor subtypes compared to luminal A subtypes, and that our data have not been stratified according to the tumor subtype. However, Dobson et al. have

reported an amplification of miR-30c gene and overexpression of the mature miR-30c in breast cancer patients, associated with poor survival (55). Consistent with this, overexpression of miR-30c-5p in breast tumor cells increased invasion by targeting NOV/CCN3, the expression of which is negatively correlated with metastasis in patients. This discrepancy may be explained by poor expression of NOV/CCN3 in MDA-B02 and that miR-30c was overexpressed in presence of others members of miRs-30. This raises the necessity to extend these studies to larger cohort patients with define sub-types of cancer and associated gene profiling.

We have also studied the association between miR-30 levels and clinic-pathological features (Table 2B). Low level of miR-30a, c, d, e and miR-30e-3p was significantly associated to higher primary tumor size. We also assessed let-7c, a tumor suppressor in breast cancer that is not involved in metastatic dissemination (56). The data obtained in the present cohort showed that let-7c was negatively correlated to tumor size confirming a tumor suppressor role but was not associated to metastatic relapse. Low level of mir-30a, c and e was also associated with high grade and negative hormonal status whereas miR-30b levels show only a tendency to be associated to hormonal levels and miR-30d was not associated with this parameter. This is in good agreement with Iorio *et al* study that reports higher expression of miR-30s in ER+ and PR+ breast tumors (16). Our results confirm that miRs-30 would maintain epithelial non-differentiated and non-invasive phenotype of cancer cells that would prone anti-metastatic effect in breast cancer. However, they point out that the five members of the family might have different regulating functions *in vivo* that need to be further investigated.

Conclusion

We have shown that low level of miRs-30 in breast tumor cells promotes bone metastasis in animal model and is associated to poor relapse-free survival in primary tumors from breast cancer patients. Increasing miRs-30 in breast tumor cells impacts negatively on osteomimicry and decreases tumor cell invasion within the bone marrow that prevents vicious cycle to promote bone metastasis. Specific to bone, miRs-30 inhibits osteoclasts and stimulated osteoblasts through regulation of IL-11 and WNT signaling DKK1, respectively. MiRs-30 lowers expression of osteomimetic genes, *CDH11*, *CTGF*, *ITGβ3* and *RunX2* and of pro-metastatic genes, *ITGα5*, *NEDD4*, *5NTE* and *TNC* that suggests potential anti-metastatic properties of miR-30 mimics. Further loss-of-function studies are necessary to find out if these proteins might be therapeutic targets through miR-30 mimic inhibition.

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Table 1: Stable over-expression of microRNA-30 family in MDA- B02 decreases bone metastasis in mice.

Cell line	Radiography		Histomorphometry			TRAP staining	
	mm ² /mouse	<i>P</i>	BV/TV (%)	<i>P</i>	TuV/STV (%)	OC.S/BS (%)	<i>P</i>
MDA-B02 WT	12.95 ± 1.14 (n=8)	NS					
MDA-B02-pmiRVec	14.09 ± 1.69 (n=10)	-----	11.88 ± 2.41 (n=13)	-----	45.25 ± 5.41 (n=13)	77.43 ± 8.13 (n=5)	-----
MDA-B02-pmiR30d-b	7.67 ± 1.17 (n=9)	0.01	20.88 ± 5.30 (n=5)	0.01	14.74 ± 5.63 (n=5)	60.25±2.19 (n=5)	0.05
MDA-B02-pmiR30b-c	6.00 ± 0.96 (n=7)	0.01	27.55 ± 2.65 (n=5)	0.01	21.75 ± 2.51 (n=5)	48.0 ± 1.50 (n=4)	0.01
B02-pmiR30e-c-d-b-a	5.74 ± 0.76 (n=6)	0.02	26.64 ± 2.70 (n=4)	0.02	21.75 ± 6.13 (n=4)	41.67 ± 16.52 (n=4)	0.02

Measurements are performed 32 days after tumor cell injection.

Values of p-two sided are for comparison with the control group (B02-pmiR Vec) using , one-way ANOVA, *post hoc Bonferroni* test (mean ± SD)

BV/TV: bone volume to tissue volume ratio. TuV/STV: tumor volume-to total soft tissue

MDA-B02-pmiR30d-b: MDA-B02 transduced with pmiR-30d-b; MDA-B02-pmiR30b-c: MDA-B02 transduced with pmiR-30b-c; MDA-B02-pmiR30e-c-d-b-a: MDA-B02 transduced with pmiR-30e-c-d-b-a. OC.S/BS: active osteoclast-resorption surface per trabecular bone surface, TRAP: tartrate-resistant acid phosphatase.

Table 2: (A) Low level of miR-30a-5p, miR-30d-5p and miR-30e-5p is a poor prognosis in recurrence of breast cancer patients. (B) Association of miR-30s levels in primary tumors with clinico-pathological features of breast cancer patients.

A

miRNAs	HR	CI	P-value
miR-30a-5p	0.17	0.04-0.65	0.010
miR-30b-5p	0.26	0.05-1.38	0.068
miR-30c-5p	0.35	0.06-1.91	0.223
miR-30d-5p	0.20	0.04-0.99	0.049
miR-30e-5p	0.26	0.07-1.02	0.050
miR-30e-3P	0.48	0.08-2.97	0.431
Let-7c	0.72	0.40- 1.30	0.279

B

Variable	N	miR-30a-5p	miR-30b-5p	miR-30c-5p	miR-30d-5p	miR-30e-5p	miR-30e-3p	let-7c
Post-menopausal status								
Yes	69	0.511	0.952	1.000	1.000	1.019	1.060	0.899
No	40	1.043 (0.895)	1.805 (0.875)	1.009 (0.692)	0.954 (0.669)	1.008 (0.597)	0.972 (0.855)	1.319 (0.245)
Tumor Size								
< 20 mm	36	1.239	1.027	1.17	1.309	1.499	1.217	1.432
>= 20 mm	69	0.865 (0.002)	0.905 (0.150)	0.880 (0.001)	0.926 (0.020)	0.938 (0.001)	0.925 (0.002)	0.762 (0.007)
Grade								
GI	11	1.210	1.042	1.238	0.973	1.689	1.306	1.553
GII	42	1.050	0.986	0.970	1.155	1.115	1.053	1.167
GIII	28	0.697 (0.071)	0.895 (0.071)	0.758 (0.028)	0.811 (0.295)	0.748 (0.006)	0.848 (0.076)	0.748 (0.071)
LN status								
pN neg	49	1.132	0.952	1.072	1.062	1.292	1.168	1.350
pN pos	60	0.812 (0.028)	0.977 (0.942)	0.799 (0.114)	0.889 (0.252)	0.948 (0.012)	0.899 (0.014)	0.747 (0.048)
ER/PR status								
ER/PR pos	69	1.082	1.012	1.050	1.000	1.219	1.082	1.195
ER/PR neg	40	0.752 (0.02)	0.872 (0.09)	0.804 (0.050)	0.975 (0.343)	0.771 (0.006)	0.901 (0.382)	0.641 (0.128)

MiR levels were quantified by TaqMan qPCR using RNU48 and snu6B, as endogenous normalizers. The expression level of a given miR in each sample was normalized to the median expression values for that miR in the 120 tumor patients. In A, these expressions were compared to the metastatic relapse by Cox regression analysis on continuous variable in 120 patients. Data are compared by Mann-Whitney and

Kruskall-Wallis tests. LN status: Lymph node status, ER/PR estrogen, progesterone receptors, HR: Hazard Ratio.

Figure legends

Figure 1. Comparative expression of miRs in a non-osteotropic human breast cancer cell line (MDA-MB-231) with an osteotropic subpopulation of MDA-MB-231 (MDA-B02) and micro-metastasis cell line from bone marrow aspirate (BC-M1).

(A) Hierarchical clustering of normalized miR expression levels for the 27 miRs that displayed the highest coefficient of variation between cell lines. C_T values and relative quantity were obtained from RQ manager (Applied Biosystems). T-test and $\log_2$ fold changes were calculated on Microsoft Excel. MiRs that are differentially expressed between the three cell lines were reported as $\log_2$ fold-change ≥ 1.5 , with P-value ≤ 0.05 considered as significant.

(B) The miR-30 family (miRs-30) was identified to be down-regulated in MDA-B02 and BC-M1 compared to the parental MDA-MB-231 cell line. Levels of miR-30s (-5p: miR-30a, -30b, 30c, -30d and -30e; and -3p: miR-30a*, -30d* and -30e*) were measured by TaqMan RT-qPCR in 3-5 independent experiments. Error bars are indicated $\pm$ SD.

Figure 2. Stable over-expression of miR-30a, miR-30b, miR-30c, miR-30d and miR-30e in MDA-B02.

Genomic sequence of a single miR-30 (miR-30d, miR-30b) or in tandem (miR-30d-b, miR-30b-C, miR-30e-c-d-b-a) was expressed in MDA-B02 by transduction with the lentiviral vector pmiRVec containing different miR-30 genomic sequences. Level of mature miR-30s-5p analyzed by TaqMan RT-qPCR was compared to MDA-B02 transduced with pmiRVec.

Figure 3. Stable over-expression of miRs-30 reduces breast cancer bone metastasis formation.

(A) Summary of the experimental bone metastasis protocol. MDA-B02wt, MDA-B02 transduced with pmiRVec (MDA-B02-pmiRVec) or with pmiR-30d-b (MDA-B02-pmiR-30d-b) were inoculated in the caudal artery of female Balb/c *nude* mice on day 0. (B,C) Mice were analyzed by radiography (RX) and bioluminescence. (D-E) RX analysis of hind limbs from mice bearing tumor burden, at scheduled intervals (day 25 and day 32). Arrow indicates osteolytic lesions in mice hind limbs. (F) Bone colonization by tumor cells as judged by bioluminescence imaging. Bioluminescence was analyzed over time in mice inoculated with MDA-B02-pmiRVec and MDA-B02-pmiR-30d-b, (Mean $\pm$ SD). (G) Goldner's trichrome staining of

tissue sections of tibial metaphysis. Bone is stained green whereas bone marrow is stained violet and tumor cells (asterisk) are stained brown. (H) TRAP-stained metastatic bone tissue sections from animals inoculated with MDA-B02-pmiRVec and MDA-B02-pmiR-30d-b. Osteoclasts are stained red.

Figure 4. MiRs-30 affects osteoclast and osteoblast formation in MDA-B02.

(A) Primary mouse bone marrow cells were cultured in the presence of RANKL and M-CSF and were treated or untreated (Ctrl) with media conditioned by MDA-B02-scramble (MDA-B02 Ctrl) or by MDA-B02 transfected by miR-30s mimics (MDA-B02 miR-30s). Mature osteoclasts were quantified as multinucleated (3 or more nuclei), TRAP-positive cells. Representative images of mature osteoclasts are shown for each group. (B) Relative expression of markers associated with osteoclast differentiation and activity as analyzed by RT-qPCR, (Mean $\pm$ SD, n=3). (C-D) Relative expression of *TWf1*, *IL-11* and *IL-8* in MDA-B02 Ctrl and MDA-B02 miR-30s, as measured by RT-qPCR (Mean $\pm$ SD n=3). Elisa measurement of IL-11 and IL-8 in conditioned media from MDA-B02 Ctrl (B02 CT) and MDA-B02 miRs-30 (B02-miRNA mimics) (Mean $\pm$ SD n=3). (F) MC3T3-E1 cells were treated or not (NT) during differentiation with conditioned media from MDA-B02 Ctrl (B02 Ctrl) and from MDA-B02 miR-30s (B02-miRNA-mimics). (G) MC3T3-E1 cells were treated with conditional media from MDA-MB-231 scramble (MDA-CT) and from MDA-MB-231 transfected with miRNA-inhibitors (MDA-miRNA inhibitors. (H) MC3T3-E1 was transfected with scramble (MC3T3-CT), with miR-30s mimics (MC3T3-miRNA-mimics) or not transfected (MC3T3 NT). Mineralisation is evidenced by Von Kossa staining. (I) Relative expression of markers associated with osteoblast differentiation and activity as analyzed by RT-qPCR, (Mean $\pm$ SD, n=3). (J-L) Relative expression of *DKK-1*, *Noggin* and *SOST* in MDA-B02-Ctrl (B02-Ctrl), MDA-B02 transfected with miR-30s mimics (high B02-miRs-30) and MDA-B02 transduced by pmiRVec30e-c-d-b-a (low B02-miRs-30) (Mean $\pm$ SD n=3), *:P<0.05, **p<0.02, ***p<0.01.

Figure 5. Stable over-expression of miR-30d-b in MDA-B02 decreases tumor growth in a mice model of orthotopic tumor xenograft.

(A-B) Tumor cells were inoculated subcutaneously in female *Balbc* nude mice. Tumor growth was obtained by measuring tumor volumes with a Vernier caliper and tumor weight at time of sacrifice. (C-F) Ki-67 and CD31 immunostaining of tissue sections of harvested tumors from mice inoculated with MDA-B02-pmiRVec and MDA-B02-pmiR-30d-b, as a measurement of proliferative index of tumor cells and number of tumor-associated blood vessels, respectively.

Figure 6. MiRs-30 reduce invasion of MDA-B02 and MDA-MB-231.

(A-B) Proliferation of MDA-B02-pmiRVec and MDA-B02-pmiR-30d-b was analysed by cell counting. Cell invasion was measured by loading MDA-B02-pmiRVec and MDA-B02-pmiR-30-d-b in inserts with porous membrane coated with basement membrane Matrigel (upper chamber) and the chemoattractant (serum) was placed in wells of a companion plate (lower chamber), (Mean $\pm$ SD).

(C-D) Cell invasion was measured in MDA-B02 transduced with different genomic sequences of miR-30, (Mean $\pm$ SD n=3). (E-F) Cell invasion was measured in MDA-B02 and MDA-MB-231 transfected with miRs-30 mimics and miRs-30 inhibitors (Mean $\pm$ SD n=3). (D-F) Representative images of invasion, with the three first wells having FBS as chemoattractant in the lower chamber and the fourth well without chemoattractant (blank). *:P<0.05, **p<0.02.

Figure 7. MiRs-30 targets osteomimetic genes and down-regulates osteomimicry.

(A) Relative expression of osteomimetic genes in MDA-B02-pmiRVec, MDA-B02-pmiR-30d-b and MDA-B02-pmiR-30e-c-d-b-a (Mean $\pm$ SD n=3-6) (B) Relative expression of genes targeted by miR-30s in MDA-B02-pmiRVec and MDA-B02-pmiR-30e-c-d-b-a (Mean $\pm$ SD n=3-6), *:P<0.05, **p<0.02, ***p<0.01. (C) Over-expression of *ITG α 5*, *CTGF*, *5NTE*, *TNC*, *NEDD4*, *Vim* and *NID1* in human bone metastasis as compared to other metastatic sites, in breast cancer patients. Data were obtained from two publically available data sets from the Gene Expression Omnibus, under IDs GSE11078 (MSK) and GSE14020 (Institut Curie). Pairwise comparisons were carried out by performing nonparametric Mann-Whitney U test. *:P<0.01, **p<0.005, ***p<0.001.

