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Titolo della tesi

"Breast cancer cell stemness and dormancy in the bone microenviroment"

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SUMMARY

This work investigated the role of N - cadherin in the interaction between dormant tumor cells and a particular subpopulation of osteoblasts, called SNOs (Spindle-shape N-cadherin positive Osteoblast) located at the level of the endosteal niche. Since the N-cadherin transduction signal requires a homophilic interaction, mouse BrCa cell line 4T1 were used to establish an interaction with primary mouse osteoblasts in vitro and in vivo. On these cells, the *Cdh2* gene encoding for N-cadherin was removed via CRISPR / Cas9, obtaining $4T1^{Cdh2KO-GFP}$ cells. Subsequently, $4T1^{Cdh2KO-GFP}$ were transfected with a murine Cdh2-turboGFP vector to restore the expression of N-cadherin in the KO cells obtaining $4T1^{Cdh2Res-GFP}$ cells. We evaluated the expression of some markers typical of Hematopoietic Stem cells, including CXCR4, SCA1 and TIE2. Only CXCR4 was expressed, with a reduced expression in $4T1^{Cdh2Res-GFP}$ compared to $4T1^{Cdh2KO-GFP}$ cells. To evaluate the stem capacity and the self-renewal ability of these cells we investigated the formation of primary and secondary mammospheres, respectively, observing a lower volume and number in the $4T1^{Cdh2Res-GFP}$ compared to $4T1^{Cdh2KO-GFP}$ cells. The formation of primary and secondary mammospheres was investigated also in 4T1 cells sorted by MACS into 4T1 N-Cadherin^{High} and 4T1 N-Cadherin^{Low} obtaining in the primary mammospheres a lower volume and number in 4T1 N-Cadherin^{High} compared to 4T1 N-Cadherin^{Low} cells. The number of secondary mammospheres was similar while a lower volume was observed in 4T1 N-Cadherin^{High} compared to the 4T1 N-Cadherin^{Low} cells. Finally, we observed that the transcriptional expression of N-cadherin did not affect the mRNA expression of Notch2, a receptor relevant to induce dormancy in BrCa cells lodging the endosteal niche. The effect of N-cadherin expression in 4T1 cells was evaluated in vivo using four-week-old Balb/c wild-type mice intratibially injected with 4T1 N-cadherin^{High} and 4T1 N-cadherin^{Low} cells, noting the absence of significant differences in the number of cells colonizing the endosteal niche.

CHAPTER 1

1. INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most frequent cancer in the female population, in which it is one of the main causes of death. This pathology develops in the lining cells of the galactophore ducts and in the lobules where the cells undergo abnormal and uncontrolled proliferation, degenerating into malignant neoplasm. These neoplasms can be divided into ductal carcinomas and lobular carcinomas.

There are several risk factors related to this pathology including age, exposure to radiation as well as hereditary and genetic factors. In fact, about 10% of cases of this tumor are caused by mutations of the BRCA1 and BRCA2 genes that encode proteins regulating the DNA repair mechanisms occuring in the cell replication phase. Genetic alterations of these two genes induce a loss of their control function, becoming responsible of the development of the disease[1,2].

1.2 BREAST CANCER: EPIDEMIOLOGICAL DATA

In Italy, according to the latest AIRTUM (Associazione Italiana Registri Tumori) publication on cancer numbers in 2018, breast cancer is the most frequently diagnosed cancer and, with about 52,800 new cases, it represents the most frequent neoplasm in women in all age groups, and about 41% of total neoplasms diagnosed under the age of 50 years against 22% over the age of 70 years (**Table 1).**

AGE	0-49	50-69	70+
1	Breast (41%)	Breast (35%)	Breast (22%)
2	Thyroid (15%)	Colorectal (11%)	Colorectal (16%)
3	Melanoma (7%)	Lung (7%)	Lung (7%)
4	Colorectal (4%)	Uterus (7%)	Pancreas (6%)
5	Uterin cervix (4%)	Thyrod (5%)	Stomach (5%)

Table 1 *Top five cancers in terms of frequency and proportion of total incident cancers (excluding skin cancers) in women by age group. POOL AIRTUM 2008-2014. AIOM (Associazione Italiana Oncologia Medica)*

Again in Italy, breast cancer represented in 2018 the first cause of death from cancer in women, with 12,274 deaths, representing 29% of causes of cancer death before the age of 50, 21% between 50 and 69 and 14% after 70 (**Table 2**).

AGE	0-49	50-69	70+
1	Breast (29%)	Breast (21%)	Breast (14%)
2	Lung (9%)	Lung (15%)	Colorectal (13%)
3	Colorectal (7%)	Colorecatl (10%)	Lung (10%)
4	Uterus (6%)	Pancreas (7%)	Pancreas (8%)
5	Nervous System (6%)	Ovary (7%)	Stomach (7%)

Table 2 *Top five causes of cancer death and proportion of total cancer deaths in women, by age group. POOL AIRTUM 2010-2014. AIOM (Associazione Italiana Oncologia Medica)*

On average, for an Italian woman, the risk of developing breast cancer in the course of life is now 13%: about one in 45 women develops this pathology by the age of 50, one in 19 between 50 and 69, and one in 23 women between 70 and 84. The incidence is very related to age. In fact, it is rare that this pathology manifests itself before the age of 25 with an incidence of about 2/100,000 cases per year. After the age of 25, the chances of developing

the disease increases slightly with an incidence rate of less than 10/100,000 cases per year, reaching 150/100,000 cases per year in woman under 50 years of age.