Figures

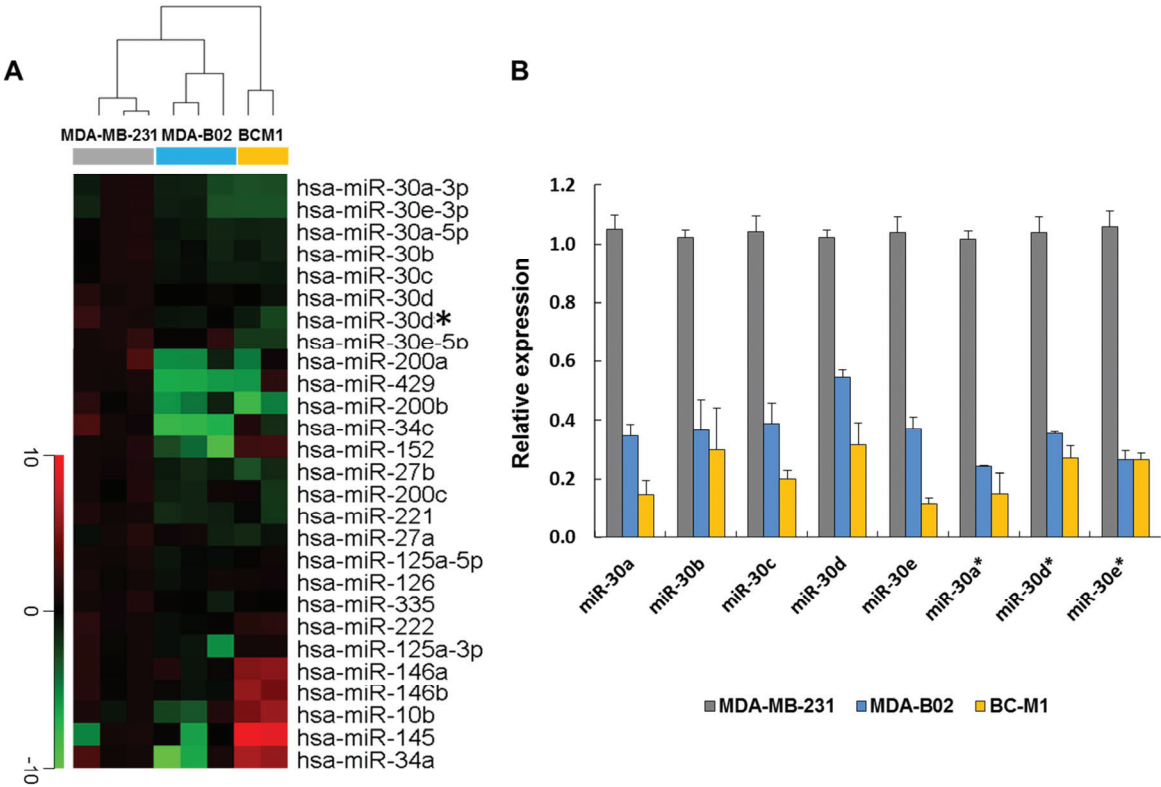


FIGURE 1

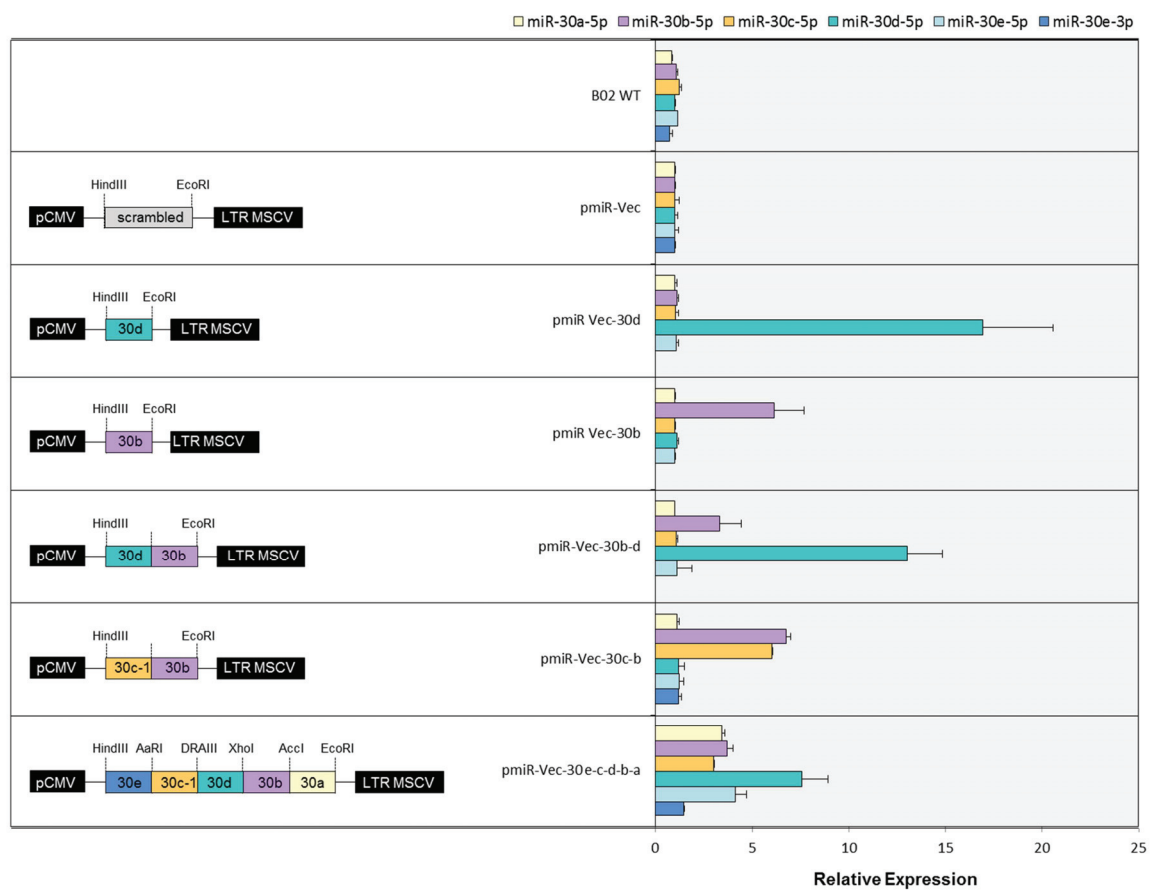


FIGURE 2

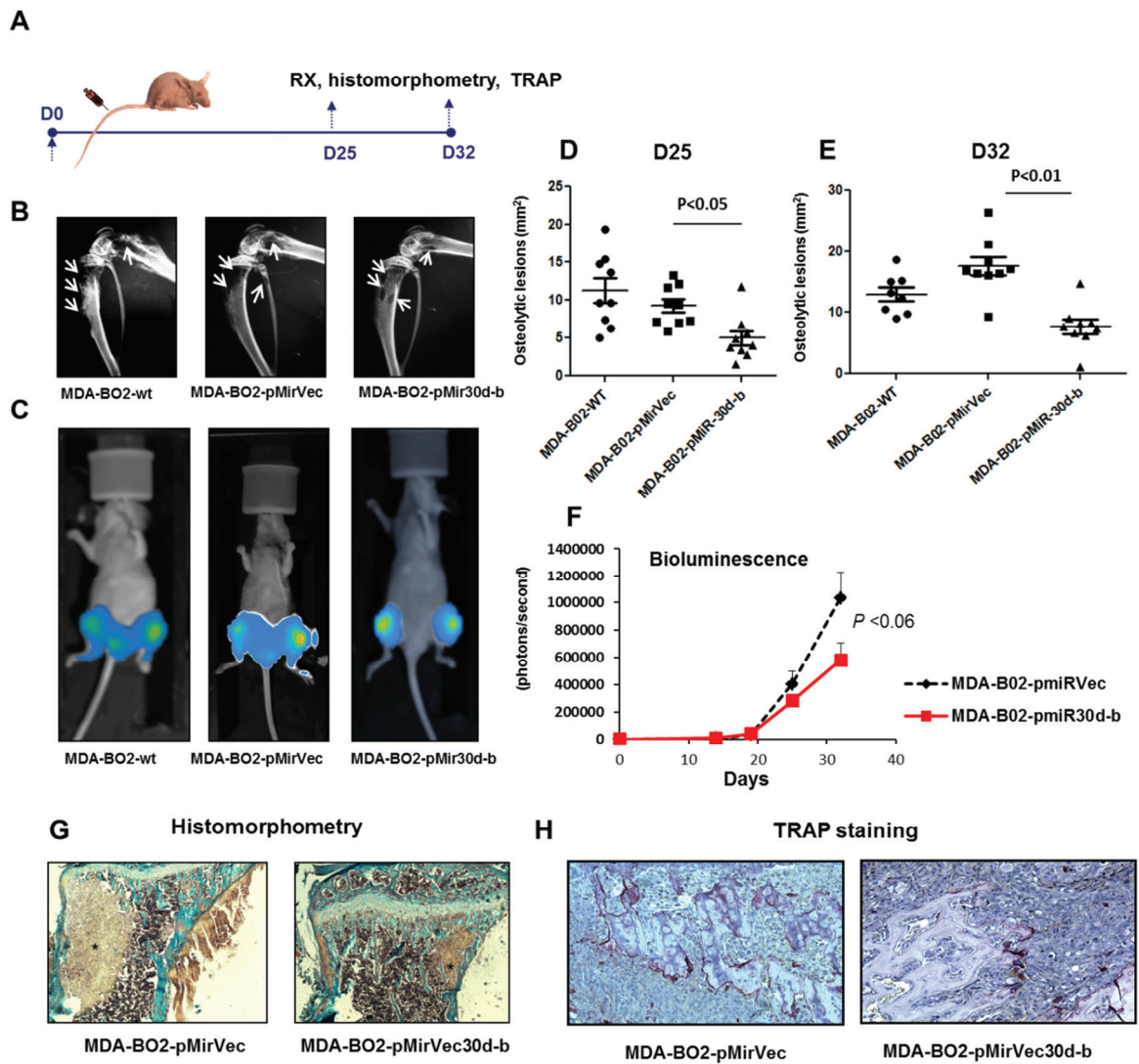


FIGURE 3

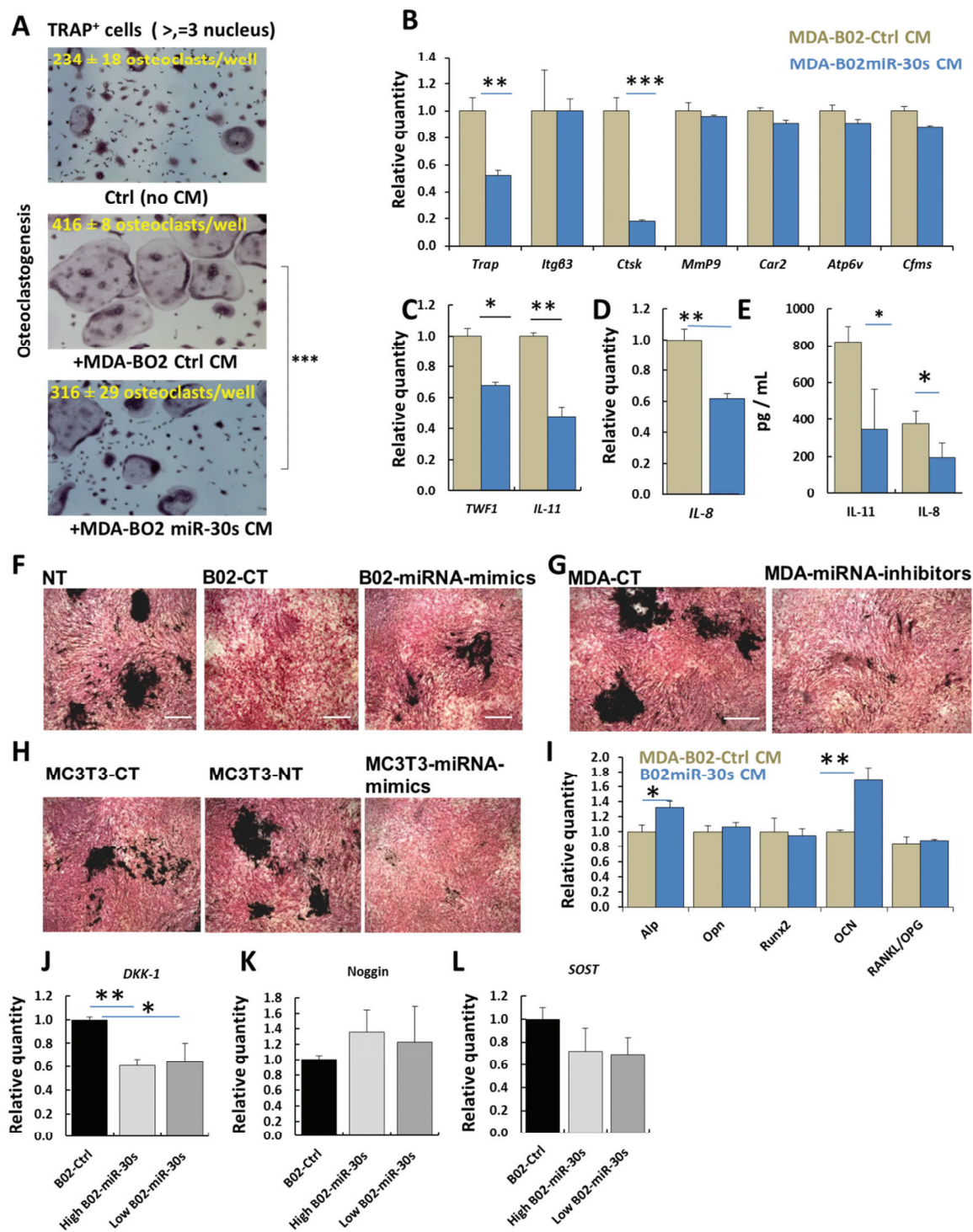


FIGURE 4

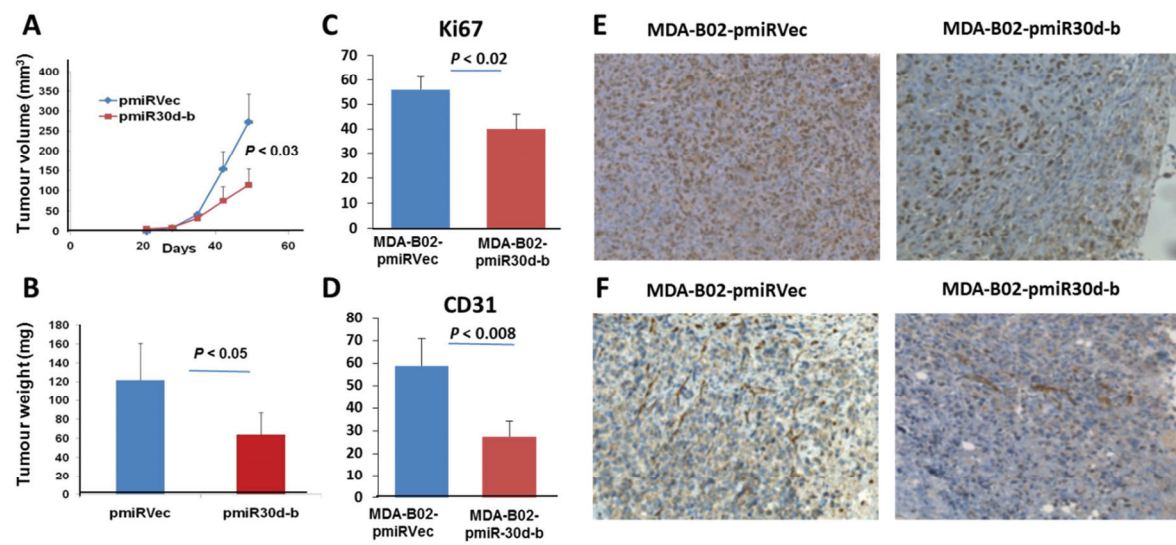


FIGURE 5

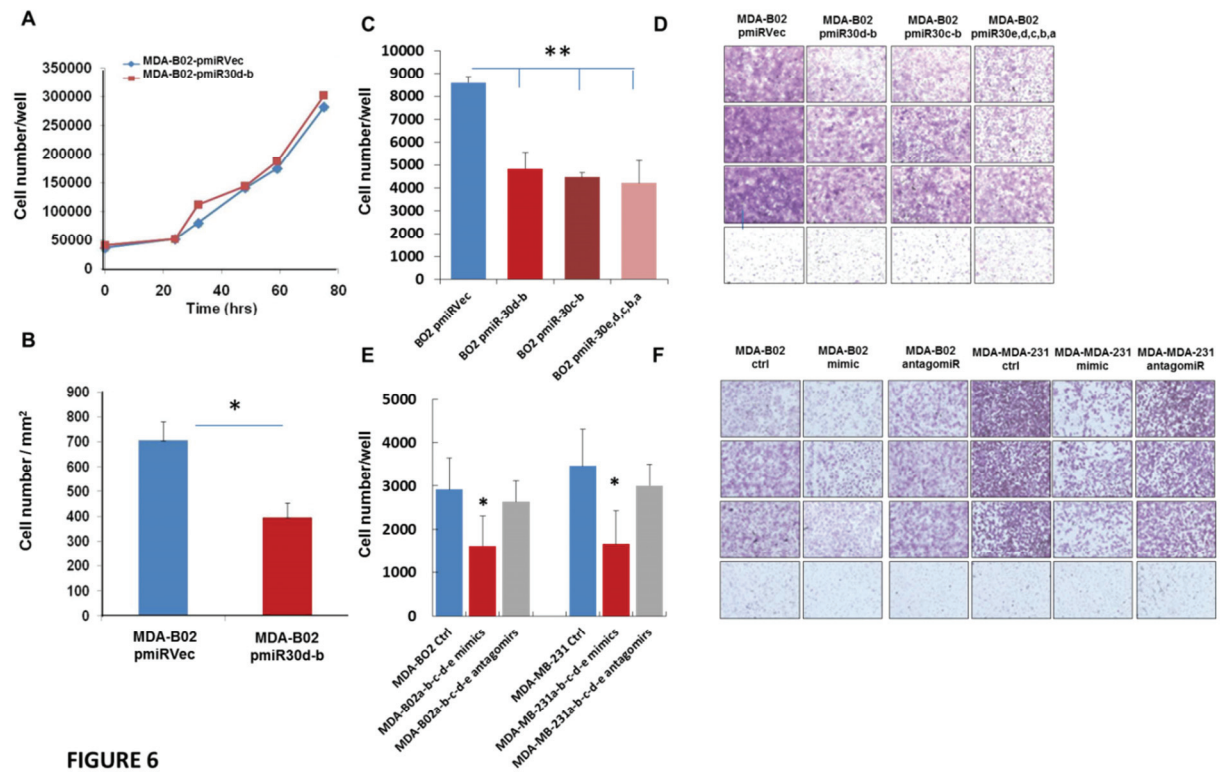


FIGURE 6

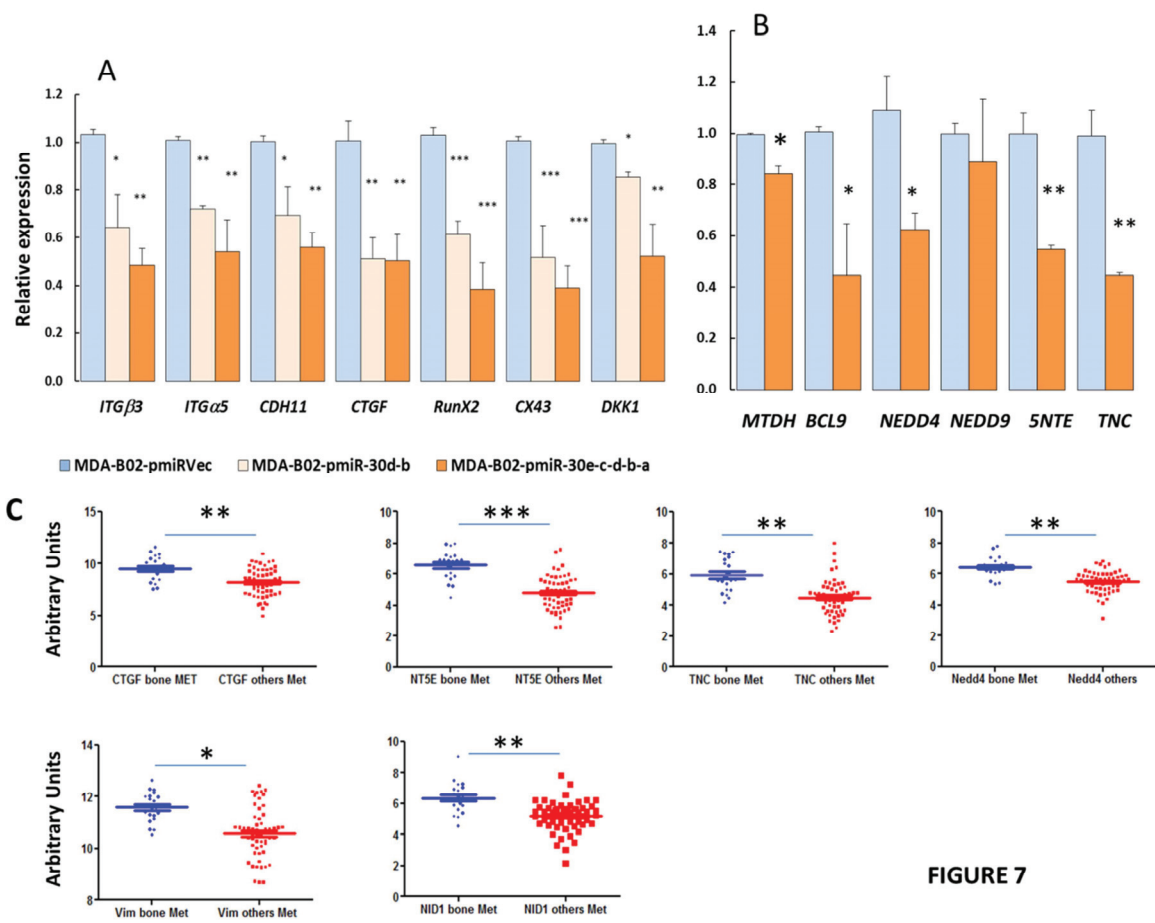


FIGURE 7

Supplementary methods

plentivector constructs

The human miR-30 genes were PCR-amplified from MDA-MB-231 genomic DNA (0.5 µg) with Taq DNA Polymerase in buffer II (Accuprime High Fidelity, Invitrogen) with appropriate primers (See above). PCR products were digested by the appropriate restriction enzymes, purified on agarose gel, and extracted (NucleoSpin, Macherey Nagel) prior to ligation in Plentivector (T4 DNA ligase, Invitrogen).

pmiRVec Construct	Primers	From	PCR Inserts
pmiR-30-e-d-c-b-a			
pmiR-30-e	5'-TAGAAGCTTCCAGCCTGTATTAATAACCAG 5'-TGAGAATTCCATCACAGAGCTCAATCT	pmiRVec HinDIII/EcoRI	30e
pmiR-30-e-d	5'-GTACACCTGCTTTCTAGGATATCAGGGTAATATC-3' 5'-TGAGAATTCTTCTCTAAGGCCAGCAGC-3'	pmiR-30-e AaRI/EcoRI	30d
pmiR-30-e-d-c	5'-GTACACCTGCTTTCTATGTGTCACACAGGTCAC-3' 5'-TAGCACTTGGTGCTCAGTGTACATCTGGTTC-3'	pmiR-30e-d AaRI/DraIII	30c
pmiR-30-e-d-c-b	5'-AGTCTCGAGACACTTTCTCTCAGGATATC-3' 5'-TGAGAATTCTAACTACTCCTACTGCAACC-3'	pmiR-30e-d-c XhoI/EcoRI	30b
pmiR-30-e-d-c-b-a	5'-CTAGTATACAGACATTTTCTAGAATATTGCTG-3' 5'-TGAGAATTCTCCTCAATGCCCTGCTGAAG-3'	pmiR-30e-d-c-b AccI/EcoRI	30a
pmiR-30d-b			
pmiR-30b	5'-TAGAAGCTTAGCCGGGCATAGTAGTAGCGTGCC-3' 5'-TGAGAATTCTACTCCTACTGAACCATGC-3'	pmiRVec HinDIII/EcoRI	30b
pmiR-30d	5'-TCACCACTGCCCTGGATTGCACTTGGTGAACCAC-3' 5'-TGAGAATTCTTTCTCTTAATTATTAGC-3'	pmiR30b BstX1/EcoRI	30d
pmiR-30d-b	5'-ATTCTCGAGAGCCGGGCATAGTAGCGTGCC-3' 5'-TGAGAATTCTACTCCTACTGCAACCATGC-3'	pmiR30d XhoI/EcoRI	30b

psiCHECK-2-CDH11 Vector

A 364 bp of CDH11 ORF was PCR-amplified from MDA-MB-231 genomic DNA (0.5 µg) with Taq DNA Polymerase in buffer II using the following primers: (F) 5'-TAGCTCGAGAGAACTACTGTTTACAAGCC-3' and (R) 5'-TCAGGCCCGGTGTACTCCT-3'. The purified PCR product was digested by XhoI and NotI prior to ligation in psiCHECK-2 vector.

MiRNA quantification in primary tumor of breast cancer patients.

MiRs were extracted from primary tumors with Trizol and quantified on TaqMan 96 wells custom plates after multiplexed RT. Total RNA was reverse-transcribed using a pool of TaqMan MicroRNA Assays RT primers. MiR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p, miR-30e-5p, miR-30e-3p, let-7c, RNU48 and Snub reverse primers were diluted at 0.05X in T/E buffer. RT reaction was carried out by adding 3 µl RNA (100ng) in a mix containing 7 µl RT primer pool, 0.3 µl dNTP, 3 µl Reverse transcriptase 50u/µl, 1.5 µl 10X RT buffer and 0.2 µl RNase inhibitor. PCR amplification was run on StepOne Applied BioSystems Real-time PCR Systems using custom plates. 10 µl TaqMan 2X Universal PCR Master Mix and 1.4 µl of 1/15 diluted RT were added to each well containing primers and probe. Results were analysed with RQ Manager, with RNU48 and Snub as endogenous controls (ΔC_T). Relative quantitation was calculated as the $\Delta\Delta C_T$ mean using both endogenous controls and expressed relative to the median values of all patients.

MiRNA profiling of breast cancer cell lines

Cellular miRs (defined in miRBase database V19, Sangers Institute) were analysed by real-time PCR on **TaqMan® Low Density Array Human MicroRNA Panels A and B** (Applied BioSystems). Single-stranded cDNA was synthesized from 800 µg total RNA by two separated RT reactions using TaqMan MicroRNA Reverse Transcription Kit and Megaplex™ RT Human Pool A, Pool B. Thermo-cycling conditions were 40 cycles: 16°C, 2 min, 42°C, 1 min, 50°C, 1 sec followed by 5 min at 85°C. RT were diluted in 0.1 X TE pH 8.0 and mixed with TaqMan® Universal PCR Master Mix, No AmpErase UNG, 2X prior to loading TaqMan MicroRNA Arrays A and B. Amplification was performed on 7900HT System equipped with TaqMan Array Block and results expressed as relative quantification ($\Delta\Delta C_t$) using RQ Manager software.

Luciferase assay

MDA-B02-pmiRVec, MDA-B02-miR-30-e-d-c-b-a and MDA-MB-231 were seeded in 48 well plates (2×10^4 cells/plate) and transiently transfected with psiCHECK-2-CDH11 Vector. Cells were harvested 48h later in lysis buffer and luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to corresponding firefly luciferase activity and plotted as a ratio between the two values.

Table S1: Identification of genes that are miRs-30 targets and might be involved in bone metastasis.

Gene symbol	Gene name	Score	PCT	8 mer	7-8 mer	Exp Valid
Tumorigenic proliferation						
LIN28B	lin-28 homolog B	-0.46	0.98	1	1	
RARG	retinoic acid receptor, gamma	-0.30	0.97	2	0	
SMAD2	SMAD family member 2	-0.25	0.91	2	0	
KLF9	Kruppel-like factor 9	-0.20	0.67	0	1	
CALB2	calbindin 2	-0.20	0.85	1	1	
ERG	v-ets erythroblastosis virus E26 oncogene homolo	> -0.03	0.93	1	0	(39)
Apoptosis / senescence / cell survival						
AVEN	apoptosis, caspase activation inhibitor	-0.34	0.15	1	0	(57)
MTDH	metadherin	-0.24	> 0.99	2	2	(40)
NFIB	nuclear factor I/B	-0.18	0.99	0	3	
GLI2	GLI family zinc finger 2	> -0.05	0.95	1	1	
Immune surveillance						
B3GNT5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	-1.07	> 0.99	3	0	
GALNT7	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase	-0.84	> 0.99	2	0	(58)
PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide	-0,23	0,92	1	0	
Cancer stemness						
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-0,25	0,7	1	0	
IL1A	interleukin 1, alpha	-0,24	0,62	1	0	
ITGA6	integrin, alpha 6	-0,2	0,85	1	0	
EMT						
EED	embryonic ectoderm development	-0.61	0.76	2	0	
SNAI1	snail homolog	-0.25	0.76	1	0	(38)
SNAI2	snail homolog 2	—	0.51	0	1	
CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila)	-0,55	> 0.99	5	0	
Adhesion						
CELSR3	cadherin, EGF LAG	-1.50	> 0.99	5	0	
S100BP	S100P binding protein	-0.53	0.74	2	1	
ITGA4	integrin, alpha 4	-0.25	0.70	1	0	
PCDH10	protocadherin 10	-0.23	0.86	1	1	
ITGA6	integrin, alpha 6	-0.20	0.85	1	0	
ITGB3	integrin, beta 3	-0.19	0.93	1	0	(59)
ITGA5	integrin, alpha 5	-0.14	0.77	1	0	

PCDH20	protocadherin 20	-0,24	0.56	1	0	
UNC5C	unc-5 homolog C (C. elegans)	-0,21	0.93	1	0	
PCDH19	protocadherin 19	-0,04	0.67	0	1	
SMAD2	SMAD family member 2	-0,25	0,91	0	2	
Migration / invasion						
NEDD4	neural precursor cell expressed, developmentally down-regulated 4	-1.00	> 0.99	2	1	
NT5E	5'-nucleotidase, ecto (CD73	-0.47	0.93	2	0	
TWF1	twinfilin, actin-binding protein	-0.42	0.98	1	1	(33)
BCL9	B-cellII lymphoma	-0.10	0.71	0	2	(49)
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3	-0.18	0.96	1	1	
PLAGL2	pleiomorphic adenoma gene-like 2	-0.10	> 0.99	2	1	
Matrix						
TIMP3	TIMP metalloproteinase inhibitor 3	-0.22	0.93	1	0	
LOX	lysyl oxidase	-0.22	0.94	1	0	
NID1	nidogen 1	-0.05	0.96	1	0	
ADAM12	ADAM metalloproteinase doMayn 12	> -0.03	0.64	0	2	
ADAM22	ADAM metalloproteinase doMayn 22	-0,17	0.73	1	1	
ADAM11	ADAM metalloproteinase doMayn 11	> -0.01	0.84	0	1	
ADAM19	ADAM metalloproteinase doMayn 19	-0,32	0.93	1	0	
ADAM9	ADAM metalloproteinase doMayn 9	-0,18	0.91	1	0	
Bone						
Osteogenesis						
RUNX2	runt-related transcription factor 2	-0.52	0.84	1	1	34,35,41
RUNX1	runt-related transcription factor 1	-0.12	0.96	1	1	
SATB1	SATB homeobox 1	-0.06	0.76	1	0	
SATB2	SATB homeobox 2	-0.05	0.44	1	1	
WNT7B	wingless-type MMTV integration site family, member 7B	-0.01	0.76	1	0	
Osteoclast						
CALCR	calcitonin receptor nuclear factor of activated T-cells	-0.26	0.81	1	0	
OSTM1	osteopetrosis associated transmembrane protein 1	> -0.03	0,84	0	2	
Osteoblast						
GJA1	gap junction protein, alpha 1	-0.06	0.84	1	0	
BMP7	bone morphogenetic protein 7	> -0.02	0,4	0	1	

Biological targets of miR-30 family have been predicted by TargetScan by searching for the presence of conserved 8 mer/7 mer sites that match the seed region of miR-30s. Prediction is marked by context+scores (Score) of the sites and probability of conserved targeting (Pct). 8 mer is an exact match

to position 2-8 of the mature miRNA (the seed+postion8) followed by an “A”, 7-8 mer is the seed + position 8.

Exp Valid indicates the predicted miRs-30 gene targets that have been already experimentally demonstrated to be down-regulated by miRs-30 (marked yellow). The experimental validations are referenced.

Table S2: Clinical pathological features of 120 breast cancer patients.

Variable		N	HR	CI	P-value
Post-Menopausal Status	Yes	69	1.00		
	No	40	1.05	0.49-2.23	0.909
Tumor size	< 20 mm	36	1.00		
	≥ 20 mm	69	2.35	0.89-6.21	0.086
Morphology	Other	20	1.00		
	Ductal	89	1.32	0.46-3.81	0.605
Grade	GI	11	No events		
	GII	42	1.00		
	GIII	28	2.18	0.94-5.05	0.070
pN	Neg	49	1.00		
	1-3 pos	36	1.54	0.49-4.86	
	>4 pos	29	6.89	2.52-18.81	<0.001
pN	Neg	49	1.00		
	Pos	65	3.39	1.29-8.92	0.014
ER	Pos	85	1.00		
	Neg	24	2.09	0.95-4.64	0.068
PR	Pos	74	1.00		
	Neg	35	1.47	0.69-3.15	0.319
ER and PR	Pos	69	1.00		
ER and/or PR	Neg	40	1.93	0.92-4.05	0.083

ER, PR: Estrogen and progesterone receptor. pN: lymph node status

P values correspond to Mann-Whitney test.

Legends of supplementary figures

Figure S1. Heat Map of the differential miR expression in three breast cancer cell lines, MDA-MB-231, MDA-B02 and BC-M1.

Values representative of the relative quantification of miRs measured by TaqMan Low Density Array in three separated cell cultures. Ct values were calculated from the RQ manager (Applied Biosystems) and the t-test and Log₂ fold change were calculated on Microsoft Excel. MiRs that are differentially expressed between the 3 cell lines are reported as log₂ fold-change ≥ 1.5 , with P-value ≤ 0.05 considered as significant. Heat-map and hierarchical clusters were performed using the Euclidean distance and pairwise average linkage on Genepattern v3.2.3 (Broad Institute).

Figure S2. Mesenchymal phenotype of MDA-B02 and BC-M1.

(A) Expression of genes associated with epithelial-mesenchymal transition is increased in MDA-B02 and BC-M1. (B) MDA-B02 and BC-M1 are CD44⁺ and CD24⁻ and have ALDH activity. Dicer expression is decreased in MDA-B02 and BC-M1 relatively to MDA-MB-231, ***: P<0.01, **: p<0.02.

Figure S3. Expression levels of miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p in breast cancer cell lines.

Levels of miR-30s mature forms were measured in breast cancer lines isolated from basal claudin-low subtypes (MDA-MB-231, MDA-B02, BC-M1 and Hs575) from hormone responsive luminal A (T47D, MCF-7), luminal B (BT-474, ZR-75) and in HER+ subtype (SK-BR3). Results are mean $\pm$ SD of triplicate determination on three different biological samples.

Figure S4. Expression of miR-30a-5p, miR-30b-5p, miR-30c-5p miR-30d-5ps and miR-30e-5p in MDA-B02 after transfection with miRNA-mimics and in MDA-MB-231 after transfection with miRNA-inhibitors.

(A) MDA-B02 were transfected by a mix of the five miR-30s-5p mimics at equimolar concentrations (MDA-B02-miR-30a,b,c,d,e) and by equivalent concentrations of a miR scramble control sequence (MDA-B02-miR-Ctrl), log₁₀ values in MDA-B02-miR-30a,b,c,d,e relative to MDA-B02-miR-Ctrl. (B) MDA-MB-231 were transfected by equimolar concentration of the five miR-30s-5p, chemically modified single-stranded RNA molecules designed to specifically inhibit endogenous miRNA and with control scramble (miRNA inhibitors, Applied BioSystems). Results are triplicate determination of three biological samples.

Figure S5. Experimental validation of cadherin11 targeting by miRs-30.

(A) A conserved 8-mer site in CDH11 ORF matches the seed region of miR-30a-5p, miR-30b-5p, miR-30c-5p, miR-30d-5p and miR-30e-5p. (B) Free energy for duplexes of CDH11 coding sequence and miR-30s seed sequence has been calculated according to RNAhybrid Software. (C) MiR-30s sequence is conserved through evolution. The alignments have been done using the publicly available data base, miRBase. (D-E) Cadherin11 is highly expressed in MDA-B02 compared to parental MDA-MB-231. (F) Firefly luciferase activity of the psiCheck2-CDH11 reporter in MDA-B02, MDA-B02-pmir-30e-d-c-b-a and MDA-MB-231. Values are normalized to that of the internal Renilla luciferase (Mean $\pm$ SEM of triplicate on two biological samples).

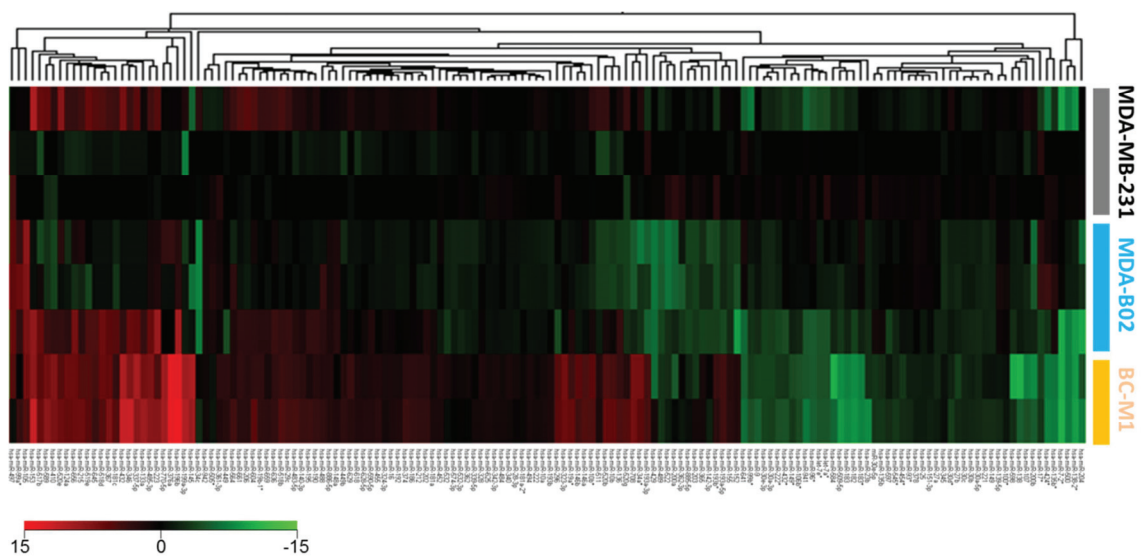


FIGURE S1

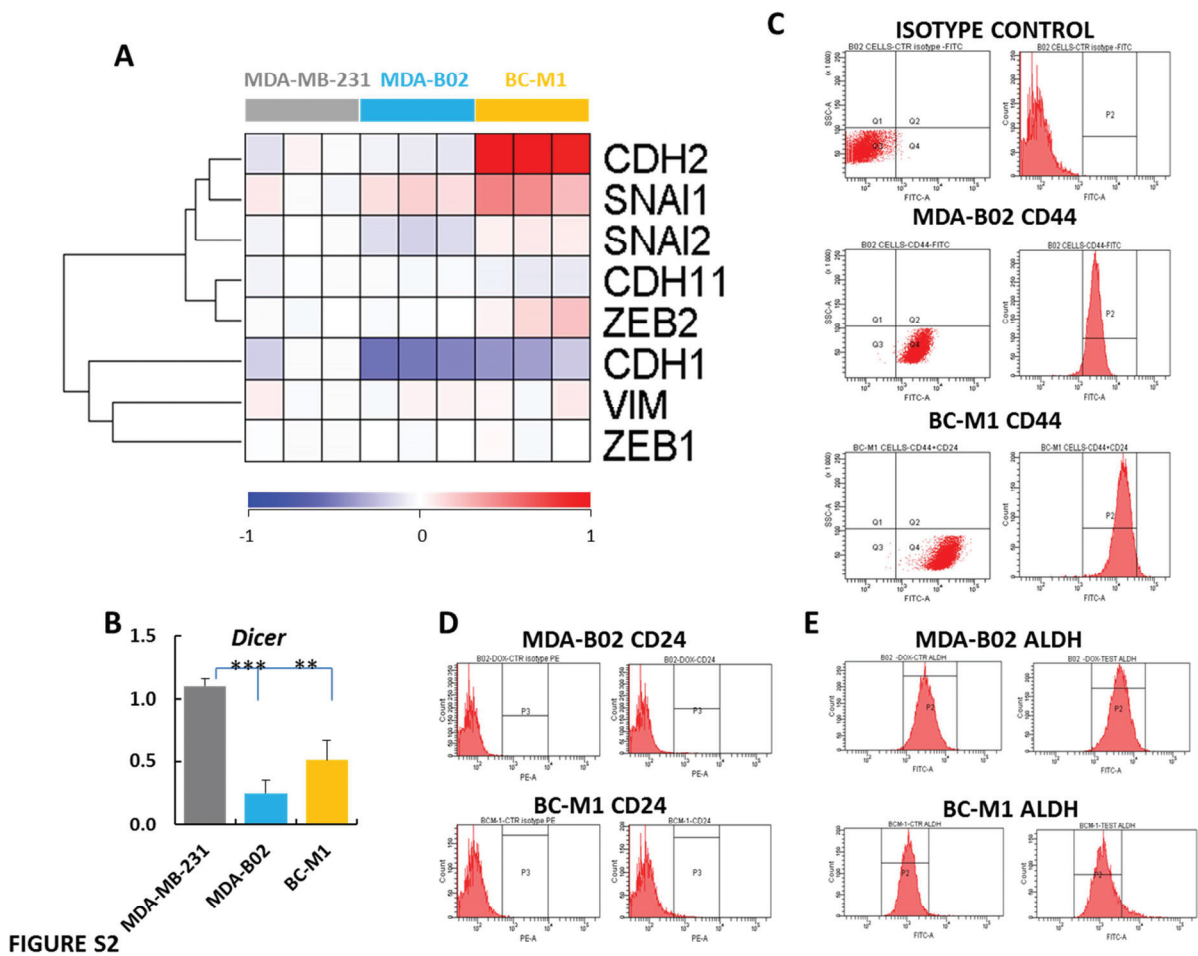


FIGURE S2

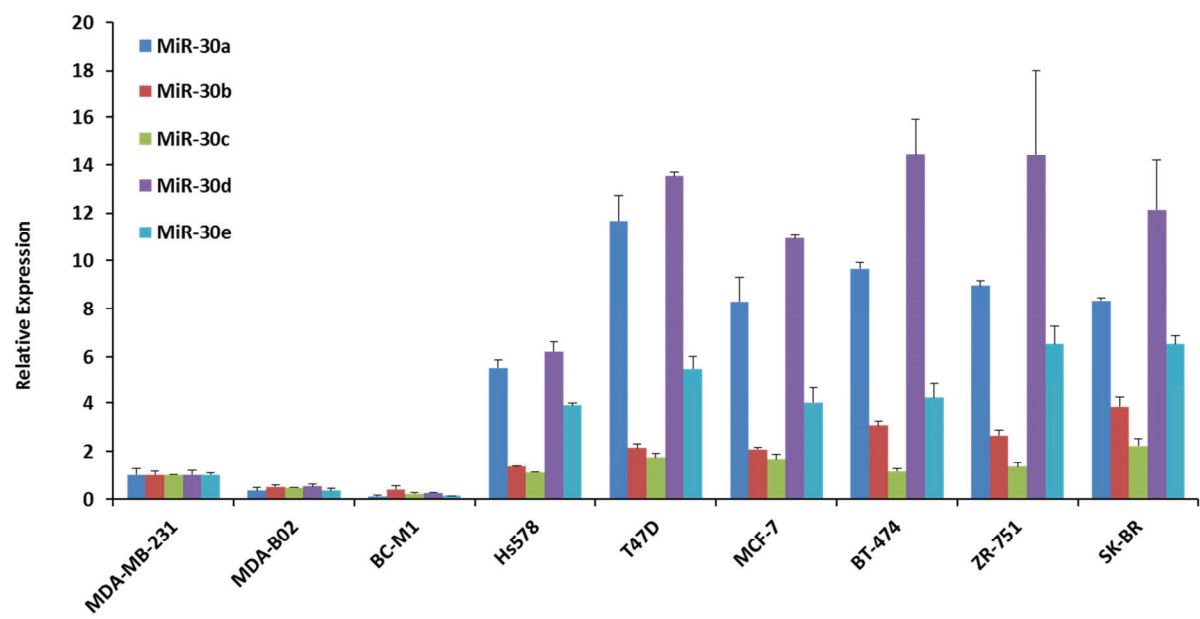


FIGURE S3

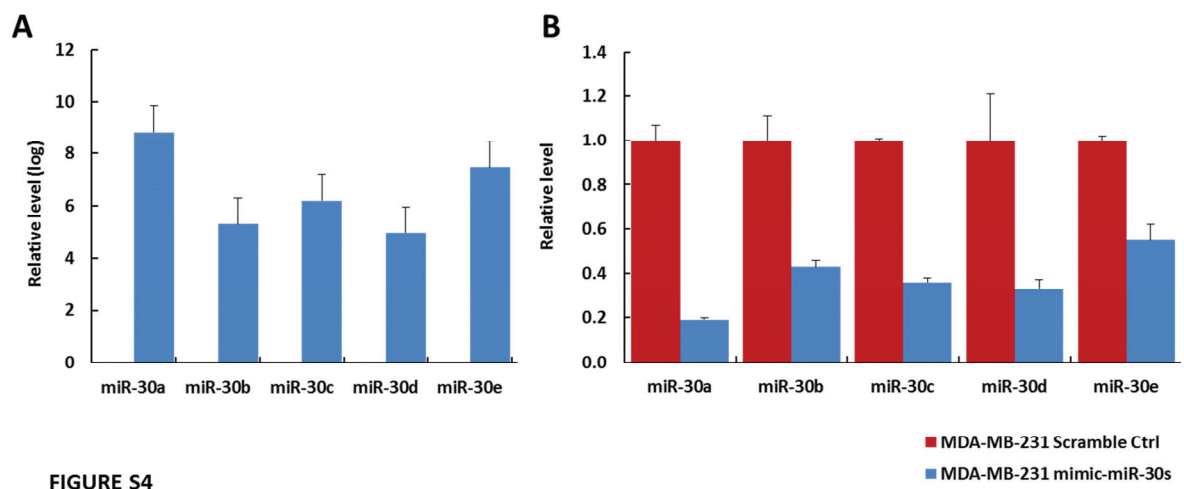


FIGURE S4

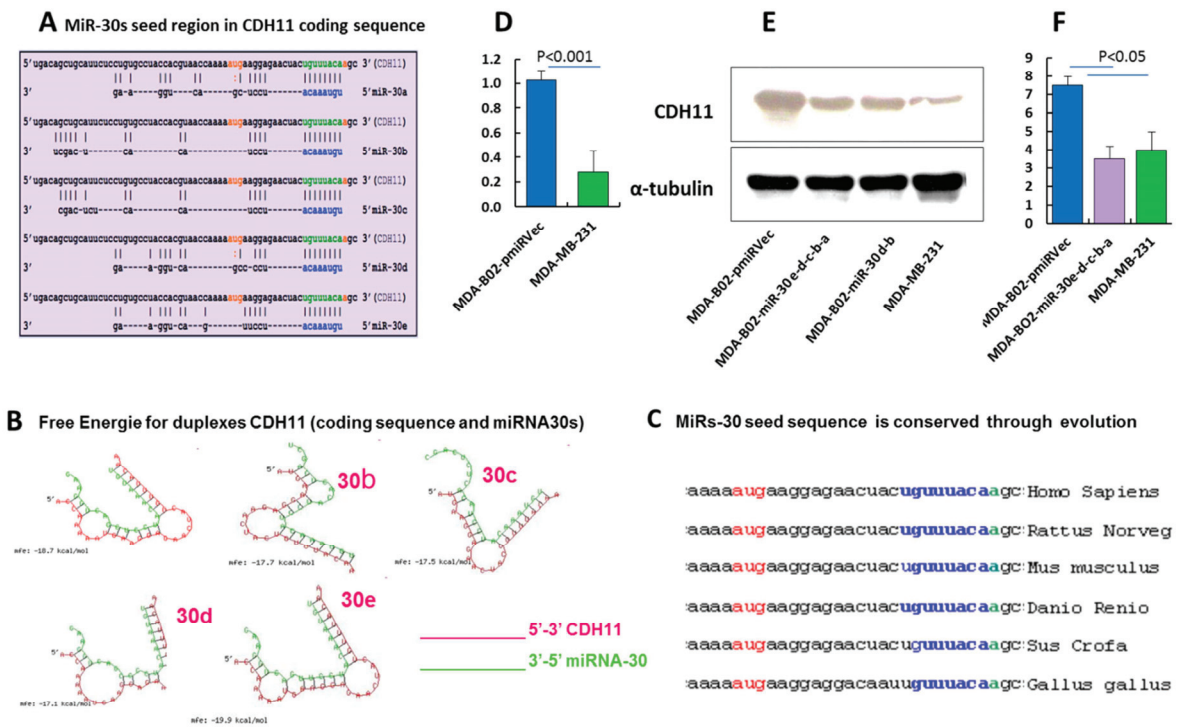


FIGURE S5

1.2. MiRs-30 activity in breast tumorigenesis and tumor cell dissemination to bone.

In the first part of the work, we have shown that the miRNA-30 family (miRs-30) regulates the expression of a set of genes which are mediators of bone metastasis formation, at the steps of bone tropism, homing, colonization and macro-metastasis development. Through the carcinoma-induced down-regulation of Dicer expression, large sets of miRNAs are underexpressed in breast human tumors compared to normal tissues and this interference with miRNA processing enhances experimental tumorigenesis (Valastyan and Weinberg, 2010). Therefore, the decrease of miRs-30 levels, which are highly expressed in epithelial tissues, in the breast primary tumor may affect breast tumorigenesis and the subsequent metastatic dissemination (Joglekar et al., 2009). Based on the analysis of a genome-wide miRNA expression in a set of normal and tumor breast tissues the pioneer work of Iorio et al. (Iorio et al., 2005) demonstrates the existence of a breast cancer-specific miRNA signature. The authors reported that miRs-30, the levels of which did not differ in tumor versus normal tissue, was expressed at higher levels in hormone-dependent and well differentiated breast tumors versus hormone-independent tumors. MiRs-30 are tumor suppressor in lung (KuMarchwamy et al., 2012b), prostate (Kao et al., 2013), breast (Zhang et al., 2013) and anaplastic thyroid (Visone et al., 2007) cancers mostly by regulating EMT and related transcription factors such as SNAIL1 and ZEB2. Their lower expression levels in lymph node metastases versus primary tumors suggest a role for miRs-30 during metastatic dissemination of breast carcinomas (Baffa et al., 2009). Moreover, miRs-30 were reduced in putative breast tumor-initiating cells (BT-ICs) inducing the up-regulation of ubiquitin-conjugating enzyme 9 (Ubc9) and ITG β 3 (Yu et al., 2010). Enforced constitutive expression of miRs-30 in BT-ICs inhibited their self-renewal capacity by reducing Ubc9, and induced apoptosis through silencing ITG β 3. Growing MCF-7 under non-attachment conditions revealed that miRs-30 level was markedly lower than in parental cells that in turn, modulated the expression of apoptosis and proliferation genes (Ouzounova et al., 2013).

In this part of work, we asked whether miRs-30 deregulated expression in breast primary tumor might modulate tumor growth and subsequent metastatic dissemination to bone. To answer this question, we used murine breast cancer cell lines that spontaneously metastasize. We have modulated the endogenous miRs-30 level of these cells by lentiviral transduction and by transfection of chemically stabilized oligonucleotides prior to inoculation in mice. We also set up a syngeneic orthotopic mouse model, based on mammary gland injection of breast cancer murine

cells. To mimic the *in vivo* phenotype of CTC, tumor cells have been grown in anchorage-independent conditions, as mammospheres. The impact of 3-dimensional culture on miRs-30 endogenous level of these cells has been analyzed in relation to their target genes. Tumor cells dissociated from mammospheres have been inoculated in caudal artery of mice to study their metastatic potential to bone.

1.2.1. Materials and Methods

1.2.1.1. Cell lines and cell culture

To study the role of miR-30s in the early steps of metastatic dissemination, we used four murine cell lines: 67NR, 66c14, 4T1 and 4T1.2. Originally, three sublines of breast cancer cells were derived from a spontaneously arising mammary tumor of a BALB/cfC3H mouse: the 67, 66 and 410 (Dexter et al., 1978) (Fig 10). The 67NR were next transfected with the bacterial pSV2 plasmid containing the neomycin resistance gene. They form primary tumors, however, they do not metastasize (Aslakson and Miller, 1992), (Fig 11). The 66 was subjected to mutagenesis with ethyl methanesulfonate. In that way, the 6-thioguanine-resistant mutant, line 66c14 was isolated from a clone of these cells (Miller et al., 1983). Later on, 66c14 were transfected with the pGL4 vector to stably express the luciferase gene and were shown to metastasize from the primary tumor to the lung and bone but also to liver (Aslakson and Miller, 1992). The last subline, the 410, was derived as a primary culture from a single nodule growing in the lung of Balb/c mouse bearing an s.c implant of the tenth *in vivo* passage of the original, unseparated tumor. The tumor 410 was serially transplanted through 4 *in vivo* passages at which time tumor cells were designated 410.4. The 4T1 is a 6-thioguanine resistant variant of 410.4 that was selected without mutagen treatment (Aslakson and Miller, 1992). From this cell line, a 4T1.2 cell line was established by a single cell cloning (Lelekakis et al., 1999). 4T1.2 was shown to closely model the sites of metastases seen in human patients including lung, liver, and bone (Fig 11). Specifically, 4T1.2 cells demonstrated a high incidence of metastasis to spine (Bolin et al., 2012).

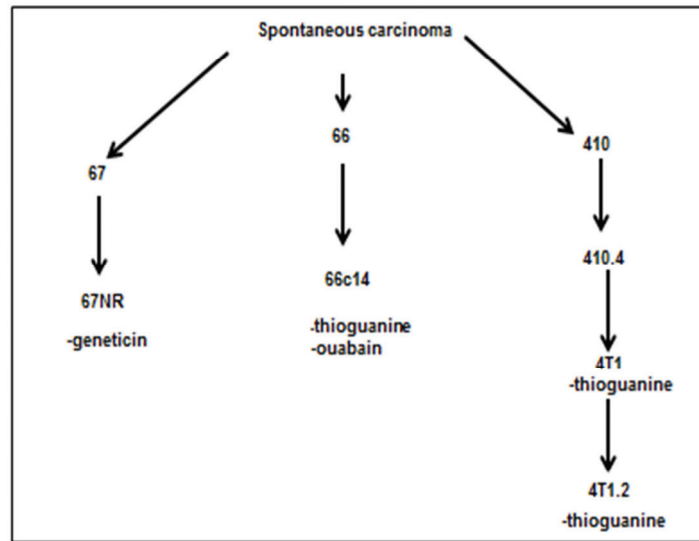


Figure 10. Derivation of murine breast cancer cell lines.

cell line	Metastatic potential				
	Dissemination route		Lung	Liver	Bone
	Hematogenous	Lymphatic			
67NR	-	-	-	-	-
66c14	+	++	++	+	+
4T1	++	+	++	+	+
4T1.2	++	+	+	-	++

Figure 11. Metastatic dissemination of breast cancer cell lines after inoculation of these cells in mice mammary gland.

The 67NR were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1 % penicillin/streptomycin. 66c14 were cultured in α MEM medium supplemented with 10 % fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium pyruvate. 4T1 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin and 0.2 % fungizone. 4T1.2 cells were cultured in α MEM medium supplemented with 10 % fetal bovine serum, 1% penicillin/streptomycin and 1% sodium pyruvate. All cells were cultured at 37°C in a humidified atmosphere in 5 % CO₂ incubator.

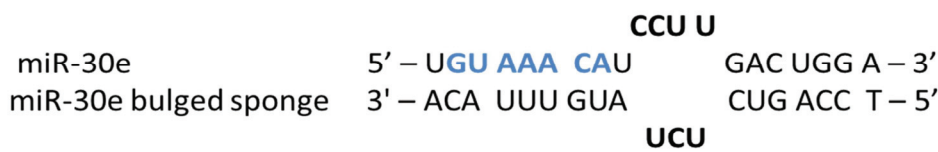
In animals, we inoculated the following cell lines: 4T1 wild-type cell line (4T1) which is from ATCC, UK, 4T1 expressing luciferase2 (4T1luc2) which was engineered in the lab, 4T1.2-luciferase2 cell line (4T1.2luc2) and 66c14-luciferase2 cell line (66c14luc2) which were a generous gift from Celeste Boilin and Cheryl Jorcyk, Boise State University, ID, USA.

1.2.1.2. Plasmid construction

The pCMV-luciferase2 retroviral vector was constructed by inserting the coding sequence of Firefly luciferase2 (luc2) into the multiple cloning site of pMiRVec. To increase expression and reliability of the firefly luciferase, a synthetic firefly luciferase gene, has been engineered by the constructor (Promega) and inserted in the cloning vector PGL4. The gene was synthetically redesigned by changing the codons to those most frequently used in mammalian cells while simultaneously removing most of the consensus sequences for transcription factor binding sites. Luc2 coding sequence was amplified by PCR using Accuprime High Fidelity (Invitrogen) and purified after agarose gel extraction (NucleoSpin, Macherey Nagel). PCR product was inserted into HindIII/EcoRI-digested pMiRVec vector using In-Fusion HD Cloning Kit (Clontech Laboratories). PCR primers for luc2 were designed with 15 base pairing extensions that are complementary to the ends of the linearized vector. In-fusion cloning reaction was as followed: 2 ml of In-Fusion HD Enzyme Premix (5X), linearized vector (25ng), PCR product (50ng), completed to 10 μ l final volume with H₂O. Reaction was incubated for 15 min. at 50°C and placed on ice, prior to transformation of Stellar Competent cells (Clontech).

The retroviral PmiRVec-miR-30sponge vector was constructed by inserting the miR-30 bulged sponge sequence in the MCS of pMiRVec. Briefly, “miRNA sponges” are miRNA competitive inhibitors that have been developed in Philipp Sharp’s laboratory, as an alternative to modified antisense oligonucleotides (Ebert et al., 2007). They function as decoy targets that anneal to the

cognate miRNA and derepress miRNA targets in mammalian cells. They are designed as oligonucleotides to miRNAs binding sites with a bulge at the position normally cleaved by Argonaute 2 and are inserted tandemly as heptameric sites into the 3'UTR of a reporter gene. MiR-30-Sponge have been constructed by Philipp Sharp laboratory by inserting six miR-30 binding sites tandemly arrayed into the 3'UTR of a reporter gene encoding destabilized GFP driven by the CMV promoter. Binding sites for miR-30 seed family were perfectly complementary in the seed region with the bulge at positions 9-12 to prevent RNA interference-type cleavage and degradation of the sponge, as followed:



The sponges specifically inhibit miRs with a complementary heptameric seed (the sequence indicated in blue, common to the five members of miRs-30), such that a single sponge can be used to block an entire miR seed family. We have digested the DNA with six bulged sequences (in red) from pcDNA5-CMV-d2eGFP vector and cloned this sequence into MCS of pmiRVec to obtain stable expression of this miRs-30 sponge.

CCATGTCTTGCGCCAGGAGAGCGGGATGGACCGTCACCCTGCAGCCTGTGCTTCTGCTAGGATCAATGT
 GTAGGCGGCCGCTCGAGCCGGTCCAGTCCCTATGTTTACACCGGTCCAGTCCCTATGTTTACCGGTCCAG
 TCCCTATGTTTACACCGGTCCAGTCCCTATGTTTACACCGGTCCAGTCCCTATGTTTACACCGGTCCAGTCCC
 TATGTTTACACCGGATCGCGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCC
 ATCTGTTGTTTGCCCTCCCCCGTGCCTTCCTTGACCTGGAAG

1.2.1.3. Cell transfection and transduction

Transient overexpression or down-regulation of miRs-30 in tumor cell lines was achieved by transfecting the cells with chemically modified double-stranded RNAs that mimic endogenous miRNAs (miR-mimics, Applied BioSystems) or chemically modified single-stranded RNA molecules designed to specifically inhibit endogenous miRNA (miRNA inhibitors, Applied BioSystems). Cell lines were transfected with 10nM of each miR-30s mimics or antagonist sequences or with 50 nM of negative scramble controls (Applied BioSystems). These oligonucleotides were incubated with 10^6 cells in appropriated medium, in the presence of

HiPerFect transfection reagent for 30 min at 37°C in a 5% CO₂ incubator, prior to cell plating. Transfected cells were then grown in culture for 48-72 hours before cell assays.

Tumor cells were transduced using the amphotropic retroviral packaging system (ClonTech Laboratories). Briefly, GP2-293 cell line which has the viral gag and pol genes incorporated in its genome were transfected with the pantropic VSV-G envelope vector and the retroviral pmiR-Vec vector (kindly provided by Dr R. Agami, The Netherlands Cancer Institute, Amsterdam). 4T1 cell line was transduced with pCMV-luciferase2 retroviral vector generating 4T1 cell line stably-expressing luciferase2 (4T1luc2). 4T1.2 and 66C14 cell lines were transduced with pmiRVec – miR-30-sponge. Virus particles were collected at 72h after transfection, filtered and used to transduce the recipient cells in presence of 8 µg/mL polybrene. Vector transduced cells were selected with blasticidine.

Finally, cell transduction was also performed with RNAi MISSION^{TR} Lenti microRNA Inhibitor particles. For these experiments, we obtained lentiViral particles from Sigma and performed 4T1.2-luc2 and 66c14-luc2 transduction by incubating 4×10^4 cells with 10 TU/cell, in presence of 8 µg/ml of polybrene for 72h. Selection of stable transfectants was further achieved by incubating the cells with puromycin. Briefly, in this technology, expression of the miRNA inhibitors is driven by the hU6 promotor upon genomic integration of the lentiviral transfer vector into the host cell post-transduction (Haraguchi et al., 2009). MiRNA inhibitors are able to competitively bind specific miRNAs and prevent them from regulating their endogenous targets. Lentiviral vectors have been constructed by integration of decoy RNA molecules in the MCS of pLKO lentiviral vector (Haraguchi et al., 2009). The entire secondary structures of these decoys and the sequence of miRNA-binding site have been optimized to establish a highly potent miRNA inhibitory system (tough decoy design).

1.2.1.4. Animal studies

Four-week-old female Balb/c mice were purchased from Janvier Laboratories. All procedures involving animals, including their housing and care, the method by which they were culled, and experimental protocols were conducted in accordance with a code of practice established by the local ethical committee of the University of Lyon.

Mice were injected orthotopically into the fat pad of the 4th mammary gland with 10^5 cells in 100 µL of 50 % PBS-50 % Matrigel for 4T1.2luc2 and 66c14luc2 and with 10^5 cells in 10 µL PBS,

for 4T1. Tumor size was calculated by external measurement of the width (m1) and length (m2) of tumors using a Vernier caliper. Tumor volume (TV) was calculated using the equation $TV = (m1^2 \times m2)/2$. On day 14 after tumor cell inoculation, mice were anesthetized and the primary tumors were resected, measured, weighted and cut into two pieces. Half of tumors was fixed into 4 % PFA for immunohistochemistry and the remaining tissue was placed into liquid nitrogen for RNA extraction.

Injection of 4T1.2luc2 and 66c14luc2 grown under non-attachment conditions and of control cells grown on plates was performed intra-arterially with 10^5 cells in 100 μ L of PBS.

Bioluminescence imaging of animals was performed weekly, by s.c injection of luciferine and was monitored by the Nightowl imaging system (Berthold, Germany), as previously described (Bolin et al., 2012; Fradet et al., 2011; Peyruchaud et al., 2001).

At the end of protocols, animals were anesthetized, sacrificed and several organs were dissected. Hind limbs and spines were placed in medium and lungs in PBS.

1.2.1.5. Bone marrow micrometastasis isolation

For *ex-vivo* bone marrow micrometastasis experiments, cells were injected into the mammary gland or into the tail artery of animals. At day of sacrifice, hind limbs, spines and lungs were collected and tibiae and femurs were minced then soaked in an enzyme cocktail containing 300U/mL type-I collagenase and 100 U/ml hyaluronidase (StemCell Technologies) in DMEM medium for 2 hours at 37°C. Lungs were minced and an enzyme solution containing 0.25 mg/mL type-I collagenase (Sigma) was added for 1 hour at 37°C. After incubation, cell suspensions from bone marrow and lungs were seeded in 6-well plates and cultured in complete medium. After 1-day culture, cells were placed under 6-thioguanine selection, allowing the selective growth of antibiotic-resistant tumor cells. Colonies of tumor cells were then fixed and stained with crystal violet.

1.2.1.6. Cell invasion assay

Experiments were conducted in 24-well cell culture plates with 8- μ m diameter pore-size inserts, coated with 100 μ L basement membrane Matrigel (0.3 mg/mL), as previously described (Fradet et al., 2011). 4T1.2luc2 or 66c14luc2 were transfected with either control or miR-30 inhibitors. After 48 h, cells were detached with trypsin/EDTA and a suspension of 5×10^4 cells in 300 μ L of

culture medium containing 0.1 % (w/v) bovine serum albumin was loaded into each insert (upper chamber). The chemoattractant [10 % (v/v) fetal calf serum] was placed in the lower chamber (750 µl /well). After a 24-h incubation at 37°C in a 5 % CO₂ incubator, inserts were collected, the non-invading cells were removed and the invading cells on the under surface of the inserts were stained with 0.5 % crystal violet for 10 minutes. Cells were then counted under microscope.

1.2.1.7. Cell culture under-non attachment condition

66c14luc2 and 4T1.2luc2 cells were cultured on ultralow attachment 10 cm plates (Nunc/Sphera) at 1×10^4 cells in 10 mL Mammocult Basal Medium, supplemented with 10 % Mammocult Proliferation Supplement, 0.0002 % heparin, 1 µM hydrocortisone, 0.5 % penicillin/streptomycin and 500 µM L-glutamine for 5 days. At this time, cell aggregates were mechanically and enzymatically disrupted, filtered through 70 or 40 µm filter, counted and cultured again in 10 cm ultralow attachment plates. The same was repeated twice and at each stage, some cells were cultured under attachment condition. RNA was extracted from both conditions to analyze miR-30s and gene expression levels.

1.2.1.8. RNA isolation

1.2.1.8. a. RNA isolation for gene expression analysis

Total RNA was extracted using Total RNA isolation NucleoSpin RNA II kit, as described in the protocol.

1.2.1.8. b. RNA isolation for microRNA expression analysis

Total RNA, including small RNAs, was extracted using miRNeasy Micro Kit, as described in the protocol.

1.2.1.9. Real time RT-PCR

1.2.1.9. a. RT-PCR for gene expression analysis

cDNA was produced from 1 µg total RNA using the iScript cDNA Synthesis Kit. Real-time quantitative PCR reactions were performed using a SYBR Green qPCR kit on a RealPlex (Eppendorf, Durham). Relative gene expression levels were normalized according to the Ct

value of the gene encoding L32 protein and results were expressed as fold differences equal to $2^{-\Delta\Delta Ct}$.

1.2.1.9. b. RT-PCR for microRNA expression analysis

TaqMan microRNA assays were used to quantify miRs-30 and snU6 levels after RNA isolation. 100 ng of total RNA were reverse-transcribed using the MultiScribe reverse transcriptase and 50 nM stem-loop RT primers specific for miR-30 members and the endogenous control snU6. Mature miRNAs were amplified using the TaqMan 2X universal PCR Master Mix and miRs-30s level were expressed as fold differences equal to $2^{-\Delta\Delta Ct}$.

1.2.1.10. Reagents

Name	Final concentration	Reference	Company
DMEM (1X) medium	(1X)	41966-029	Gibco
MEM Alpha Medium (1X)	(1X)	22561-021	Gibco
RPMI-1640 (1X)	(1X)	A10491-01	Gibco
MammoCult® Basal Medium (Human)	(1X)	5621	STEMCELL TECHNOLOGIES
Mammocult Proliferation Supplement (human)	10%	5622	STEMCELL TECHNOLOGIES
Heparin	0.0002%		
Hydrocortisone (10 µg/µl)	1 µM		
L-Glutamine 200 nM (100X)	500 µM	25030	Gibco
Sodium Pyruvate 100nM	1nM	11360-039	Gibco
PenicillinStreptomycin	1nM	15140-122	Gibco
0,05% Trypsin-EDTA (1X)		25300-054	Gibco
Fungizone® Amphotericin B 250 mg/ml	0.2%	15290-026	Gibco
6-thioguanine 50mg/ml	0.01 mg/ml	A4882-1G	Sigma
Crystal violet			
Collagenase from Clostridium Histolyticum 1mg/ml	250 µg/ml	C1639-50MG	Sigma

Collagenase/hyaluronidase	300U/100U/ml	79919	STEMCELL TECHNOLOGIES
Fetal Bovine Serum	10%	FB-1001	BioSera
Non attachment culture plate			Thermo Scientific
MycoKill AB (50X)	10 µg/ml		PAA
Growth Factor Reduced BD Matrigel Matrix	300 µg/ml	354230	BD Biosciences
rDNase	1/10	D1109R	Macherey-Nagel
EXPRESS SYBR® Green ER™ qPCR Super Mix Universal	10µl	A10314	Invitrogen
TaqMan Universal PCR Master Mix	10µl	4324018	Applied Biosystems
HiPerFect®	12% (incubated for 30 min)	301705	Qiagen
Buprécare 0,3mg/ml	1,8 µl	F15	Axience
Kétamine 1000 130mg/kg	100 mg/ml	4F3R	Virbac France
Rompun 2% 8,8mg/kg	20 mg/ml	KP08FN1	Bayer
NaCl solution	0.9%	3400936340824	Lavoisier
Vetflurane	3%	4FTR	Virbac France
Luciferine for <i>in vivo</i> use 10mg/ml-Luciferine-EF™	200 µl	E655X	Promega
luciferine for <i>in vitro</i> use- Luciferase Assay Reagent	0.1%	E1483	Promega
pmiR-Vec vector			Generous gift from Reuven Agami.
Amphotropic Systems			ClonTech Laboratories, Takara Bio Company
RNAi MISSION ^{TR} Lenti microRNA Inhibitor		HLTUD0001...	Sigma

1.2.2. Results

1.2.2.1. Orthotopic murine model of metastatic dissemination from breast tumor cells: 4T1

We have first set up a mouse model of spontaneous breast cancer metastasis. In this animal model, tumor cells form a primary tumor that is resected, mimicking clinical situation, and tumor cells disseminate to secondary organs to form micro- or macro- metastasis. 4T1-luc2 form primary tumors that are detected by bioluminescence imaging. However, we could not detect tumor cells in bone and lungs by this technique. In regard of the difficulty to detect bioluminescence in lungs, we isolated the DTC from legs and lungs. Micro-metastases were detected in lungs but not in legs. Indeed, when injected in mammary gland in a syngeneic model, some investigators report that 4T1 highly disseminates to lungs, but not to bone. Our results were in accord with these data. We further asked if 4T1 tropism might have been disturbed by luciferase2 labeling or transduction. Therefore, we performed another study using the same mice model, in which 35 mice have been inoculated with 10^5 4T1 cells. Every 7 day, 7 mice were sacrificed, lungs and bone were collected and DTC were isolated from lungs and bone. Despite the fact that lungs were highly metastatic, the presence of micro-metastases in bones was found in only one out of 35 mice, meaning that in our hands, 4T1 metastasizes very weakly, if not at all, to bones.

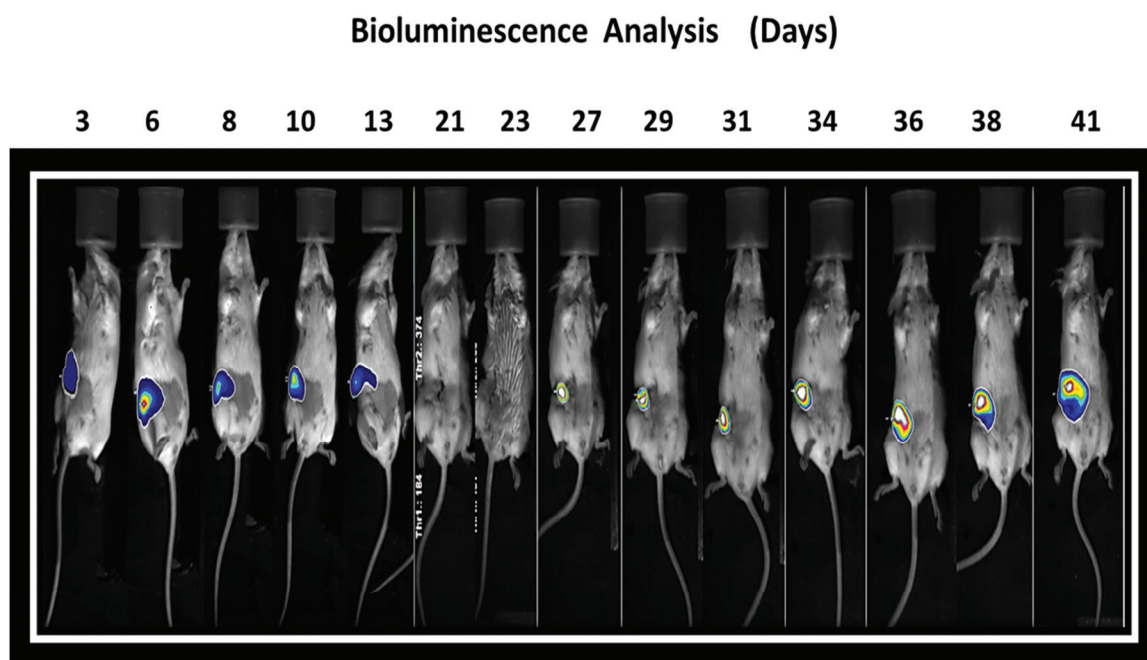


Figure 12. Follow-up of bioluminescence imaging from a mouse inoculated with 4T1luc2 in mammary gland.

Numbers indicate the day of BLI imaging. At day 14, the primary tumor was resected. The colourful spot on mouse indicates the bioluminescent 4T1-luc2 which forms the primary tumor. After 14 days, tumor was resected and tumor regrowth was detected over time.

1.2.2.2. Orthotopic murine model of metastatic dissemination from breast tumor cells: 4T1.2luc2, 66c14luc2

4T1.2luc2 was injected in 10 μ l PBS, according to the Lab's procedure. However, even by increasing the cell amount over 10^5 , we did not get formation of primary tumor, indicating probably a weaker tumorigenic propensity of these cells. Therefore, we have adapted a protocol (from Christophe Ginestier, Centre de Recherche en Cancérologie de Marseille, Laboratoire d'Oncologie Moléculaire, UMR891 Inserm/Institut Paoli-Calmettes, Marseille, France), in which 4T1.2luc2 was injected in 100 μ l of 50 % / 50 % mixture of PBS and Matrigel. In these conditions, 4T1.2luc2 forms primary tumor and disseminate to secondary organs (Figures 13).

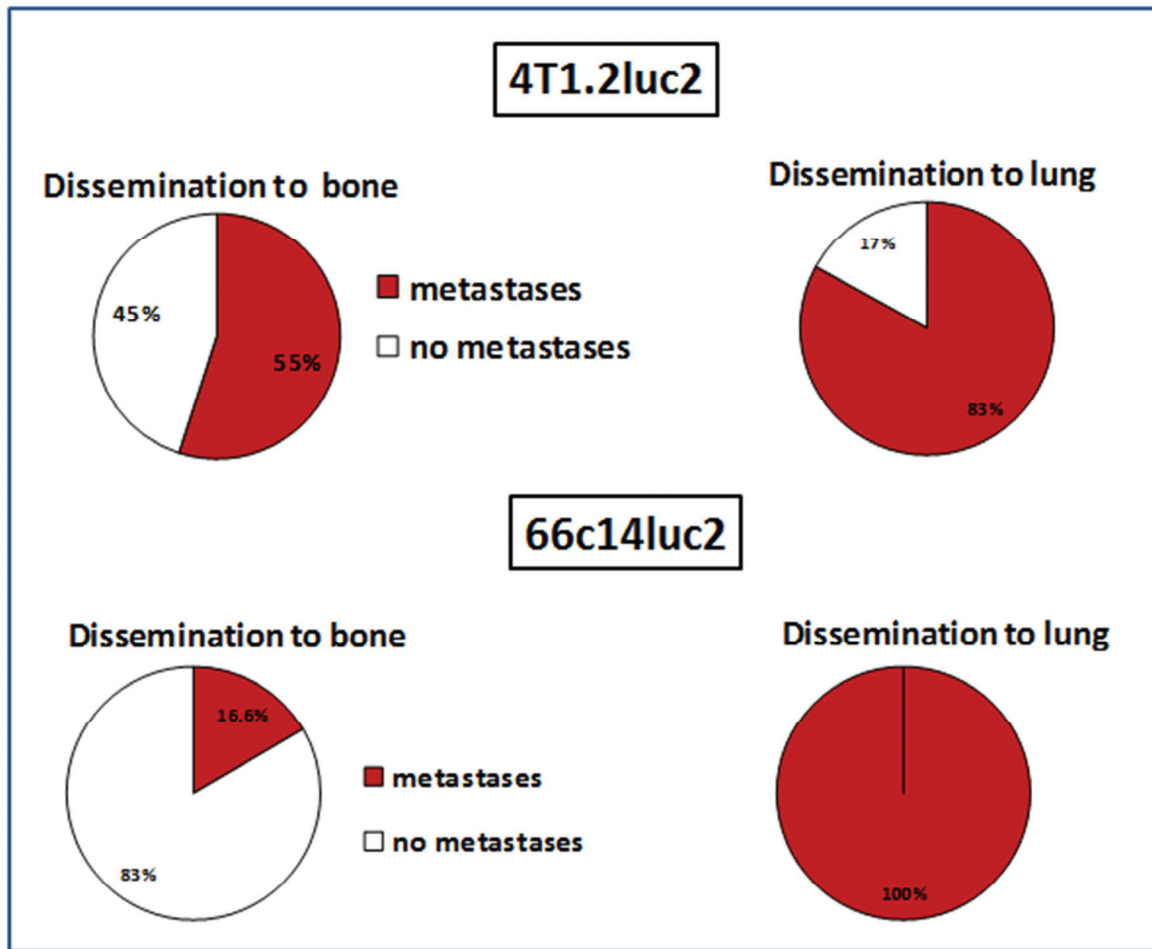


Figure 13. Spontaneous metastatic dissemination of 4T1.2luc2 and 66c14luc2 to bone and lung after cell inoculation into the mice mammary fad pad.

The diagram represents the percentage of mice having developed bone and lung metastases. (n=12 animals).

Despite the fact that injection of 66c14luc2 in 10 μ l PBS, according to the Lab's procedure induced primary tumor formation, we used the same procedure than the one used for 4T1.2luc2 study to compare the metastatic behavior between the two cell lines. 66c14luc2 was injected at range from 0.25×10^5 to 1×10^5 cells in 100 μ l of 50 % / 50 %, PBS / Matrigel. Finally, injection of 1×10^5 66c14luc2 induced strong dissemination to lungs whereas few mice developed bone metastasis. Moreover, for both cell lines, only the incidence was plotted. It should be noticed that the number of bone micro-metastasis was high in mice inoculated with 4T1.2luc2 but not in lung and inversely for 66c14luc2.

1.2.2.3. Expression of miRs-30 in breast cancer cell lines

MiRs-30 expression was investigated in the murine cell lines and their levels compared to human breast cancer cell lines, MDA-B02 and its parental MDA-MB-231. MiRs-30 were highly expressed in the three murine cell lines, and this is in agreement with the epithelial phenotype of these cell lines (not shown). Thus, we designed experiments to decrease miRs-30 activity in these cells and to conduct loss-of-function study.

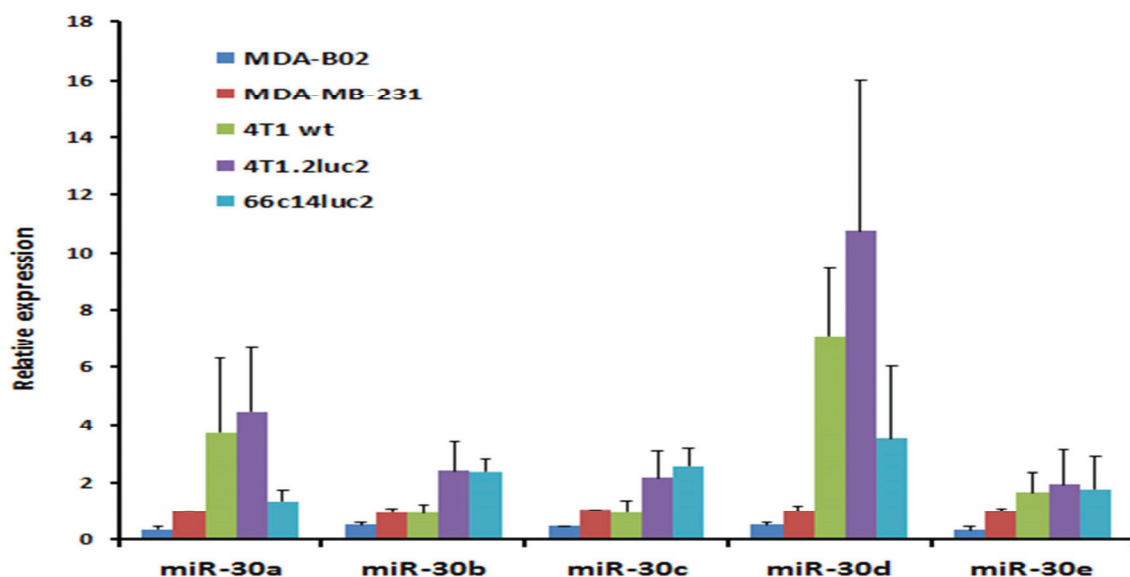


Figure 14 . Expression of miRs-30 in human and murine breast cancer cell lines.

MiRs-30 level in breast cancer cell lines was analyzed by TaqMan RT-qPCR and expressed as relative level compared to MDA-MB-231.

1.2.2.4. MiRs-30 silencing in 4T1.2luc2 and 66c14luc2 by overexpressing miR-30-sponge

4T1.2luc2 and 66c14luc2 were transduced with pmiRVec-miR-30-sponge vector to obtain the stable expression in these cells of miR-30 antagonists synthesized from genomic DNA. The expression level of miRs-30 target genes, Kruppper-like factor 10 (KLF10) and RUNX2 was analysed in 4T1.2luc2 and 66c14luc2 transduced with pmiRVec-miR-scramble vector (4T1.2luc2-Ctrl and 66c14luc2-Ctrl) and in the cells transduced with PmiRVec-miR-30-sponge vector (4T1.2luc2-sponge-30 and 66c14luc2-sponge-30).

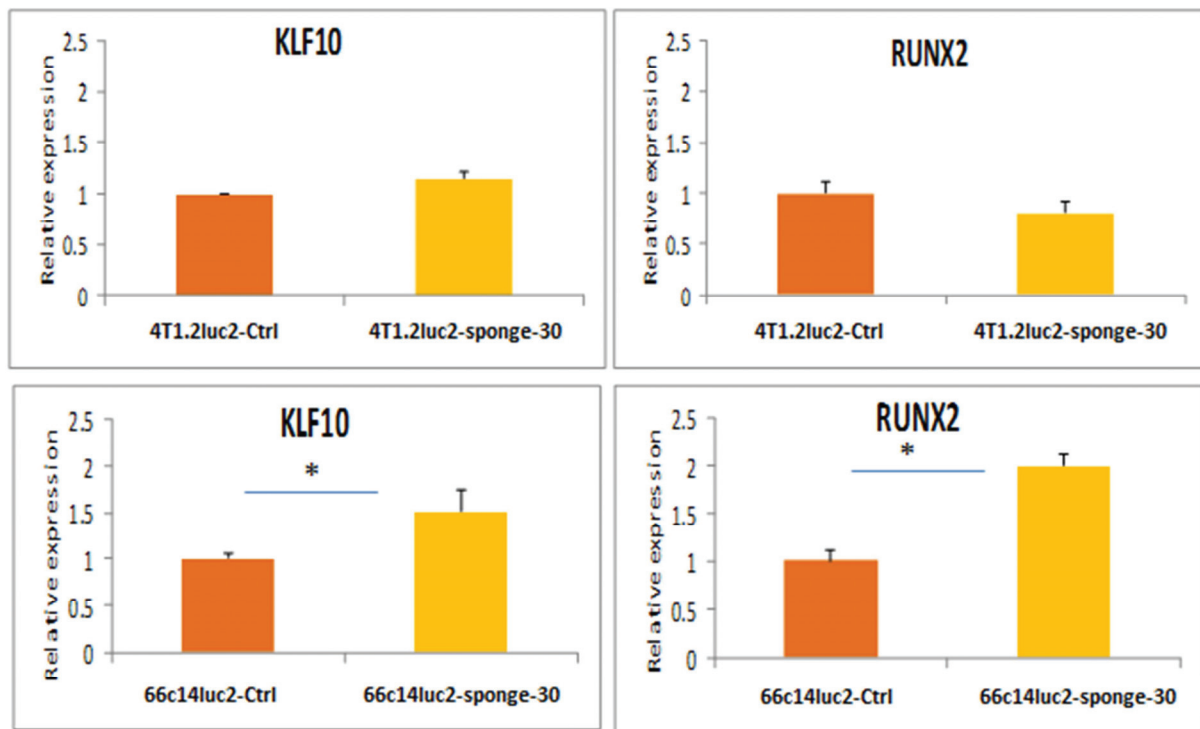


Figure 15. Expression of miRs-30 target genes in 4T1.2luc2 and 66cluc2 expressing miRs-sponge-30.

Both cell lines were transduced by pMiRVec-sponge-30 and pMiRvec-Ctrl to stably express miR-sponge-30 (4T1.2luc2-sponge-30, 66cluc2-spone-30) or miRs-scramble (4T1.2luc2-Ctrl, 66cluc2-Ctrl). Gene expression was measured by TaqMan-RT-qPCR. (*: $p < 0.05$).

Repressing miRs-30 activity was effective to derepress KLF10 and RunX2 expression in 66c14luc2 but was ineffective to do so in 4T1.2luc2.

1.2.2.5. Mir-30 silencing in 4T1.2luc2 and 66c14luc2 overexpressing miR-30-decoy

4T1.2luc2 and 66c14luc2 were transduced with lentiviral particles to obtain the stable expression in these cells of miR-30 decoys synthesized from genomic DNA. The expression level of miR-30 target genes, KLF10 and RUNX2 was analysed in 4T1.2luc2-Ctrl and 66c14luc2-Ctrl and in the cells transduced with 4T1.2luc2-decoy-miR-30 and 66c14luc2-decoy-miR-30. The silencing of miRs-30 did not induce any changing in this expression levels.

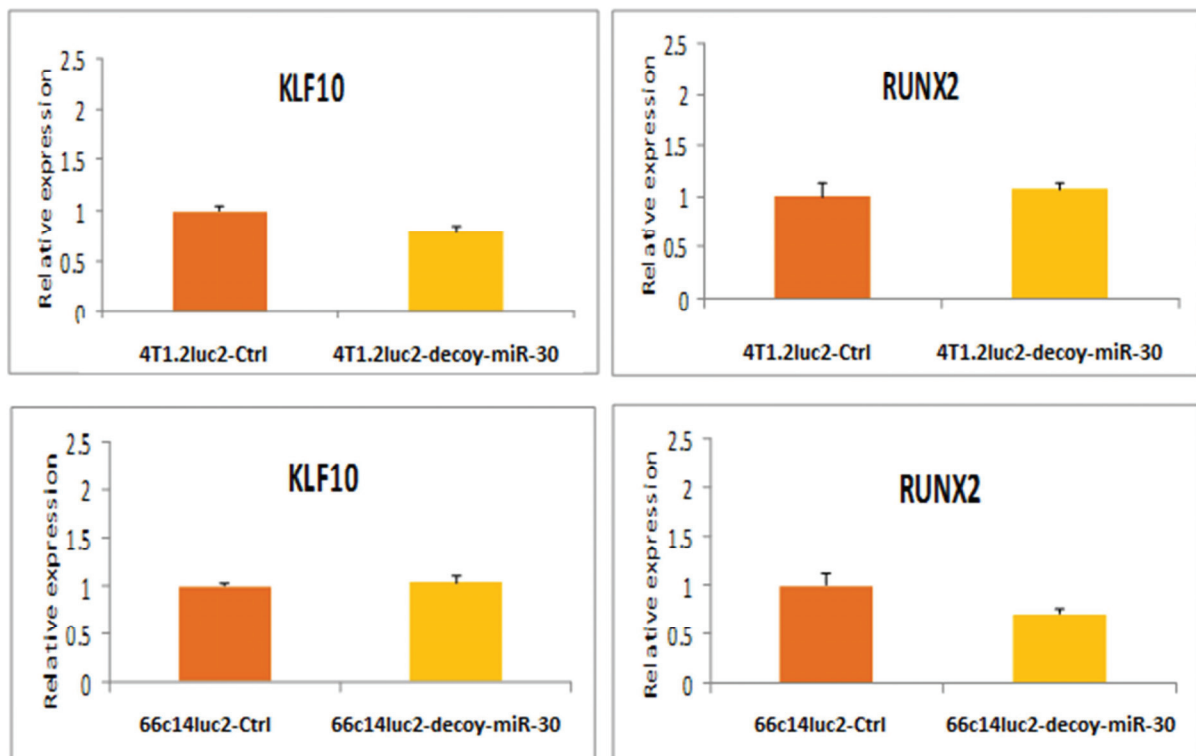


Figure 16. Expression of miRs-30 target genes in 4T1.2luc2 and 66cluc2 stably transduced with lentiviral miR inhibitors.

Both cell lines were transduced by lentiviral miR scramble control (Ctrl) and by lentiviral miR inhibitors (-decoy-miR-30) Ctrl or miR-30 inhibitors (4T1.2luc2-Ctrl, 4T1.2-decoy-miR-30, 66c14.luc2-Ctrl, 66c14luc2-decoy-miR-30). Gene expression was measured by TaqMan-RT-qPCR. (*: $p < 0.05$).

Repressing miR-30 activity was ineffective to de-repress KLF10 and RunX2 expression in 66c14luc2 and in 4T1.2luc2.

1.2.2.6. Effect of miR-30 antagonism on 4T1.2luc2 invasiveness and tumor growth in murine syngeneic model

4T1.2luc2 and 66c14luc2 were transiently transfected with a mixture of each miR-30 that mimics (miR-30 mimics) or antagonizes (miR-30 inhibitors) endogenous miR-30 or with their respective negative scramble controls. Measurement of oligonucleotide delivery into tumor cells, using Alexa-fluor488-conjugated oligonucleotides followed by flow cytometry analysis of fluorescently-labelled transfected cells, showed a transfection efficiency of 100 % (not shown). Expression level of miRs-30 delivered in the cells, by mimics or reMayning untrapped by the inhibitors was analyzed by TaqMan RT-qPCR (Fig 17A).

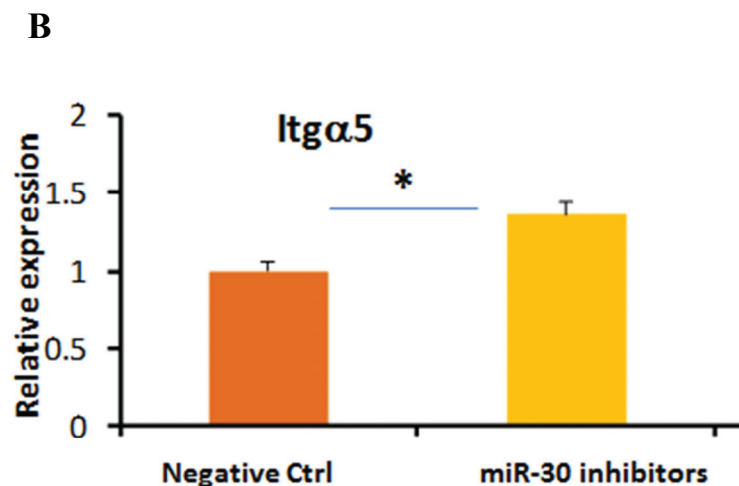
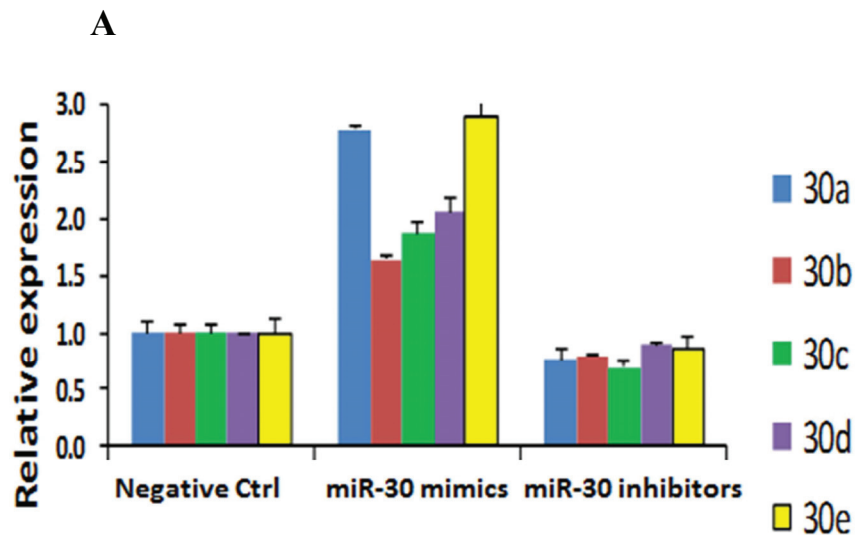


Figure 17. Expression of miRs-30 in 4T1.2luc2 after transient transfection of miR-30 mimics and miR-30 inhibitors.

(A) 4T1.2luc2 was transfected with chemically modified double-stranded RNAs that mimic endogenous miRs-30 (miR-30 mimics) or with chemically modified single-stranded RNA designed to specifically inhibit endogenous miRs-30 (miR-30 inhibitors) or with scramble controls (Negative Ctrl). MiRs-30 expression was analyzed by TaqMan RT-qPCR.

(B) Integrin $\alpha 5$ expression was measured by TaqMan-RT-qPCR. (Mean $\pm$ SD, *: $p < 0.05$).

Transfection of 4T1.2luc2 with miR-30 mimics increased the miR-30 level over endogenous level whereas miR-30 inhibitors moderately decreased this level (from 31 to only 15 % of inhibition). For data reported on fig 17A, high number of cells was transfected prior to inoculation in mice mammary gland, and we obtained a lower inhibition than in previous transfections. This might be due to the fact that these cells have high dividing rate, thus the amounts of transfectant is diluted very quickly during cell division. Despite this, the expression level of *Itga5*, a miR-30 target gene, was increased in 4T1.2luc2 transfected with miR-30 inhibitors. Modulation of invasiveness was analyzed, *in vitro* on plate coated with Matrigel. Increasing miRs-30 level inhibited significantly the invasiveness of 4T1.2luc2 whereas trapping miR-30 with miR-30 inhibitors increased cell invasiveness. This was not observed in 66c14luc2 (Fig 18).

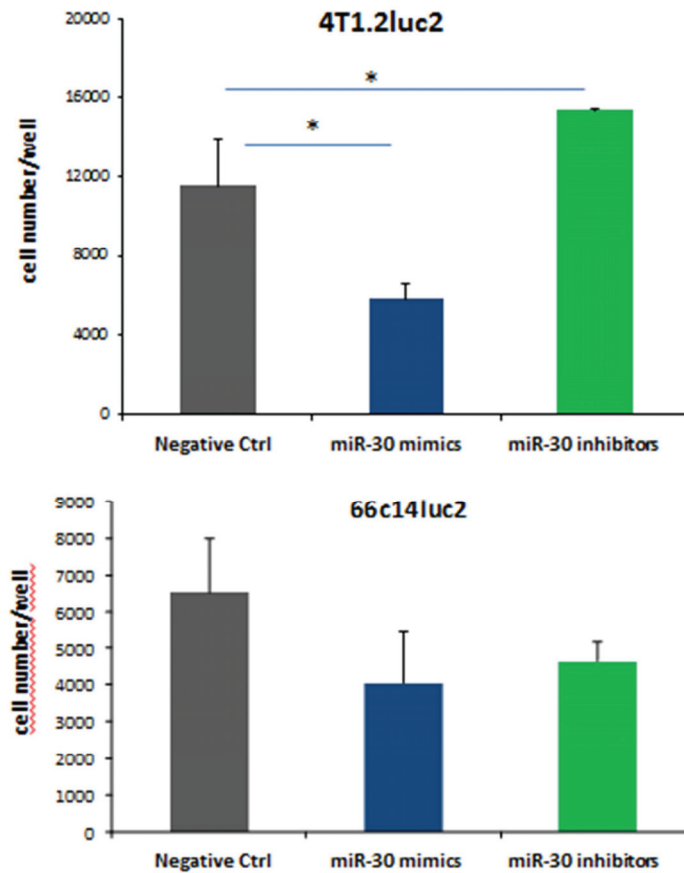


Figure 18. MiRs-30 decreased 4T1.2luc2 invasion whereas trapping miRs-30 increased 4T1.2luc2 invasion.

4T1.2luc2 was cultured in 24-well culture plates with 8- μ m diameter pore-size inserts, coated with basement membrane Matrigel. (Mean of $n=3$, $p<0.05$). No significant effect was observed with 66c14luc2.

To investigate miRs-30 activity *in vivo* in syngeneic model, Balb/c mice were injected orthotopically, with 4T1.2luc2 transfected with negative control (10 mice), or with 4T1.2luc2 transfected with miR-30 inhibitors (10 mice). No difference was observed in the growth of primary tumor (Figure 19). Mice were sacrificed 11 days after tumor resection and micrometastasis were isolated from legs, spine and lungs. We did not observe any occurrence of metastasis in lungs in the two groups of mice. The incidence of metastasis in bone and spine was only of 2 mice having metastasis in the group of mice inoculated with 4T1.2luc2 transfected with miR-30 inhibitors (not shown). The reason of the absence of dissemination may be explained by the short time that we kept the animal after resection, due to the fact that we inoculated cells that were transiently transfected. This study appears only as preliminary experiment.

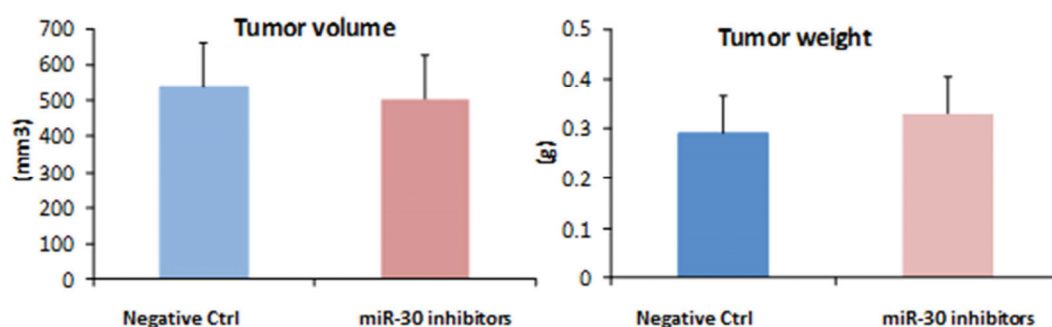


Figure 19. MiRs-30 did not affect 4T1.2luc2 growth in mice mammary fat pad.

4T1.2luc2 was transfected with miRs-30 inhibitors (miR-30 inhibitors) and scramble control (Negative Ctrl), prior to inoculation in mammary fat pad. Tumors were resected 11 days after cell inoculation and weighted.

1.2.2.7. Expression of miRs-30 is down-regulated in 4T1.2luc2 and 66c14luc2 under non-attachment growth conditions

Preclinical evidence suggests that metastasis arises from a small subset of tumor cells, the cancer stem-like cells (CSCs) that have acquired the capacity to self-renew and to differentiate into cell with limited proliferative capacity. CSCs, also named tumor initiating cells (TICs) have migratory capacity and high metastatic potential. Here, we asked if miR-30 level might be modulated in breast TIC (BT-ICs) and impact on their metastatic potential. To enrich 4T1.2luc2 and 66c14luc2 in BT-ICs, we have grown these cell lines without anchorage, in serum-free medium, conditions in which they form mammospheres.

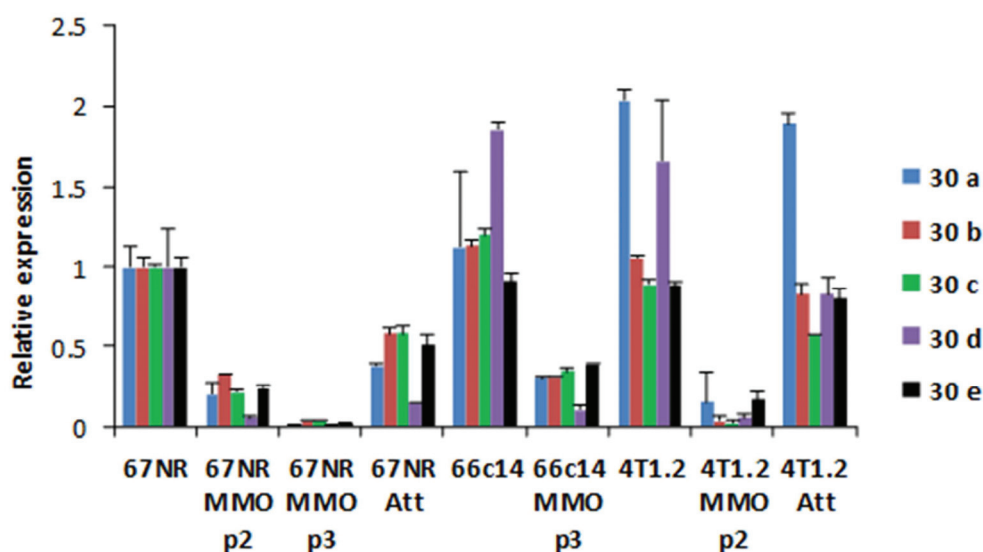


Figure 20. Expression of miRs-30 in mammospheres obtained by growing 67NR, 66c14luc2 and 4T1.2luc2 without anchorage and without serum.

MiR-30 level in cells grown under non-attachment conditions (MMO) was compared to the corresponding cell line cultured on plastic and in medium supplemented with FBS (Att). p is the number of passages for MMO, (Mean $\pm$ SD of 3 measurements).

The three cell lines harbored reduced levels of the five miRs-30 when grown as mammospheres and this was partly reversed when these mammospheres were dissociated, plated and cultured in presence of FBS. Moreover, the CSCs phenotype was markedly up-regulated in tumor cells dissociated from mammospheres (Fig 21). The CSC-like markers, CD44 that binds hyaluronan and osteopontin, was up-regulated in both cell lines whereas CD24 remained unchanged showing Cd44^{high}/Cd24^{low} character and CSC enrichment of these cultures. The expression of the three transcription factors POU domain, class 5, Transcription Factor 1 (*Oct4*), SRY (sex determining region Y)-Box 2 (*Sox2*) and *Nanog* was also increased, confirming the CSC enrichment.

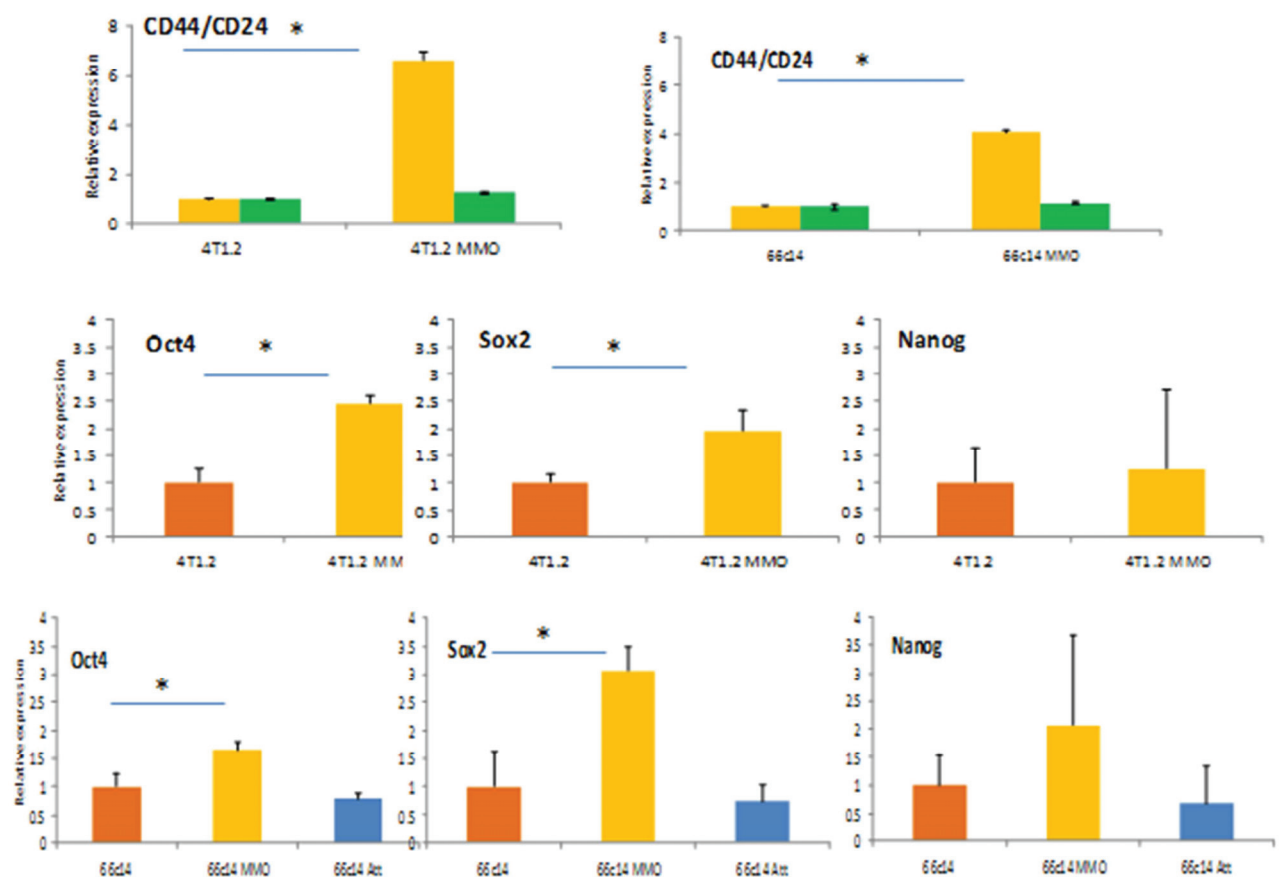


Figure 21. Expression of cancer stem cell markers in 4T1.2luc2 and 66c14luc2.

Gene expression in 4T1.2luc2 and 66c14luc2 grown under non-attachment conditions (4T1.2 MM, 66c14 MM) was compared to that in 4T1.2luc2 (4T1.2) and 66c14luc2 (66c14) grown in regular culture conditions. 66c14 Att: dissociated mammospheres that have been put back in regular culture conditions (Mean $\pm$ SD, * $p < 0.05$).

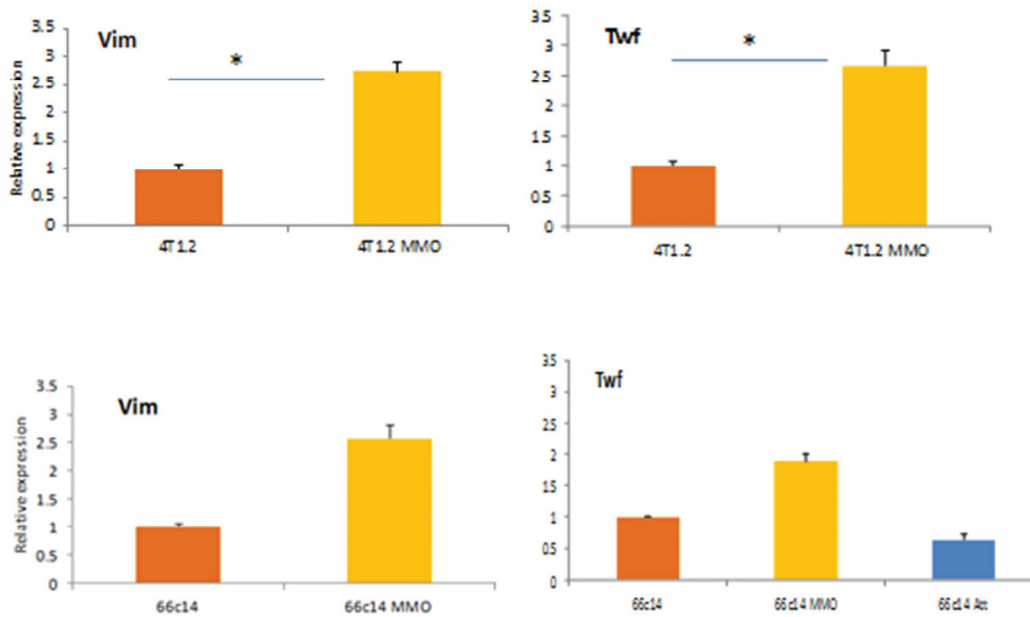
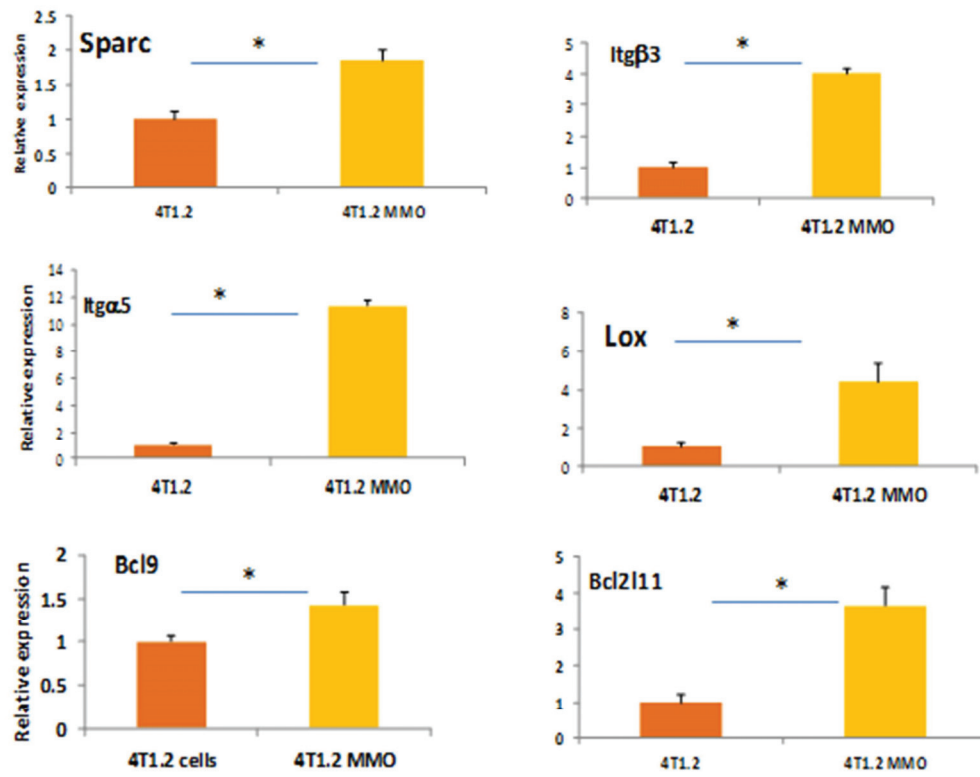


Figure 22. Expression of Vimentin and Twifilin in 4T1.2luc2 and 66c14luc2.

See abbreviations on figure 21. (*: Mean $\pm$ SD, $p < 0.05$)

Vimentin and Twifilin are proteins that regulate EMT by promoting F-actin formation, a key component of cellular transition to a more invasive mesenchymal phenotype. Both genes are targets of miRs-30 at the 3'UTR level and their expression is up-regulated in tumor cells that have low levels of miRs-30 (see paper page 59-111 of this manuscript). This result suggests that the miRs-30 depletion in mammospheres promotes Vim and Twf expression and thus would stimulate the invasive mesenchymal phenotype of mammospheres. In primary tumor, BT-ICs acquire mobility to migrate into the surrounding stroma, circulate in blood stream and seed at distant sites to grow. The specific development of bone metastasis requires the recruitment of CTC in bone marrow and their adaptation to survive in the surrounding microenvironment. We hypothesized that miRs-30-depleted mammospheres mimic CTC that have undergone EMT and escaped primary tumors to reach blood flow. To anticipate their propensity to migrate to bone, the expression of genes involved in bone metastasis has been analyzed in these cells.

A



B

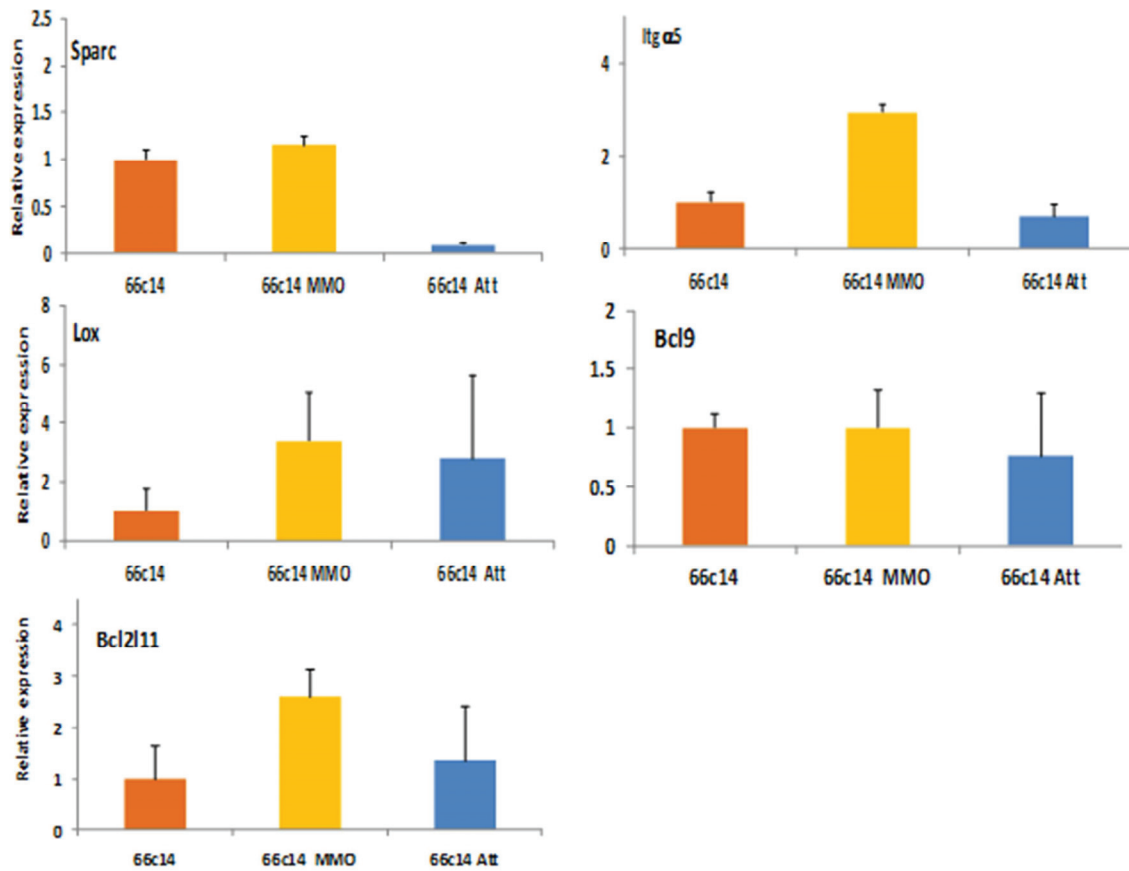


Figure 23. Expression of bone metastasis-related genes in 4T1.2luc2 (A) and 66c14luc2 (B).

See abbreviations on figure 21, (Mean $\pm$ SD, $p < 0.05$).

Secreted protein, acidic, cysteine-rich (SPARC, osteonectin), Integrin β 3 and Integrin α 5 that are involved in bone tropism and bone homing were up-regulated in miRs-30-depleted tumor cells, suggesting that miRs-30 would decrease bone-seeking of these tumor cells. Regarding genes which might be involved in bone invasive properties of CTCs, we analyzed the expression level of *Bcl9*, an experimentally validated miRs-30 target. BCL9 is a transcriptional coactivator of the WNT signaling pathway known to promote multiple myeloma cell proliferation, survival, migration, drug resistance, and formation of multiple myeloma cancer stem cells (Zhao et al., 2014). The up-regulation of *Bcl9* in 4T1.2luc2 MMO suggests that this protein might stimulate invasion of CTC in bone marrow. Lysyl oxidase (LOX) is also a critical factor for bone homing of tumor cells that enhances tumor cell engraftment in the pre-metastatic niche (Erler et al.,

2009). *Lox* expression was increased in miRs-30-depleted-4T1.2luc2, in accord with the fact that this gene is a miRs-30 target. In this model, lysyl oxidase would also stimulate the engraftment of tumor cells in bone marrow. Finally, the expression level of Bcl2-like 11 (*Bcl2l11*) which is a proapoptotic gene, belonging to Bcl-2 family member (Li et al., 2014), and a predicted target of miR-30s, was also increased in these cells, suggesting a higher level of apoptosis in mammospheres. This was confirmed by preliminary study showing that late apoptosis was increased in mammospheres as compared to cells grown in attachment (not shown). The reason for that is not clear, but it is reasonable to think that the non-attachment culture conditions without serum alter the cells. Despite this, we decided to investigate if miRs-30-induced modulation in expression of these genes would modify metastatic dissemination of 4T1.2luc2 and/or 66c14luc2 in animal model.

1.2.2.8. Metastatic dissemination of miRs-30-depleted 4T1.2luc2 and 66c14luc2 in mice.

Tumor cells dissociated from mammospheres and tumor cells detached from culture wells were injected intra-arterially in mice. Metastasis dissemination to lung, spine and legs was assessed by occurrence of micro-metastasis in organs. We choose this type of injection that bypasses escape of primary tumor to investigate the steps of bone tropism and homing of CTC.

	LUNGS	%	LEGS	%	SPINE	%
4T1.2 MM	3/5	60	4/5	80	4/5	80
66c14 MM	5/5	100	5/5	100	5/5	100
4T1.2	4/5	80	4/5	80	4/5	80
66c14	5/5	100	3/5	60	4/5	80

Table 5. Incidence of metastasis in mice inoculated with 4T1.2luc2 and 66c14luc2 grown under non-attachment conditions and with 4T1.2luc2 and 66c14luc2 cultured in regular conditions.

Abbreviations are as in figure 21. (%) represent the percentage of mice that developed micro-metastasis in organ.

MiRs-30-induced regulation of gene involved in bone metastasis did not impact on metastatic dissemination *in vivo* (Table 5). We hypothesized that growing cells as mammospheres may induce a loss-of-functions of these cells by stimulation of apoptosis and/or necrosis. In this model, there was a huge amount of tumor cells in lungs that we cannot numerate, making difficult to figure out the impact of gene modulation. Moreover, tumor cells seemed also to have grown outside organs, especially in bone. We concluded, on these preliminary data, that 4T1.2luc2 and 66c14luc12 grown as mammospheres is not adapted to study bone tropism and bone homing in a mice model of bone metastasis.

1.2.3. Discussion

In the course of this study we have first confirmed that 4T1 is not a model of metastatic dissemination to bone after cell inoculation in fat pad since animals developed strong macrometastasis in lung after tumor resection but not in bone. Moreover, mice would suffer rapidly from lung metastasis before developing bone metastasis. We found that 4T1.2luc2 forms reproducible breast primary tumors when inoculated in mammary gland mixed to Matrigel and disseminates more in spine and leg than in lung. 66c14luc2 disseminate more in lungs than in bone. In regard of high levels of miRs-30 expression in these murine cells, we used a loss-of-function approach to study miRs-30 activity. The miRs-sponge technology that has been specifically design to target entire miR family seemed very attractive for our study, in regard of the five members of the family. From the sponge sequences in a cloning vector designed by Philippe Sharp laboratory, we obtained a stable cell line from the entire 4T1.2luc2 and 66c14luc2 population. This technical approach allows avoiding clonal selection of these very heterogeneous cell populations. We did not obtain the derepression of miRs-30 target genes in 4T1.2luc2. The reason for that is not clear, since the technology is efficient to derepress expression of several genes (RUNX2, ITG β 3, CDH11 and CX43) in human MDA-MB-231 and MCF-7. It is possible that the transduction was not efficient in 4T1.2luc2 or that these genes were not efficiently regulated by miRs-30 in these cells. We used the miR-30s mimics and miR-30s inhibitors to modulate gene expression in these cells. Here we choose to target the 5 miRs-30 by mixing equimolar concentrations of each. Both, mimics and inhibitors modulate 4T1.2luc2 invasiveness efficiently indicating that exogenous miRs-30 can still repress gene targets, despite high levels of endogenous miRs-30. In this, miRs-30 in these murine cell lines might be not directly linked to their spontaneous dissemination. This would agree with results reporting that overexpression of miR-30a suppresses the migration and invasive phenotype of breast cancer cell line, MDA-MB-231. When we transfected MDA-MB-231 with miR-30 mimics, cell invasiveness was decreased but transfection with miR-30 inhibitors did not increase cell invasiveness (See paper page 59-111). Another complexity of miRs-30 activity is that there are five members that share similar seed regions but that may have difference in activity. They are also expressed at very high levels in cells that would explain why miR-30 inhibitors were inefficient to modulate tumor growth *in vivo*. Further, we found that growing these cells without anchorage, to mimic CTC *in vivo*,

induced a depletion of endogenous level of miRs-30. In preliminary results, this miRs-30 depletion up-regulated the expression of several genes involved in bone metastasis. Although these experiments need to be repeated, we hypothesize that the down-regulation of miRs-30 is not occurring only in primary tumor, through the occurrence of EMT and/or Dicer down-regulation. This process may be modulated during dissemination steps. *DICER* expression will be analyzed in mammospheres to find out if the tumorigenicity was altered. It also turn out that such grown cells are not suitable to be inoculated to mouse, probably due to an enhanced oncogenic-induced failsafe program in cells.

Overall, the orthotopic mouse model set up with 4T1.2luc2 is suitable to investigate metastatic dissemination to bone. However, we did not succeed to efficiently downregulate miRs-30 in these cells. Transient transfection of short oligonucleotides is not suitable, in regard of the rapid high dilution of these molecules in highly dividing cells. In 4T1.2luc2, the efficiency of sponge technology that allows continuous and stable expression of miRs-30 inhibitors, counteracting all members of the family need to be improved.

PART 3

**GENERAL DISCUSSION AND
PERSPECTIVES**

According to data reported in literature, miRs-30 appear as tumor suppressors, in regard of their EMT repressor properties. These miRs inhibit tumorigenesis, tumor cell invasion and survival. We have shown that they inhibit breast cancer bone metastasis, mostly by repressing the expression of osteomimetic genes. To show that, we have increased miRs-30 in MDA-B02, a cell line that specifically invades bone, in animal model. These cells have been previously selected by selection pressure in animal and exhibit a mesenchymal phenotype. They disseminate to bone after injection in blood circulation and mimic breast cancer circulating tumor cells that have escaped primary tumor. In accord to its mesenchymal phenotype, this cell line is only weakly tumorigenic and do not escape primary tumor to disseminate in mice. Therefore, our observation is still restricted to a specific cell line with osteomimicry competence and need to be extended to others cell lines. We need to find out if miRs-30 would decrease bone metastasis in rather less osteomimetic cell lines before concluding that this miR family is negatively associated to a specific bone metastasis signature. This is also sustained by the fact that some members of miRs-30 prevents tumor growth and/or cancer cells escape and lung metastasis in animal models. In the clinic, we show that miR-30a-5p, miR-30d-5p and miR30e-5p in primary tumors from patients with breast cancer is negatively associated with poor distant metastasis-free survival. From this observation that also partially confirms previously published data, we conclude that a miRs-30 signature exists *in vivo* in breast tumors. Whether this confers the CTC to have an enhanced ability to invade bone rather than lungs or others metastatic sites reMayns to be investigated. It seems also, from our results, obtained with 4T1.2 model, that the expression of pro-metastatic genes is increased in breast cancer CTC. Therefore, searching for a gene signature of bone metastasis in CTC from breast cancer patients would be of interest. It would also inform on whether osteomimicry is relevant for all types of breast cancer subtypes or restricted to hormone-responsive breast cancer. We and others have also shown that miRs-30 in primary tumors from patients with breast cancer are negatively associated with hormone-insensitive status. For further study, we will work with the hormone-responsive and epithelial cell line, MCF-7 that expresses relatively high levels of miRs-30 and does not present osteomimicry competency. The aim will be to find out if modulation of miRs-30 levels in these cells would modulate osteomimetic genes and bone metastasis, in an hormone-responsive model.

Another point that needs to be addressed is to find out whether the five different members of the miR family differ in their anti-tumoral and anti-metastatic activity. This has been suggested by

some discrepancy in the literature. It is of special importance in regard of the very high expression of each miR-30. This means that for a loss-of-function study, a high amount of “trapping activity” is needed to derepress the expression of targeted genes. In these experiments, we are going to use the sponge technology that seems promising, using MCF-7 cells. Surprisingly, preliminary study conducted with MDA-MB-231 and MCF-7 showed that increasing miRs-30 levels by transfecting these cells with equimolar concentrations of each miR-30 modulate gene expression, in a way that resembles that obtained with MDA-B02. Nevertheless, this approach will allow to compare the activity of each miR-30, as they may modulate differently the expression of specific genes.

In the course of our study, we have also shown that genes associated to various solid cancer metastases were down-regulated in MDA-B02-miR-30s by direct miRs-30 targeting (NEDD4, 5NTE) or indirect regulation (TNC). These genes were also up-regulated in bone metastasis at the transcriptomic level and appear as bone metastasis targets. We also found that the oncogenes Nid1 and Vimentin that are in-silico predicted direct targets of miR-30s are up-regulated in bone metastasis at transcriptomic levels and could promote bone metastasis. We are going to investigate the role of some of these targets in bone metastasis.

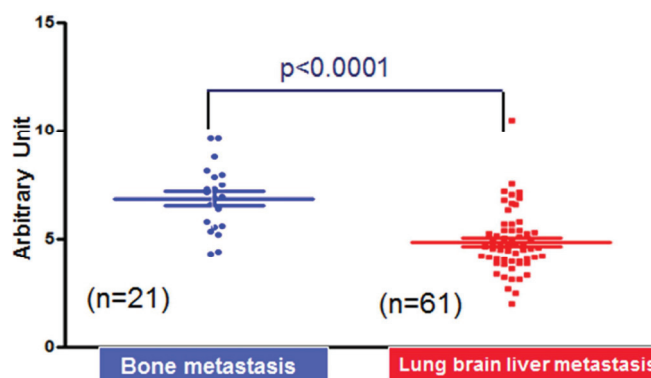
Finally, another interesting approach to characterize the role of miRs-30 in bone metastasis would be to treat mice with miRs-30 mimics, using our mice model of human bone metastasis.

PART 4

ADDITIONAL WORK

1. *Twist1* Expression in Breast Cancer Cells Facilitates Bone Metastasis Formation.

Twist1 is a basic helix-loop-helix transcription factor initially identified as a major regulator of cell movement and tissue reorganisation during early embryogenesis that is aberrantly reactivated in human cancers. Twist1 promotes tumour cell invasion through activation of miR-10b, facilitating the intravasation of breast cancer cells in the systemic circulation and their dissemination to the lungs. Twist1 has been reported as a link between EMT and stemness in breast carcinomas. We have shown, by microArray transcriptomic analysis, that Twist1 expression level is significantly higher in bone metastasis from breast cancer patients than in others type of metastasis.



Twist1 expression in bone metastasis from breast cancer patients (from Croset M, Pantano F, Driouch K and Clezardin P; unpublished results)

(from the Gene Expression Omnibus, under IDs GSE11078 (MSK) and GSE14020 (Institut Curie)).

We have studied the activity of Twist1 in bone metastasis formation in a mice model of human bone metastasis.

We have shown that Twist1 enhances the engraftment of tumor cells to bone marrow without affecting osteomimicry and EMT/MET-dependent functions but through a miR-10b dependent mechanism.

TWIST1 Expression in Breast Cancer Cells Facilitates Bone Metastasis Formation

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ABSTRACT

The transcription factor TWIST1 induces epithelial-mesenchymal transition and/or escape to the oncogenic-induced failsafe program, facilitating the intravasation of breast cancer cells in the systemic circulation and their dissemination to the lungs. Its involvement in breast cancer bone metastasis is unknown. To address this question, human osteotropic MDA-MB-231/B02 breast cancer cells were stably transfected with a Tet-inducible vector encoding for *TWIST1*, whose expression was specifically repressed in the presence of doxycycline (dox). The intra-arterial inoculation of transfectants expressing *TWIST1* in immunodeficient mice substantially increased the extent of osteolytic lesions in these animals, being 50% larger than that of animals bearing mock-transfected tumors, as determined by radiography. This difference was accompanied by a sharp reduction of the bone volume (indicating a higher bone destruction) and a twofold increase in the tumor volume compared with mice bearing mock-transfected tumors, as determined by histomorphometry. Importantly, the suppression of *TWIST1* expression in MDA-MB-231/B02 cells in the presence of dox abolished the stimulatory effect of *TWIST1* on bone metastasis formation in vivo. Additionally, examination of the bone marrow from untreated and dox-treated animals on day 7 after tumor cell inoculation, at which time there was no evidence of radiographic osteolytic lesions, revealed that the number of tumor cell colonies that were recovered from the bone marrow of untreated mice was dramatically increased compared with that of dox-fed animals. In vitro, *TWIST1* expression promoted tumor cell invasion and enhanced microRNA 10b (miR-10b) expression, a proinvasive factor, but was dispensable for growth of tumor cells. In vivo, the repression of miR-10b substantially decreased the presence of *TWIST1*-expressing breast cancer cells in the bone marrow. Overall, these results establish that TWIST1 facilitates breast cancer bone metastasis formation through a mechanism dependent of miR-10b, which leads to increase tumor burden and bone destruction. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: TWIST1; BONE; METASTASIS; BREAST CANCER; MIR-10B; INVASION

Introduction

Breast cancer is prone to metastasize to bone: around 70% to 80% of patients with advanced disease exhibit osteolytic bone metastases.⁽¹⁾ Once metastatic breast cancer cells are in the bone marrow, they cannot destroy bone on their own. Instead, they alter the functions of bone-resorbing cells (osteoclasts) and bone-forming cells (osteoblasts) and hijack signals coming from the bone matrix.⁽¹⁾ Specifically, metastatic breast cancer cells enhance bone resorption and inhibit bone formation, and bone-derived growth factors released from resorbed bone stimulate skeletal tumor outgrowth.⁽¹⁾ The realization that breast cancer cells in the bone marrow promote skeletal destruction has led to

the use of therapies that inhibit the activity of bone-resorbing osteoclasts (bisphosphonates, denosumab).⁽²⁾ However, these treatments are only palliative. They do not provide a life-prolonging benefit to patients, and a significant proportion of these patients with advanced breast cancer still experience bone complications.⁽²⁾ Thus, cellular and molecular mechanisms implicated in the early dissemination of breast cancer cells in the bone marrow need to be better understood in order to improve current treatment modalities.

The blood of many patients with advanced breast carcinomas contains circulating tumor cells, at least a subset of which may extravasate and disseminate in the bone marrow.⁽³⁾ These tumor cells disseminating in the bone marrow, in all probability,

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represent the earliest sign of development of metastatic disease in patients. Indeed, their detection in the bone marrow correlates with a poor clinical outcome in breast carcinomas.⁽⁴⁾ Additionally, a current hypothesis gaining ground is that disseminated tumor cells in the bone marrow (and circulating tumor cells in the blood) display a full or partial epithelial-to-mesenchymal transition (EMT), leading to mesenchymal traits such as loss of E-cadherin-mediated cell-cell adhesion, induction of invasiveness, and anchorage-independent growth.^(3,5,6) EMT can also induce circulating and disseminating breast cancer cells to enter into a stem cell-like state, as exemplified by the expression of stem cell markers (such as CD44^{high}/CD24^{low}).^(3,5,6) Interestingly, Twist1 has been reported as a link between EMT and stemness in breast carcinomas.⁽⁷⁾ It is expressed in disseminated breast cancer cells that persist in the bone marrow after chemotherapy.⁽⁸⁾ Yet its involvement in breast cancer bone metastasis formation is unknown.

Twist1 is a basic helix-loop-helix transcription factor initially identified as a major regulator of cell movement and tissue reorganization during early embryogenesis.⁽⁷⁾ However, *Twist1* is aberrantly reactivated in human cancers.⁽⁷⁾ In clinical studies, *Twist1* expression in primary breast tumors has been associated with disease aggressiveness and poor survival.⁽⁹⁾ Indeed, *Twist1* mediates escape to the oncogenic-induced failsafe program by preventing oncogene-induced senescence and apoptosis, which provides tumor cells with a growth advantage.⁽¹⁰⁾ In mouse and human breast tumor xenograft models, *Twist1* also promotes tumor cell invasion through activation of microRNA 10b (miR-10b), facilitating the intravasation of breast cancer cells in the systemic circulation and their dissemination to the lungs.^(11–13) Additionally, the prometastatic effects of *Twist1* are dependent on the activation of a CD44-lysyl oxidase (LOX) pathway that stimulates *Twist1* transcription in breast cancer cells.⁽¹⁴⁾ Thus, a number of experimental findings have been gathered demonstrating that *Twist1* expression in breast cancer is associated with tumorigenesis and lung metastasis.

In the present study, we sought to explore the function(s) of *Twist1* in experimental human breast cancer bone metastasis.

Materials and Methods

Cell lines, cell culture and transfection

The human B02 breast cancer cell line is a subpopulation of the MDA-MB-231 cell line that was selected for the high efficiency with which it metastasizes to bone in animals.⁽¹⁵⁾ This cell line was authenticated using short tandem repeat analysis. The human MCF-7 and mouse 4T1 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human mammary epithelial cell (HMEC) lines expressing the control vector pBABE-PURO or pBABE-PURO/*Twist1* and characteristics of Tet-off-expressing B02 cells were previously described.^(11,16) The cDNA encoding the entire human *Twist1* was amplified by PCR and inserted into the pCi-Neo plasmid (Promega, Charbonnières, France) to generate the pCi-Neo-*Twist1*. The bidirectional vector pBiL/*Twist1* was constructed by inserting the *Twist1* encoding sequence digested by *NheI*/*NotI* from the pCi-Neo into the pBiL plasmid (Clontech). Tet-off-expressing B02 cells were cotransfected with pBiL/*Twist1* together with a vector conferring puromycin resistance. Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (2 µg/mL). Luciferase induction upon doxycycline (dox) withdrawal was used to select

inducible clones among stable transfectants. Two *Twist1*-inducible transfectants (B02-Twist#112 and B02-Twist#135) and one negative control-inducible transfectant (B02-GFP/ β GAL) encoding for β -galactosidase (β GAL) and luciferase were used in the present study. Inducible transfectants were routinely cultured in DMEM medium (Invitrogen, France) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO₂ incubator.

For the silencing of miR-10b in B02-Twist#135 and 4T1 cells, an antagomiR-10b and a negative control antagomiR were purchased from Applied Biosystems. Both cell lines were transfected with 50 nM of antagomiR-10b or negative control antagomiR using the HiPerFect transfection reagent (Qiagen, Courtabeuf, France). These antagomiRs were incubated with 1×10^6 cells for 30 minutes at 37°C in a 5% CO₂ incubator. Transfected cells were then grown in culture for 72 hours before tumor cell inoculation to animals. The efficiency of oligonucleotide delivery into these tumor cells was assessed using cell transfection with Alexa-fluor488-conjugated oligonucleotides followed by flow cytometry analysis of fluorescently-labeled transfected cells.

Immunofluorescence

Dox-treated and untreated B02 transfectants were fixed in culture wells with 2% paraformaldehyde (Sigma) in PBS for 10 minutes and permeabilized with 0.2% (vol/vol) saponin in PBS for 10 minutes. The samples were washed with 10 mM glycine in PBS prior to blockage of aspecific sites with 10% (vol/vol) goat serum. Immunodetection was performed using a mouse monoclonal antibody against *Twist1* (Twist2C1a; Abcam; dilution 1/100) or a rabbit polyclonal antibody against YB-1 (Abcam; dilution 1/1,000). Following overnight incubation at 4°C, cells were incubated for 1 hour with a secondary antibody (Alexa 488-conjugated goat anti-mouse for *Twist1* and Alexa 488-conjugated goat anti-rabbit for YB-1) at a dilution of 1/1000 (Rockland, Tebu-bio). Nuclei were labeled for 10 minutes at room temperature with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 mg/mL in PBS. The distribution of F-actin was visualized after incubation of permeabilized cells for 50 minutes at room temperature with phalloidin (1/40 dilution; Molecular Probes), according to the manufacturer's instructions. Cells were observed using a LSM510 laser scanning confocal microscope (Zeiss, Le Pecq, France) with a 63 $\times$ (numerical aperture 1.4) Plan Neo Fluor objective. To prevent contamination between fluorochromes, each channel was imaged sequentially, using the multi-track recording module, before merging.

Western blotting

Total protein cell extracts were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing a protease cocktail inhibitor (Roche). Nuclear and cytoplasmic proteins were extracted from 4×10^6 cells using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific), according to the manufacturer's instructions. Protein cell extracts were electrophoresed on a 4% to 12% gradient SDS-polyacrylamide gel (Life Technologies), then transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA) and proteins were probed with a primary antibody against *Twist1* (twist2C1a, 1/200 dilution; Abcam), YB1 (rabbit polyclonal antibody, 1/2000 dilution; Abcam), E-cadherin (goat polyclonal antibody, 1/2000 dilution; R&D Systems), vimentin (rabbit polyclonal antibody, 1/1000 dilution; BioVision), β -tubulin (CI B-512, 1/5000 dilution;

Sigma) or H4 histone (mabF-9, 1/1000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), according to the manufacturers' instructions. After incubation with primary antibodies, membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and anti-mouse secondary antibodies (Amersham; 1/2000 dilution), and immunostaining was performed with enhanced chemiluminescence (ECL) detection system (Perkin Elmer).

Real-time RT-PCR

Total RNA from human breast tumor cells was extracted using the Nucleospin RNA kit based on RNA adsorption on silica membrane further treated by rDNAse (Macherey Nagel, Duren). cDNA was produced from 1 µg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time quantitative PCR reactions were performed using a SYBR Green qPCR kit (Qiagen, Courtabeuf, France) on a Light-Cycler (Roche Diagnostics, Mannheim, Germany). Relative gene expression levels were normalized according to the threshold cycle (Ct) value of the gene encoding the ribosomal protein L32 and results were expressed as fold differences equal to $2^{-\Delta\Delta C_t}$. In some experiments, *Twist1* levels in B02 clones were expressed as copy number/µg RNA by absolute quantification, using a standard curve set up from 2×10^{11} to 2×10^2 copies. PCR reactions were performed with the following primers: *Twist1* forward 5'-GCAGGACGTGTCAGCTC-3', *Twist1* reverse 5'-CTGGCTCTTCCTCGCTGT-3'; *VEGF* forward, 5'-AGGAGAGGGCAGAATCAGAC-3', *VEGF* reverse 5'-TCTATCTTTCTTGGTCTGCATT-3'; *DKK1* forward 5'-TAGCACCTGGATGGGTATT-3', *DKK1* reverse 5'-ATCCTGAGGCACAGTCTGAT-3'; *YB-1* forward 5'-GGAGATGAGACCAAGGTCA-3', *YB-1* reverse 5'-GGTAAGCCGGCATTACTCA-3'; *LOX* forward 5'-GGATACGGCATGGCTACTTC-3', *LOX* reverse 5'-TTGGTCGGCTGGCTAAGAAAT-3'; *LOXL2* forward 5'-CGGAGGATGTCGGTGTGGT-3', *LOXL2* reverse 5'-TGGCAGTCGATGTCCGCAT-3'; *CDH2* forward 5'-CCATCACTCGGCTTAATGGT-3', *CDH2* reverse 5'-ACCCA-CAATCTGTTCACAT-3'; *RUNX2* forward 5'-GGAGTGGACGAGG-CAAGA-3', *RUNX2* reverse 5'-AGCTTCTGTCTGTCTCTGG-3'; *ZEB1* forward 5'-AGCAGTGAAAGAGAAGGAATGC-3', *ZEB1* reverse 5'-GGTCCTCTTCAGGTGCCTCAG-3'; *ZEB2* forward 5'-CAAGAGGCG-CAAACAAGC-3', *ZEB2* reverse 5'-GGTTGGCAATACCGTCATCC-3'; *SNAI1* forward 5'-TCGGAAGCCTAACTACAGCGA-3', *SNAI1* reverse 5'-AGATGAGCATTGGCAGCGAG-3'; *SNAI2* forward 5'-AAGCATTT-CAACGCTCCAAA-3', *SNAI2* reverse 5'-GGATCTCTGGTTGGTATGACA-3'; and *RPL32* forward 5'-CAAGGAGCTGGAAGTGTGC-3', *RPL32* reverse 5'-CAGCTCTTCCACGATGGCT-3'.

TaqMan microRNA assays (Applied Biosystems, Life Technologies) were used to quantify miR-10b and RNU48 levels after extraction by cell homogenization in TRIzol reagent (Gibco). Ten (10) ng of total RNA were reverse-transcribed using the MultiScribe reverse transcriptase and 50 nM stem-loop RT primers specific for miR-10b and the endogenous control RNU48. Mature miRNAs were amplified using the TaqMan 2× universal PCR Master Mix and miR-10b levels were expressed as fold differences equal to $2^{-\Delta\Delta C_t}$.

Cell enumeration assay

Cell growth curves were done after seeding the cells (1×10^4 per well) into 12-well plates and cultured them up to 96 hours. Cells were harvested at various time points following trypsinization and single-cell suspensions were then counted by the True Volumetric Absolute Counting technique of flow cytometry (Partec Instruments).

Cell apoptosis assay

Cells in culture were serum-starved for 24 hours using DMEM medium supplemented with 0.1% (wt/vol) BSA then treated or not treated with camptothecin (a proapoptotic drug) for 6 hours. These cultured cells were then detached with Accutase, washed twice with cold PBS and resuspended in Annexin V binding buffer at a concentration of 1×10^6 cells/mL, according to the manufacturer's instructions (BD Pharmingen). Cells in suspension were stained with phycoerythrin (PE)-AnnexinV and the vital dye 7-amino-actinomycin (7-AAD). Early apoptotic cells will exclude 7-AAD, whereas late-stage apoptotic cells will stain positively, due to the passage of the dye into the nucleus where it binds to DNA. Cell apoptosis was measured by flow cytometry.

Cell invasion assay

Experiments were conducted in 24-well cell culture plates with 8-µm-diameter pore-size inserts, coated with 100-µL basement membrane Matrigel (0.3 mg/mL), as described.⁽¹⁷⁾ Untreated and dox-treated B02 transfectants (1.6×10^5 cells/mL) and control- and TWIST1-expressing HMEC cells (5×10^5 cells/mL) were resuspended in culture medium containing 0.1% (wt/vol) bovine serum albumin and 300 µL of this cell suspension were loaded into each insert (upper chamber). The chemoattractant (10% v/v fetal calf serum) was placed in the lower chamber (750 µL/well). After a 24-hour incubation at 37°C in a 5% CO₂ incubator, inserts were collected, the non-invading cells were removed, and the invading cells on the under surface of the inserts were fixed and stained with crystal violet. Cells were then counted under a microscope.

Animals

Four-week-old female Balb/c immunocompromised mice were purchased from Charles River Laboratories (St. Germain sur l'Arbresle, France). All procedures involving animals, including their housing and care, the method by which they were culled, and experimental protocols were conducted in accordance with a code of practice established by the local ethical committee of the University of Lyon.

Animal studies

Orthotopic tumor xenograft experiments were conducted, as described.⁽¹⁷⁾ Four-week-old female *nude* mice treated with dox or left untreated were injected in the fat pad of the fourth mammary gland with dox-treated or untreated transfectants (1×10^6 cells in 50 µL PBS), respectively. Tumor size was calculated by external measurement of the width (m_1) and length (m_2) of tumor xenografts using a Vernier caliper. Tumor volume (TV) was calculated using the equation $TV = (m_1^2 \times m_2)/2$. At the end of the protocols, mice were euthanized and tumors were collected then prepared for immunohistochemistry.

Bone metastasis experiments were conducted in female Balb/c *nude* mice, as described.^(15–18) Dox-treated or untreated transfectants (5×10^5 cells in 100 µL PBS) were injected into the tail artery of anesthetized *nude* mice treated with dox or left untreated, respectively. Animals received or did not receive dox in the water supply (containing sucrose) for the duration of the experiments. Radiographs of anesthetized animals were taken weekly with the use of MIN-R2000 films (Kodak, Rochester, NY, USA) in an MX-20 cabinet X-ray system (Faxitron X-ray Corporation, Wheeling, IL, USA). Osteolytic lesions were identified on radiographs as radiolucent lesions in the bone. The area

of osteolytic lesions was measured using a computerized image analysis system, and the extent of bone destruction per leg was expressed in square millimeters. For measurement of skeletal tumor burden, bioluminescence imaging of animals was performed weekly using the Nightowl imaging system (Berthold, Germany), as described.^(15–18) On day 32 after tumor cell inoculation, anesthetized mice were euthanized by cervical dislocation after radiography and bioluminescence imaging.

For ex-vivo bone marrow micrometastasis experiments, dox-treated or untreated B02 transfectants (5×10^5 cells in 100 μ L PBS) were injected into the tail artery of animals treated with dox or left untreated, respectively. Alternatively, animals were inoculated with B02-Twist#135 or 4T1 cells transfected with antagomiR-10b (5×10^5 cells in 100 μ L PBS). Animals were then culled on day 7 after tumor cell inoculation. Hind limbs were collected and tibias and femurs were minced then soaked in an enzyme cocktail containing 300 U/mL type-I collagenase and 100 U/mL hyaluronidase (StemCell Technologies) in DMEM medium for 2 hours at 37°C. Lungs were minced and an enzyme solution containing 0.25 mg/mL type-I collagenase (Sigma) was added for 1 hour at 37°C. After incubation, cell suspensions from bone marrow and lungs were seeded in six-well plates and cultured in complete medium. After 1 day of culture, B02-Twist#135 and 4T1 cells were placed under antibiotic selection for 2 weeks (puromycin and 6-thioguanine, respectively), allowing the selective growth of antibiotic-resistant tumor cells. Colonies of tumor cells were then fixed, stained with crystal violet, and counted.

Bone histology, histomorphometry, and immunohistochemistry

Bone histology and histomorphometric analysis of bone tissue sections were performed as described.^(15–18) Histomorphometric measurements (bone volume [BV]/tissue volume [TV] and tumor volume [TuV]/soft tissue volume [STV] ratios) were performed in a standard zone of the tibial metaphysis, situated at 0.5 mm from the growth plate, including cortical and trabecular bone. The BV/TV ratio represents the percentage of bone tissue. The TuV/STV ratio represents the percentage of tumor tissue. The in situ detection of osteoclasts was performed on tartrate-resistant acid phosphatase (TRAP)-stained longitudinal paraffin-embedded medial sections of tibial metaphysis with the use of a commercial kit (Sigma). Osteoclast resorption surface was calculated as the ratio of TRAP-positive trabecular bone surface to the total trabecular bone surface at the tumor-bone interface.

For immunohistochemistry, tumor sections were incubated with a mouse anti-human Ki67 monoclonal antibody (DakoCytomation) or a rabbit polyclonal anti-CD31 antibody (AnaSpec) that specifically recognize proliferative cells and blood vessels, respectively. The mitotic index and tumor microvessel density were quantified, as described.⁽¹⁸⁾

Osteoclastogenesis assay

Experiments were conducted as described,^(16–18) with minor modifications. Briefly, murine bone marrow cells were cultured in α -MEM medium containing 10% (vol/vol) fetal calf serum supplemented with macrophage colony-stimulating factor (M-CSF) (20 ng/mL) and RANK-L (30 ng/mL), in the presence of conditioned medium from transfectants (20 μ g/mL). A low concentration of RANKL was chosen in this study in order to maximize potential differences between conditioned media

from B02-Twist#135 cells expressing or not expressing TWIST1. After 7 days, mature osteoclasts were enumerated under a microscope on the basis of the number of nuclei (more than three nuclei) and TRAP activity. Results were expressed as the number of osteoclasts per well. The expression of genes involved in osteoclast differentiation and activity was analyzed by RT-qPCR, as described.⁽¹⁹⁾

Cytokine array

A commercial antibody-based protein microarray designed to detect 23 proteins including growth factors, cytokines, and chemokines (RayBio Human Cytokines Array 1, RayBiotech) was used. Array membranes were incubated for 2 hours with the conditioned medium from cultured transfectant cells (150 μ g/mL). After washing, membranes were incubated with a cocktail of 23 biotinylated antibodies, and the remaining experimental procedure was carried out following the manufacturer's instructions.

Flow-cytofluorometric analysis

Untreated and dox-treated B02-Twist#135 cells (1×10^6 cells in 500 μ L PBS supplemented with 1% BSA) were stained with a phycoerythrin-conjugated human anti-CD24 or a fluorescein-isothiocyanate-conjugated human anti-CD44 monoclonal antibody (BD Biosciences). Stained cells were then immediately analyzed by flow cytometry (Canto II; Becton Dickinson).

Statistical analysis

All data were analyzed using StatView software (version 5.0; SAS Institute Inc, Cary, NC). Pairwise comparisons were carried out by performing a nonparametric Mann-Whitney *U* test. *p* values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

Results

Structural and functional characterization of TWIST1-expressing B02 transfectants

To investigate the role of TWIST1 in bone metastasis, we chose the human osteotropic MDA-MB-231/B02 breast cancer cell line (named B02 for brevity). The B02 breast cancer cell line is a subpopulation of the MDA-MB-231 cancer line that was selected for the high efficiency with which it metastasizes to bone in animals.⁽¹⁵⁾ Like many other basal breast cancer cell lines, MDA-MB-231 (Supporting Fig. S1) and B02 cells did not express Twist1. B02 cells were stably transfected with a Tet-inducible bidirectional vector encoding for TWIST1 and luciferase, whose expression was specifically repressed in the presence of dox. A negative control cell line was obtained by introducing a pBI-L bidirectional Tet-inducible vector encoding for β -galactosidase and luciferase (B02-Ctrl).⁽¹⁶⁾ Two TWIST1-inducible clones (B02-Twist#112 and B02-Twist#135) were selected on the basis of a specific TWIST1 expression upon dox withdrawal. TWIST1 levels in B02-Twist#112 and #135 cells were moderate and high, respectively (Supporting Fig. S2). B02-Twist#135 cells expressed $2 \pm 0.014 \times 10^8$ copies/ μ g RNA of TWIST1, as assessed by RT-qPCR analysis, whereas B02-Twist#112 cells had 3.5 fewer copies. Analysis of B02-Twist#135 cells by confocal microscopy showed that TWIST1 was specifically localized in the nucleus. Dox treatment of B02-Twist#135 cells led to an almost complete

repression of *Twist1* , as judged by both confocal microscopy and immunoblotting (Fig. 1A, B).

Although B02 cells did not express *Twist1* , many other genes encoding for EMT-inducing transcription factors, including *Snai1* (Snail), *Snai2* (Slug), *Zeb1* and *Zeb2* , were highly expressed in these mesenchymal cells, compared with that observed for MCF-7 breast cancer cells, which have an epithelial phenotype (Supporting Table 1). Additionally, B02 cells lost the expression of the epithelial marker E-cadherin, whereas they expressed the mesenchymal marker vimentin (Fig. 1C). The expression of these transcription factors and vimentin, and the loss of E-cadherin

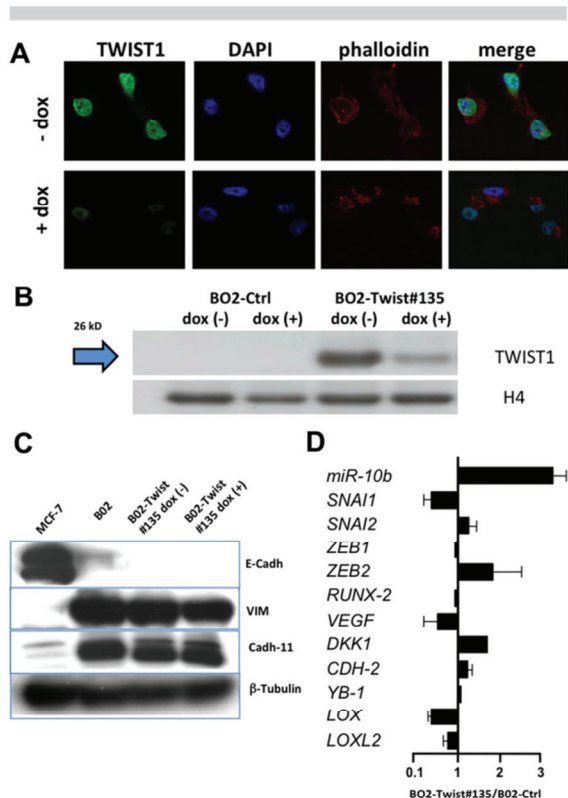


Fig. 1. Ectopic expression of *Twist1* in mesenchymal human B02 breast cancer cells upregulates microRNA-10b. (A) Representative images by confocal microscopy showing the expression of *Twist1* (green) in untreated [dox (-)] and doxycycline-treated [dox (+)] B02-Twist#135 cells. Dox almost completely repressed the nuclear expression of *Twist1* in B02#135 cells, compared with that observed with untreated cells. DAPI (blue) and phalloidin (red) were used as reporters for the nuclei and cytoskeleton, respectively. (B) Mock-transfected B02 cells (MDA-B02-Ctrl) and B02-Twist#135 cells, treated or not treated with dox, were subjected to Western immunoblotting with antibodies against *Twist1* or H4 histone (control for equal loading). B02-Ctrl cells do not express *Twist1* . Dox repressed *Twist1* expression in B02-Twist#135 cells. (C) Immunoblotting of E-cadherin (E-Cadh), vimentin (VIM), cadherin-11 (Cadh-11), and β -tubulin in MCF-7 and parental B02 breast cancer cells, and untreated and dox-treated B02-Twist#135 cells. (D) Effects of *Twist1* on target gene expression in B02 cells. Relative transcript levels are represented as the ratios between B02-Twist#135 and B02-Ctrl real-time PCR values.

likely explained the spindle-like, fibroblastic morphology of B02 cells (not shown). This morphological change is one of the hallmarks of an EMT. Ectopic *Twist1* expression in B02-Twist#135 cells did not, however, further modify the morphological appearance of these cells (not shown) and, at the molecular level, it did not alter cadherin-11 expression (Fig. 1C). Cadherin-11 is an osteoblast cadherin whose expression correlates with the osteotropic properties of B02 cells.⁽²⁰⁾ Conversely, *Twist1* regulated the expression of zinc-finger transcription factors. An inverse transcriptional link between *Twist1* and *Snai1* in MCF10A mammary epithelial cells has been suggested.⁽²¹⁾ Additionally, ectopic *Twist1* expression in human HMLE mammary epithelial cells induces *Snai2* transcription.⁽²²⁾ In agreement with these findings,^(21,22) we observed here that *Twist1* had a slight impact on expression of these two transcription factors, regulating *Snai1* and *Snai2* in a negative and positive manner, respectively (Fig. 1D). It also increased *Zeb2* transcription, but not *Zeb1* transcription (Fig. 1D). Compared with parental B02 cells, *Twist1* ectopic expression did not affect or merely modestly modulated expression of other target genes,^(23–26) such as transcription factor *YB-1* (a transcription factor associated with breast cancer cell proliferation), Dickkopf-1 (*DKK-1* ; an inhibitor of canonical Wnt signaling), vascular endothelial growth factor (*VEGF*), *CDH-2* (N-cadherin), and *RUNX-2* (an osteoblastic transcription factor) (Fig. 1D). By contrast, it induced a 3.5-fold increase of miR-10b expression in B02#135 cells (Fig. 1D). *LOX* , but not *LOXL2* (LOX-like 2), was modestly repressed (Fig. 1D). In agreement with previous studies,⁽⁷⁾ ectopic *Twist1* expression also modestly but significantly increased stemness traits of B02 cells, which were already CD44^{high}/CD24^{low}, increasing the percentage of CD44-positive cells from 75% to 91.4% upon dox withdrawal (Supporting Fig. S3). All of the B02 cells were CD24^{low}, irrespective of *Twist1* expression (Supporting Fig. S3).

We next examined whether ectopic *Twist1* expression affects B02 breast cancer cell functions in vitro. B02-Ctrl and B02-Twist#135 cells grew at a similar rate in the presence or absence of dox, indicating that *Twist1* was not required for the proliferation of B02 breast cancer cells, when growing in vitro (Fig. 2A). These results were in line with the observation that *Twist1* did not affect *YB-1* production, as determined by immunoblotting and confocal microscopy (Fig. 2B; data not shown). *Twist1* did not affect tumor cell apoptosis either, whether B02-Twist#135 cells, when deprived of serum, were treated or not treated with camptothecin (a proapoptotic agent) (Supporting Fig. S4). By contrast, *Twist1* ectopic expression in B02#135 cells substantially enhanced tumor cell invasion and promoted miR-10b expression, when compared with B02-Ctrl cells (Fig. 2C, D). Furthermore, both the increased cell invasiveness and enhanced miR-10b expression were abrogated in dox-treated B02-Twist#135 cells (Fig. 2C, D). Thus, *Twist1* contributed to the invasive phenotype of B02 breast cancer cells in vitro. Similar results were obtained with immortalized HME mammary epithelial cells where ectopic *Twist1* expression also promoted both cell invasiveness and miR-10b expression (Fig. 2E, F), indicating that the proinvasive effect of *Twist1* was not restricted to B02-Twist#135 cells.

Twist1 ectopic expression enhances breast cancer bone metastasis formation

To determine whether *Twist1* plays a causal role in bone metastasis, luciferase-expressing B02-Ctrl or B02-Twist#135 cells

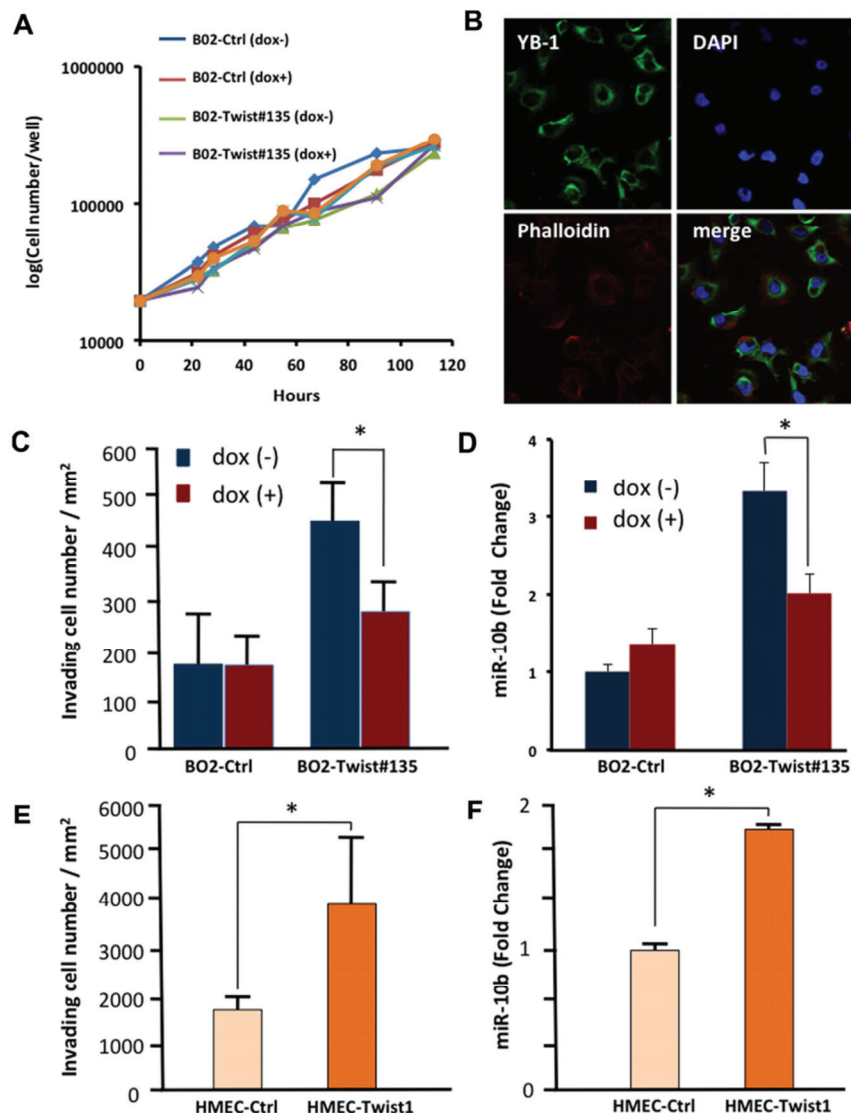


Fig. 2. *Twist1* ectopic expression promotes invasion but is a dispensable transcription factor for the proliferation of human B02 breast cancer cells in vitro. (A) Proliferation rates of untreated and dox-treated mock-transfected (B02-Ctrl) and *Twist1*-expressing B02 cells (B02-Twist#135). (B) Representative images by confocal microscopy showing the expression of YB-1 (green) in B02-Twist#135 cells. DAPI (blue) and phalloidin (red) were used as reporters for the nuclei and cytoskeleton, respectively. (C) Cell invasion assay. Untreated and dox-treated B02 clones (B02-Ctrl and B02-Twist#135) were loaded in inserts with a porous membrane coated with basement membrane Matrigel (upper chamber) and the chemoattractant (serum) was placed in wells of a companion plate (lower chamber). After a 24-hour incubation at 37°C, the non-invading cells were removed and the invading cells on the under surface of the inserts were fixed, stained, and counted under a microscope. * $p < 0.05$. (D) miR-10b expression in untreated and dox-treated B02-Ctrl and B02-Twist#135 cells. miR-10b expression level in untreated B02-Ctrl cells was set to 1. Data were then expressed as a fold-change of miR-10b expression compared with that observed in untreated B02-Ctrl cells. * $p < 0.05$. (E, F) Same as C and D, showing the effects of *Twist1* on invasion and miR-10b production by immortalized human mammary epithelial cell (HMEC) lines expressing the control vector (HMEC-Ctrl) or *Twist1* (HMEC-Twist1), respectively.

were injected into the tail artery of immunodeficient mice. Bioluminescence imaging of animals bearing B02-Twist#135 tumors showed tumor take in hind limbs as early as on day 7 after tumor cell inoculation, then skeletal tumor burden progressively increased over time (Fig. 3A). There was no

bioluminescence in organs other than bone, indicating that ectopic *Twist1* expression did not modify the bone tropism of B02 breast cancer cells in vivo. However, tumor take in hind limbs of animals bearing B02-Twist#135 tumors occurred much earlier than that observed in animals bearing B02-Ctrl tumors

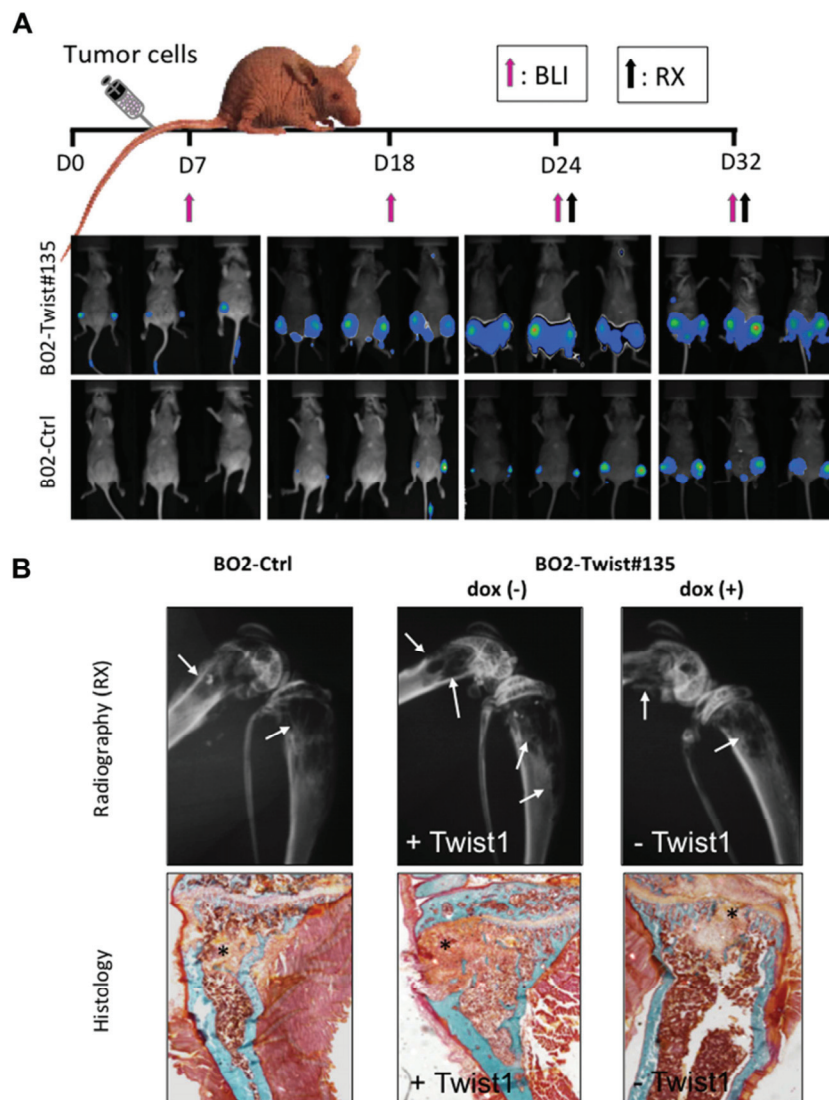


Fig. 3. *Twist1* ectopic expression promotes breast cancer bone metastasis formation. (A) Upper panel: Summary of the experimental bone metastasis protocol. B02-Ctrl cells or B02-Twist#135 cells [treated or not treated with doxycycline (dox)] were inoculated into female Balb/c *nude* mice on day 0. Animals then received or did not receive dox in the water supply for the duration of the experiment. Animals were analyzed by bioluminescence imaging (BLI) and radiography (RX) at scheduled intervals. Bottom panels: For BLI, representative images of animals inoculated with B02-Twist#135 cells or B02-Ctrl cells were shown at different time intervals after tumor cell inoculation. These images best illustrate the effect of *Twist1* on skeletal tumor burden. (B) Upper panels: Radiographic analysis of hind limbs from mice bearing B02-Ctrl or B02-Twist#135 tumors. *Twist1* expression in B02-Twist-135 cells was turned off by dox [dox (+)]. Arrows indicate osteolytic lesions. Bottom panels: Goldner's trichrome staining of tissue sections of tibial metaphysis. Bone is stained green whereas bone marrow and tumor cells (asterisk) are stained red. All images were obtained from different mice on day 32 after tumor cell inoculation. The images shown are examples that best illustrate *Twist1*'s effects on bone destruction and tumor burden.

(Fig. 3A). This observation suggested that the expression of *Twist1* in B02 cells promoted tumor cell invasion in the bone marrow microenvironment.

Radiographic analysis on day 32 after tumor cell injection revealed that the extent of osteolytic lesions in hind limbs of

animals bearing B02-Twist#135 tumors was increased, being 50% larger than that of animals bearing B02-Ctrl tumors (Table 1, Fig. 3B). Compared with B02-Ctrl tumor-bearing mice, there was also a substantial increase in the extent of osteolytic lesions in animals bearing B02-Twist-112 tumors (Supporting Fig. S5).

Table 1. Effects of *Twist1* Expression in B02 Breast Cancer Cells on Bone Metastasis Formation in Animals Treated or Not Treated With Doxycycline

Cell line	Radiography		Histomorphometry				TRAP staining	
	mm ² /mouse	<i>p</i>	BV/TV (%)	<i>p</i>	TuV/STV (%)	<i>p</i>	OC.S/BS (%)	<i>p</i>
B02-Ctrl	4.1 ± 0.3 (<i>n</i> = 11)	–	21.4 ± 8.1 (<i>n</i> = 11)	–	41.7 ± 12.9 (<i>n</i> = 11)	–	45.9 ± 22.8 (<i>n</i> = 5)	–
B02-Twist#135	8.2 ± 0.5 (<i>n</i> = 10)	0.01	7.7 ± 4.2 (<i>n</i> = 10)	0.001	72.4 ± 16.6 (<i>n</i> = 10)	0.0007	33.5 ± 6.1 (<i>n</i> = 5)	NS
B02-Twist#135 + doxycycline	3.2 ± 0.4 (<i>n</i> = 5)	0.02	20.4 ± 2 (<i>n</i> = 5)	0.533	25.8 ± 16.2 (<i>n</i> = 5)	0.027	36.9 ± 11.4 (<i>n</i> = 5)	NS

Values are mean ± SD. Values of *p* (two sided) are for pairwise comparison with the control group using the Mann-Whitney U test. All measurements were performed 32 days after tumor cell injection.

TRAP = tartrate-resistant acid phosphatase; BV/TV = bone volume-to-tissue volume ratio; TuV/STV = tumor volume-to-total soft tissue volume ratio; OC.S/BS = active osteoclast-resorption surface per trabecular bone surface; B02-Ctrl = control B02 breast cancer cells expressing the doxycycline-inducible vector encoding for β-galactosidase (Tet-Off system); – = not applicable (referent); B02-Twist#135 = B02 breast cancer expressing the doxycycline-inducible vector encoding for *Twist1* ; NS = not significant.

Additionally, on day 24 after tumor cell injection, osteolytic lesions in hind limbs of animals bearing B02#Twist-135 tumors were statistically significantly larger than those of animals bearing B02#Twist-112 tumors, indicating that there was a correlation between *Twist1* expression levels in B02 cells and the extent of bone destruction in vivo (Supporting Fig. S5). This difference was accompanied with a sharp reduction of the BV/TV ratio (indicating a higher bone destruction) and a twofold increase in the TuV/STV ratio (a measure of the skeletal tumor burden) (Table 1, Fig. 3B). Importantly, the *Twist1* -related stimulatory effects on bone destruction and skeletal tumor burden were abolished in dox-fed animals, indicating that *Twist1* expression in tumor cells was responsible for enhanced bone metastasis formation in vivo (Table 1, Fig. 3B).

Compared with dox-fed animals, the overall TRAP staining of bone tissue sections of metastatic legs from untreated mice bearing B02-Twist#135 tumors was increased (Fig. 4A). However, ratios of TRAP staining surfaces over bone surfaces at the tumor-bone interface were similar, irrespective of *Twist1* expression (Table 1). This showed that bone destruction in metastatic legs from animals bearing B02-Twist#135 tumors increased as a function of the skeletal tumor burden; the higher the tumor burden, the higher the bone destruction (Table 1). To further address this question, a human cytokine antibody array was used to measure cytokines in the conditioned medium from B02#Twist-135 cells, treated or not treated with dox. Several cytokines and interleukins known to stimulate osteoclast differentiation (MCP-1, GM-CSF, IL-6, IL-8) were produced by these cells (Fig. 4B). Cytokine profiles produced by B02#Twist-135 cells were, however, similar, irrespective of *Twist1* expression (Fig. 4B). Additionally, to directly test whether ectopic *Twist1* expression in B02 cells could influence osteoclast differentiation, we treated primary mouse bone marrow cell cultures with RANKL and M-CSF, which are two hematopoietic factors both necessary to induce osteoclastogenesis, together with the conditioned medium of untreated or dox-treated B02#Twist-135 cells. Consistent with in vivo data (Fig. 4A), the conditioned media of B02-Twist#135 cells treated or not treated with dox stimulated the formation of TRAP-positive multinucleated osteoclasts to a similar extent (Fig. 4C). Therefore, ectopic *Twist1* expression did not enhance the intrinsic ability of B02 cells to stimulate osteoclast differentiation. Compared with the conditioned medium of dox-treated B02-Twist#135 cells, the conditioned medium of untreated B02-Twist#135 cells did, however, stimulate osteoclast activity as judged by a twofold increase in *Ctsk*

expression (Fig. 4D), a gene encoding for cathepsin K, an osteoclast-derived cysteine protease enabling degradation of the collagenous matrix.⁽¹⁾ Conversely, the expression of other markers associated with osteoclast differentiation (eg, *Trap* , *Calcr* , *Car2*) and morphology (eg, *Itgb3*) was not affected (Fig. 4D). Additionally, *Twist1* in B02 cells did not modulate osteoblast differentiation in vitro (data not shown). Hence, our observations pointed to a specific role of *Twist1* in promoting skeletal tumor outgrowth and osteoclast activity.

Twist1 ectopic expression accelerates tumor establishment but has no effect on growth rates of breast tumors into the mammary gland

To determine whether *Twist1* affects B02 cells to form primary tumors in vivo, B02#Twist-135 tumor cells were injected into the mammary fat pads of untreated or dox-fed female *nude* mice. *Twist1* expression induced a rapid tumor take in untreated animals compared with dox-fed animals (Fig. 5A). Untreated B02#Twist-135 tumor cells formed tumors within 5 to 6 weeks of inoculation, whereas it took an additional 4 weeks for dox-treated B02#Twist-135 cells to grow as palpable tumors. However, once B02-Twist#135 tumors were established, those expressing *Twist1* grew at the same rate as tumors where *Twist1* was repressed (Fig. 5A). Immunostaining of B02-Twist#135 tumors for Ki67 (a measure of tumor cell proliferation) and CD31 (a measure of the microvessel density) showed, respectively, that the proliferative index and the vascularization of tumors were similar regardless of *Twist1* expression (Fig. 5B). In the same vein, B02#Twist-112 cells, which produced moderate *Twist1* levels (Supporting Fig. S2), only modestly accelerated tumor establishment in untreated animals, compared with that observed in dox-fed animals (Supporting Fig. S6). Additionally, once tumors were established, untreated and dox-treated B02#Twist-112 cells formed primary mammary tumors at similar rates (Supporting Fig. S6). Hence, *Twist1* was not required for primary tumor growth formation. However, *Twist1* expression levels correlated with the onset of tumor establishment into the mammary gland.

Twist1 promotes the engraftment of breast cancer cells in the bone marrow through a process dependent of miR-10b

Because *Twist1* promoted breast tumor establishment into the mammary fat pad of animals, we hypothesized that this

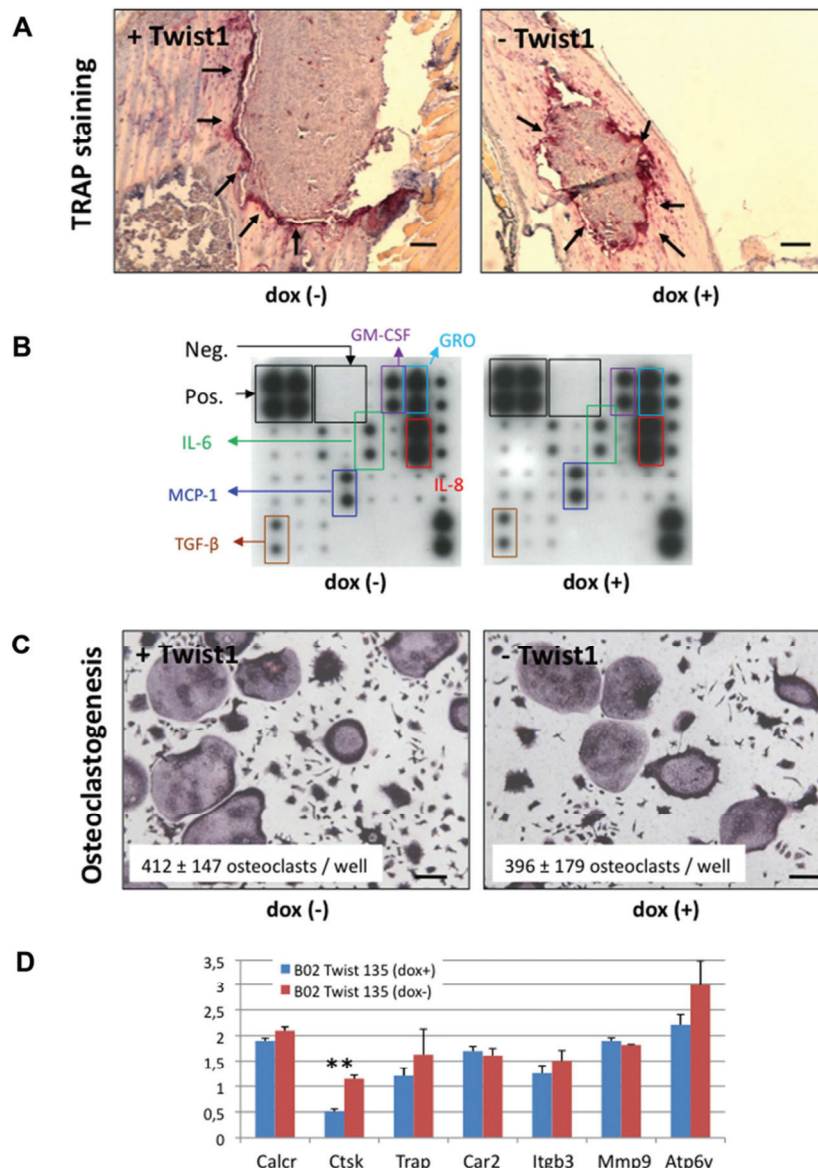


Fig. 4. *Twist1* ectopic expression in B02 breast cancer cells does not affect osteoclast differentiation, but it stimulates osteoclast activity. (A) TRAP-stained metastatic bone tissue sections from animals treated (+) or not treated (-) with doxycycline (dox). Osteoclasts are stained red. (B) Antibody-based protein microarray enabling detection of cytokines produced in the conditioned medium from B02-Twist#135 cells that were cultured in the presence or absence of dox. (C) In vitro osteoclast differentiation of murine bone marrow cells treated with M-CSF + RANKL in combination with the conditioned medium from untreated or dox-treated B02-Twist#135 cells. Mature osteoclasts were quantified as multinucleated (more than 3 nuclei), TRAP-positive cells. Representative images of mature osteoclasts are shown for each group. (D) RT-qPCR analysis of murine osteoclasts treated with the conditioned medium from untreated or dox-treated B02-Twist#135 cells. Data are representative of three separate experiments. ** $p < 0.005$. Calcr = calcitonin receptor; Ctsk = cathepsin K; Trap = tartrate-resistant acid phosphatase; Car2 = anhydrase carbonic; Itgb3 = integrin beta-3; Mmp9 = matrix metalloprotease 9; Atp6v = V-ATPase.

transcription factor might also facilitate the engraftment of B02 cells in the bone marrow microenvironment. To address this question, dox-treated or untreated B02-Twist#135 cells were injected into the tail artery of animals treated with dox or left

untreated, respectively, and these animals were then culled on day 7 after tumor cell inoculation, at which time there was no evidence of metastases, as judged by radiography. The bone marrow was collected from the hind limbs of these animals and

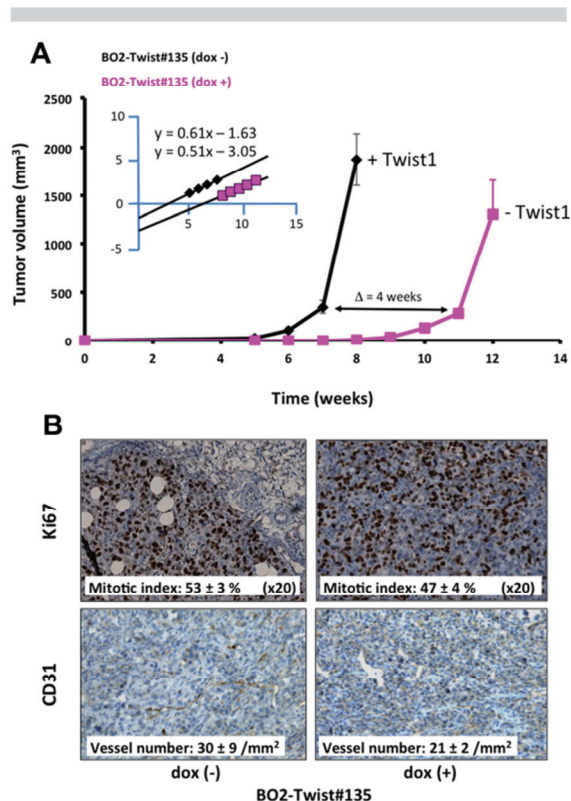


Fig. 5. *Twist1* ectopic expression in B02 breast cancer cells accelerates tumor establishment in animals. (A) B02-Twist-135 tumor cells were injected orthotopically in female mice receiving or not receiving doxycycline (dox) in drinking supply. *Twist1* expression in B02-Twist-135 cells was turned off by dox. Tumor volumes were measured with a Vernier caliper. Inset: Logarithmic conversion of the growth curves. (B) Ki67 and CD31 immunostaining of tissue sections of harvested B02-Twist#135 tumors from untreated and dox-treated animals at weeks 8 and 12, respectively, as a measurement of the proliferative index of tumor cells and number of tumor-associated blood vessels.

placed in culture under antibiotic selection, allowing the selective growth of antibiotic-resistant tumor cells. The incidence and number of colonies formed by B02 cells were then quantified. We recovered tumor cell colonies from 4 of 5 untreated animals injected with B02-Twist#135 cells (Fig. 6A). In sharp contrast, under the same experimental conditions, tumor cell colonies were recovered from the bone marrow of 1 of 5 dox-fed animals. Moreover, the average number of colonies recovered from the bone marrow of the 4 mice bearing B02-Twist#135 cells (89 colonies/well) was much higher than that recovered from the single mouse bearing dox-treated B02-Twist#135 cells (2 colonies/well) (Fig. 6A). This observation strongly suggested that *Twist1* expression enhanced the presence of B02 cells in the bone marrow.

We next undertook to determine how *Twist1* could promote engraftment of B02 breast cancer cells in the bone marrow. The observations that miR-10b was upregulated by *Twist1* in B02-Twist#135 cells (Fig. 2D) and that miR-10b induces breast tumor

invasion *in vivo*⁽¹³⁾ led us to ask whether miR-10b might mediate *Twist1*-dependent engraftment of B02 cells in the bone marrow. We therefore silenced miR-10b in B02-Twist#135 cells using an antagomiR. As assessed by RT-qPCR, miR-10b expression was suppressed by more than 70% in the presence of antagomiR-10b (data not shown). Transfected cells silenced for miR-10b were then inoculated into animals. On day 7 after tumor cell inoculation, colonies were recovered from the bone marrow of only 2 of 5 mice, whereas tumor cell colonies were recovered from all of the 5 animals inoculated with control B02-Twist#135 cells (Fig. 6B). Additionally, the average number of colonies recovered from the bone marrow of the 2 mice bearing B02-Twist#135 cells silenced for miR-10b (26 colonies/well) was much smaller than that recovered from the 5 mice bearing control B02-Twist#135 cells (65 colonies/well) (Fig. 6B). Hence, miR-10b silencing in *Twist1*-expressing B02 cells led to a substantial reduction of the incidence and number of tumor cell colonies in the bone marrow.

We wished to determine whether the ability of miR-10b to mediate engraftment of B02 cells in the bone marrow could be extended to another breast cancer cell line (4T1) that spontaneously metastasizes to the bone marrow and lungs in animals. *Twist1* directly regulates miR-10b expression in 4T1 cells.⁽¹³⁾ Mouse 4T1 cells were therefore transfected with an antagomiR-10b then inoculated into the tail artery of immunocompetent mice. At day 7 after tumor cell inoculation, the bone marrow and lung extracts of these animals were collected and cells derived from these tissues were placed in culture under 6-thioguanine selection (Fig. 7A). We recovered tumor cell colonies from the bone marrow in 3 of 5 animals inoculated with control 4T1 cells (Fig. 7B). By contrast, colonies were recovered from only 1 of 5 mice inoculated with 4T1 cells silenced for miR-10b (Fig. 7B). Similarly, tumor cell colonies were recovered from the lungs in only 2 of 5 mice inoculated with 4T1 cells silenced for miR-10b, whereas tumor cell colonies were recovered from the lungs of 4 of 5 mice bearing control 4T1 cells (Fig. 7C). Taken together, these data show that miR-10b contributes to the engraftment of *Twist1*-expressing breast cancer cells in the bone marrow and lungs.

Discussion

There are a number of experimental findings providing evidence that aberrant *Twist1* reactivation in human breast cancer is associated with tumorigenesis and lung metastasis formation.^(7,9,11-13) The contribution of *Twist1* in the development of bone metastasis *in vivo* was, however, unknown. In the present study, we demonstrated that *Twist1* facilitates experimental breast cancer bone metastasis formation. Specifically, skeletal tumor outgrowth and bone destruction in metastatic animals were both markedly increased when *Twist1* expression in B02 breast cancer cells was turned on by dox withdrawal. In osteolytic lesions, there is a vicious circle whereby metastatic breast cancer cells in the bone marrow stimulate osteoclast-mediated bone destruction, and bone-derived growth factors released from resorbed bone stimulate skeletal tumor outgrowth.⁽¹⁾ We showed here that *Twist1* promoted this vicious circle by enhancing skeletal tumor outgrowth, which in turn stimulated bone destruction. It has been previously reported that *Twist1* in PC-3 prostate cancer cells promotes PC-3-mediated osteoclast differentiation *in vitro* and stimulates *DKK-1* expression.⁽²⁴⁾ Here, the conditioned medium from B02 cells promoted

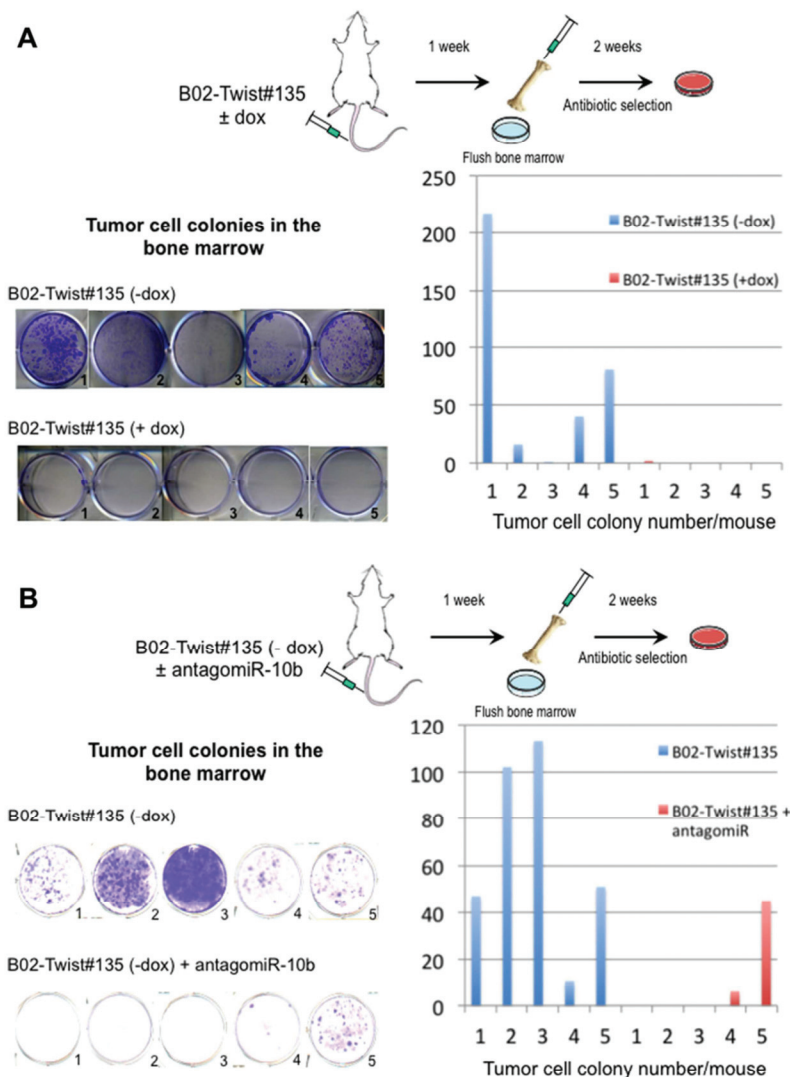


Fig. 6. *Twist1* expression enhanced the presence of B02 breast cancer cells in the bone marrow. (A) Doxycycline (dox)-treated or untreated B02-Twist-135 cells were injected into the tail artery of animals treated with dox or left untreated. *Twist1* expression in B02-Twist-135 cells was turned off by dox. One week after tumor cell inoculation, the bone marrow was collected from the hind limbs of these animals and placed in culture under antibiotic selection, allowing the selective growth of antibiotic-resistant tumor cells. After 2 weeks in culture, tumor cell colonies recovered from the bone marrow were fixed, stained and counted. Left-hand panels: Each well shows outgrowth of tumor cell colonies from the bone marrow of a single animal. There were 5 mice per group. Right-hand panel: Graph showing the total number of tumor cell colonies formed in the bone marrow from each mouse that received dox or was left untreated. (B) Untreated B02-Twist-135 cells, that were mock-transfected or transfected with antagomiR-10b, were injected into the tail artery of animals. One week after tumor cell inoculation, the bone marrow was collected from the hind limbs of these animals and placed in culture under antibiotic selection. Remaining of the experiment was performed as described in A. Left-hand panels: Each well shows outgrowth of tumor cell colonies from the bone marrow of a single animal. There were 5 mice per group. Right-hand panel: Graph showing the total number of tumor cell colonies formed in the bone marrow from each mouse bearing B02-Twist-135 cells that were mock-transfected or transfected with antagomiR-10b.

osteoclast differentiation in vitro regardless of *Twist1* expression. Additionally, *DKK-1* was only modestly upregulated in *Twist1*-expressing breast cancer cells, whereas several other cytokines (MCP-1, GM-CSF, IL-6, IL-8) that stimulate osteoclast differentiation were also produced by these tumor cells

irrespective of *Twist1*. Yuen and colleagues⁽²⁴⁾ used a murine macrophage cell line, RAW264.7, whereas we gave greater importance to the use of mouse primary bone marrow cells as a model to induce the formation of osteoclasts. The use of different cellular models for osteoclast differentiation could account for

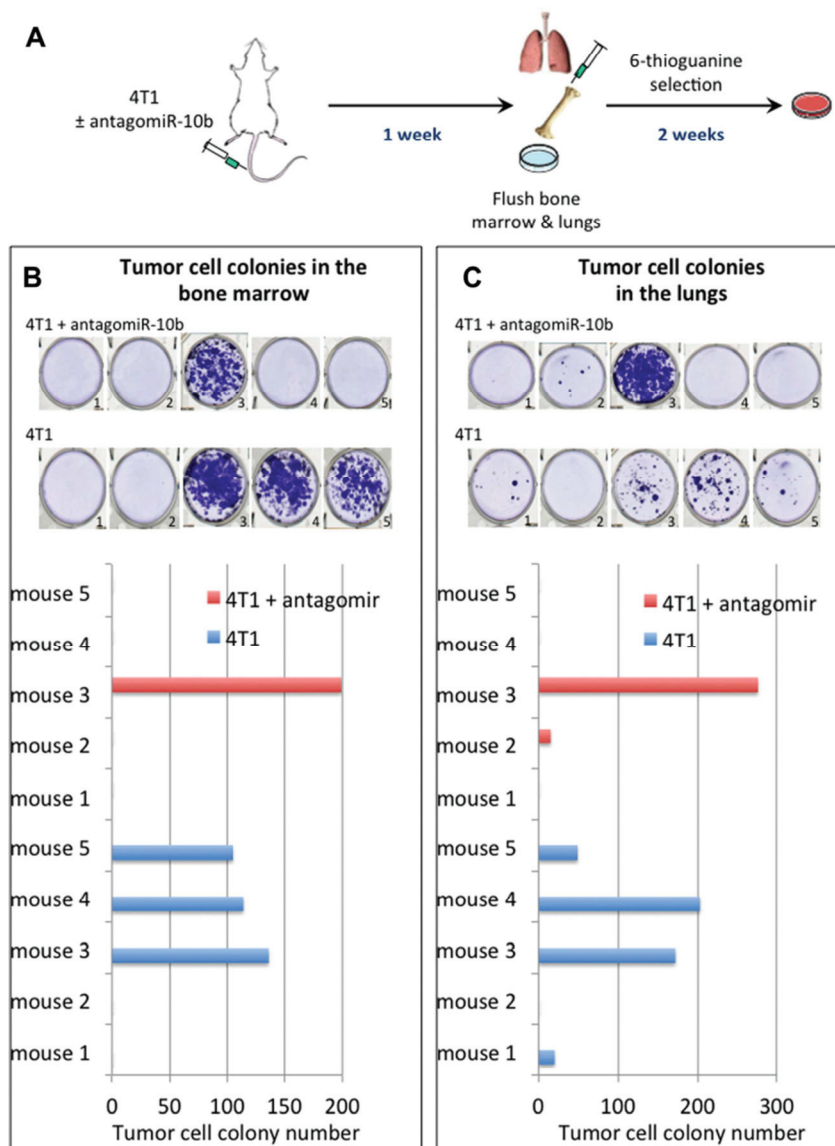


Fig. 7. Suppression of miR-10b reduced the presence of 4T1 breast cancer cells in the bone marrow and lungs. (A) 4T1 cells, that were mock-transfected or transfected with antagomiR-10b, were injected into the tail artery of animals. One week after tumor cell inoculation, the bone marrow and lungs were collected and cell suspensions were placed in culture under 6-thioguanine selection, allowing the selective growth of antibiotic-resistant tumor cells. After 2 weeks in culture, tumor cell colonies recovered from the bone marrow and lungs were fixed, stained, and counted. (B) Upper panels: Each well shows outgrowth of tumor cell colonies from the bone marrow of a single animal. There were 5 mice per group. Lower panel: Graph showing the total number of tumor cell colonies formed in the bone marrow from each mouse bearing 4T1 cells that were mock-transfected or transfected with antagomiR-10b. (C) Same as B for the presence of tumor cell colonies in the lungs.

the discrepancy between the two studies. Although TWIST1 did not enhance the intrinsic ability of B02 cells to produce cytokines that stimulate osteoclast differentiation, we observed a stimulatory effect on osteoclast activity in vitro, as judged by increased *Ctsk* (Cathepsin K) expression in osteoclasts, further indicating that TWIST1 promoted both skeletal tumor burden and bone

destruction in vivo. The effect of TWIST1 on PC3-induced osteolytic lesions in animals has not yet been investigated, making any additional comparison with our experiments difficult. Such a study would be of interest, as the role of TWIST1 in bone metastasis formation may vary according to the type of cancer.

Having shown that TWIST1 promoted the development of breast cancer osteolytic lesions, we then attempted to determine the specific steps of bone metastasis to which TWIST1 could contribute. Twist1 has been reported as a link between EMT, the intravasation of breast cancer cells in the systemic circulation, and their dissemination to the lungs.⁽¹¹⁾ Our present results establish that TWIST1 promoted breast cancer cell dissemination to the bone (Fig. 3A). *Twist1* expression in B02 cells did not modify the morphological appearance of these already mesenchymal cells and it only modestly affected the expression of major EMT-inducing transcription factors (*SNAI1*, *SNAI2*, *ZEB1*, and *ZEB2*), indicating that TWIST1 in B02 cells did not contribute to breast cancer bone metastasis formation by promoting an EMT. However, *Twist1* is expressed in disseminated breast cancer cells that persist in the bone marrow after chemotherapy,⁽⁸⁾ suggesting this transcription factor must have some robust EMT-independent functions that promote the seeding, adaptation, and/or survival of metastatic cells in this bone microenvironment. A current hypothesis gaining ground is that disseminated tumor cells in the bone marrow must acquire bone-like properties to adapt and thrive in the bone microenvironment, a process called osteomimicry.^(1,20) For example, human bone metastatic breast cancer cells, including MDA-MB-231 and B02 cells, express osteoblast-related factors, such as *RUNX2* and *CDH-11* (cadherin-11).^(20,26) However, the expression of *RUNX2* and *CDH-11* in B02-Twist#135 cells remained unchanged compared with control B02 cells, suggesting that TWIST1 did not affect osteomimicry. Additionally, TWIST1 was not required for the proliferation of B02 cells when grown in culture and as xenografts in vivo. TWIST1 did not modulate tumor cell apoptosis either. These results were in agreement with previous findings showing that suppression of *Twist1* does not affect the proliferation of 4T1 breast cancer cells in vitro and in vivo.⁽¹¹⁾ Moreover, *Twist1* depletion in 4T1 breast cancer cells does not affect cell survival.⁽¹¹⁾ Our data may be explained by the fact that B02 cells (and the MDA-MB-231 parental cell line) carry a *KRAS*(G13D) mutation that leads to the constitutive phosphorylation of YB-1 and extracellular signal-regulated kinase (ERK)1/2, which in turn endows these tumor cells with a cell growth advantage.^(27,28) This observation likely explains why TWIST1 in B02 cells did not modulate the activity of YB-1 (Fig. 2B), which is a target gene.⁽²³⁾

Although TWIST1 was dispensable for growth of B02 cells in vivo, tumor establishment of *Twist1*-expressing B02 cells in the mammary gland of animals was markedly accelerated. It has been previously reported that ectopic *Twist1* expression in MCF-7 breast cancer cells stimulates VEGF production and tumor vascularization, enhancing the establishment of MCF-7/*Twist1* tumors in the mammary gland of *nude* mice, when compared with MCF-7/control tumors.⁽²⁵⁾ TWIST1 in B02 cells did not, however, increase the microvessel density of tumor xenografts (Fig. 5B). Indeed, we have previously shown that B02 cells, which do not express *Twist1*, produce VEGF levels that are high enough to ensure a correct tumor vascularization.⁽¹⁶⁾ Besides accelerating tumor establishment in the mammary gland, our results also showed that TWIST1 markedly enhanced the incidence and number of tumor cell colonies, which were recovered in the bone marrow from animals inoculated with *Twist1*-expressing B02 cells. It is possible that TWIST1 promoted the survival of B02 cells in the mammary gland and bone marrow. For example, TWIST1 in MCF-7-I4 and MDA-MB-453-I4 breast cancer cells mediates prosurvival and proinvasive functions, at least in part, through the activation of the cell-survival AKT2 kinase.⁽²⁹⁾ However, the

effects of TWIST1 on cell survival are cell-type-specific.^(10,29) In our study, TWIST1-mediated effects on B02 cells rather resemble those reported on 4T1 and MDA-MB-231 breast cancer cells.^(11,13,30) For instance, we (this study) and others^(11,13,30) have shown that TWIST1 endowed B02, MDA-MB-231 and 4T1 cells with higher invasiveness in vitro, and that this TWIST1-dependent invasive process was associated with a robust expression of miR-10b, a prometastatic factor. Additionally, we showed that TWIST1 enhanced stemness traits of B02 cells, increasing CD44 expression. Interestingly, CD44 facilitates the arrest of CD44-expressing MDA-MB-231 cells in the bone marrow and the treatment of MDA-MB-231 cells with an anti-CD44 antibody reduces miR-10b production.^(30,31) Additionally, we showed that the repression of miR-10b in *Twist1*-expressing breast cancer cells substantially decreased the incidence and number of tumor cell colonies recovered from the bone marrow and lungs of animals. Hence, our results align with previous studies^(30,31) and strongly suggest that TWIST1 promotes the seeding and colonization of breast cancer cells in the bone marrow through, at least in part, CD44-dependent and miR-10b-dependent mechanisms.

In summary, we have provided evidence that TWIST1 enhances the presence of breast cancer cells in the bone marrow, which in turn leads to an increase in tumor burden and bone destruction.

Disclosures

All authors state that they have no conflicts of interest.

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PART 5

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PART 6

**CONFERENCE PRESENTATIONS AND
AWARDS**

1. Conference Presentations

1.1. BONE-NET Meetings:

- ✓ *19-21 January 2012, Liege, Belgium*
- ✓ *18-22 June 2012, Nantes, France*
- ✓ *4-9 February 2013, Kuopio, Finland*
- ✓ *18-21 June 2013, Leiden, Netherlands*
- ✓ *3-6 February 2014, Sheffield, UK*
- ✓ *18-20 June 2014, Hamburg, Germany*
- ✓ *7 October 2014, Crete, Greece*
- ✓ *18 November 2014, Barcelona, Spain*

1.2. International Conferences:

- ✓ *ECTS PhD training Course, 27-30 June 2014 at St Catherine's College, Oxford, UK, ORAL PRESENTATION*
- ✓ *The 2nd International Symposium on Advances in Circulating Tumor Cells (ACTC) From Basic Research to Clinical Practice, (ACTC 2014) 8–11 October 2014, Crete, Greece, POSTER*

2. Awards

- ✓ *La journée des DOctorants de la SFR Santé Lyon-Est, 5 September 2013, Lyon, France, POSTER, AWARD for the best poster*

Implication des MicroRNAs-30 dans la Dissémination Métastatique des Cellules Tumorales de Cancer du Sein au Site Osseux.

Les métastases osseuses sont des complications fréquentes des tumeurs solides, notamment du cancer du sein. Sur le plan clinique, elles sont associées à une morbidité sévère, induisant une hypercalcémie, des compressions médullaires, des douleurs osseuses, une fragilité osseuse et des fractures. Ces complications entraînent une réduction importante de la qualité de vie mais aussi de l'espérance de vie des patientes. Avec 50 000 à 70 000 personnes concernées par an en France, les métastases osseuses constituent un problème majeur de santé publique pour lequel il faut envisager des traitements curatifs, car il n'existe à l'heure actuelle que des traitements palliatifs. Les cellules tumorales de carcinomes mammaires qui métastasent au site osseux expriment des gènes qui favorisent le tropisme osseux de ces cellules ainsi que leur ancrage et développement dans la moelle osseuse. Au niveau de l'os, les cellules métastatiques altèrent la fonction des cellules osseuses formatrices, les ostéoblastes, mais aussi résorbantes, les ostéoclastes, de la matrice osseuse. En perturbant ainsi l'équilibre entre formation et résorption osseuse, les cellules métastatiques induisent la destruction de l'os. Les mécanismes moléculaires sous-jacents à ces processus sont contrôlés par l'expression génique des cellules tumorales qui interagissent avec le microenvironnement et les cellules osseuses. Dans ce contexte, les microARNs (miRs) en tant que régulateur endogène de l'expression génique, interfèrent avec les différentes étapes de la formation des métastases osseuses, incluant l'échappement des cellules tumorales de la tumeur primaire, la dissémination et l'invasion du site osseux, ainsi que l'apparition de lésions ostéolytiques.

Notre travail expérimental a débuté par l'analyse des profils transcriptomiques des miRs dans des cellules de tumeurs mammaires à fort tropisme osseux. Nous montrons que l'expression d'une famille de miRs (miRs-30), composée de miR-30a, miR-30b, miR-30c, miR-30d et miR-30e, est inhibée dans ces cellules. Sur le plan clinique, la faible expression des miRs-30 dans la tumeur de patientes atteintes de cancer du sein est associée à un mauvais diagnostic de rechute métastatique, à la taille élevée de la tumeur primaire, à l'envahissement ganglionnaire, au stade avancé de la maladie et au statut hormono-résistant. Nous avons alors étudié l'activité des miRs-30 dans le développement des métastases osseuses en utilisant un modèle expérimental de métastases osseuses humaines dans lequel des cellules tumorales sont inoculées dans la circulation sanguine des souris. L'expression forcée des miR-30s dans ces cellules tumorales qui métastasent fortement et spécifiquement à l'os, inhibe la formation des métastases osseuses. Nous montrons que les miRs-30 inhibent l'invasion et stimulent l'ostéoblastogenèse, *in vitro* et réduisent la charge tumorale et l'ostéoclastogenèse, *in vivo*. En accord avec ces résultats, l'expression de gènes qui stimulent les métastases osseuses est inhibée par les miRs-30. Parmi ces gènes, l'expression du CTGF (connective tissue growth factor) est augmentée dans les métastases osseuses humaines.

Ces résultats sont présentés dans la première partie du travail expérimental d'un papier intitulé : « The Tumor Suppressor miRs-30-5p Family Regulates Human Breast Cancer Cell Colonization in Bone », qui est en préparation de soumission à publication.

Dans la seconde partie du travail, nous avons étudié l'activité des miRs-30 dans la formation de la tumeur primaire et dans la dissémination métastatique des cellules tumorales au site osseux. Des expériences de xénogreffes tumorales orthotopiques ont été menées, en inoculant des cellules tumorales qui métastasent spontanément dans la glande mammaire de la souris. Dans ce modèle, les miRs-30 n'altèrent pas la croissance tumorale et l'apparition des micro-métastases dans l'os. Cependant les miRs-30 inhibent l'invasion et le caractère de cellules souches tumorales de ces cellules métastatiques, *in vitro*.

Globalement, cette étude montre que les miRs-30 inhibent les étapes du tropisme, de l'ancrage de l'invasion et de la formation de macro-métastases dans l'os, mais pas de la croissance tumorale et/ou de l'échappement des cellules cancéreuses de la tumeur primaire.

Ce travail nous permet d'envisager plusieurs perspectives. Nous allons modifier l'expression des miRs-30 dans des cellules de cancer du sein hormono-sensibles et étudier l'impact de cette modification sur le phénotype cellulaire, la croissance tumorale et la dissémination métastatique vers les sites secondaires. L'intérêt thérapeutique des miRs-30 sera testé en injectant les miRs-30 sous forme d'oligonucléotides stabilisés chimiquement, chez la souris, en présence ou non d'agents pharmacologiques utilisés dans le traitement des ostéolyses malignes, les bisphosphonates. Finalement, ce travail a mis en évidence des gènes fortement exprimés dans les cellules tumorales à tropisme osseux et dont l'expression est inhibée par les miRs-30. L'expression de certains de ces gènes est élevée dans les métastases osseuses humaines. Nous étudierons le rôle de ces gènes au niveau des métastases osseuses.

La dernière partie de la thèse est consacrée à l'activité de l'oncomir miR-10b, un miR dont l'expression est augmentée par le facteur de transcription Twist1, ce qui facilite la formation des métastases osseuses. Le papier intitulé « TWIST1 expression in breast cancer cells facilitates bone metastasis formation » est inséré en dernière partie du manuscrit.

Après avoir replacé ce travail dans son contexte bibliographique en présentant l'état des connaissances actuelles sur le cancer du sein, le tissu osseux, les métastases osseuses et les microARNs, nous présentons les résultats obtenus au cours des travaux de thèse, ainsi que les perspectives qui en découlent.