There is also a correlation between mortality and age, noting a reduced chance of survival for women over the age of 70 with a death rate of 60%. (**Figure 1**).

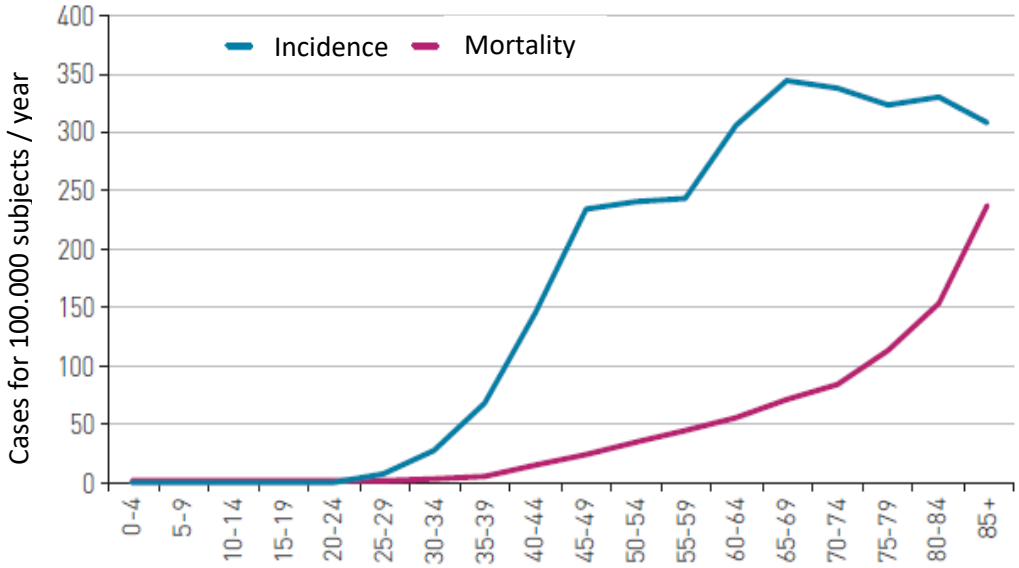

Figure *1* *Breast cancer. Age-specific rates. POOL AIRTUM 2008-2014.*

AIOM (Associazione italiana Oncologia Medica)

During the five years following the diagnosis of the disease, the probability of death increases in both young and old women. It is estimated that 5-year survival is approximately 90% for patients under 50 years of age and about 80% in women over 70 years of age. The increase in survival is due to the diagnostic anticipation linked to screening, and to the improvement of therapies, especially the adjuvant one. Today, there are several mammography screening programs available for women between 50 and 69 years of age and in some regions they are also extended to women between 45 and 49 years with an annual interval, and between 70 and 74 years with an biennial interval. Numerous studies have shown that mammography screening reduces mortality of about 20% (**Figure 2**) and increases treatment options[3,4].

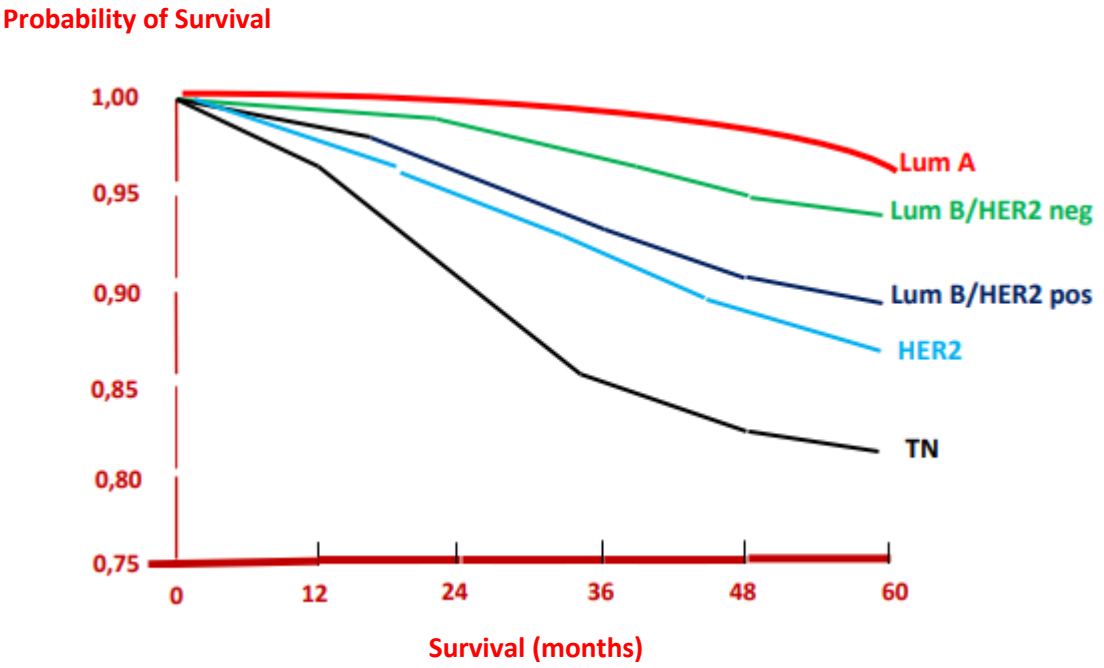

Figure 2 *Survival by breast cancer subtype https://www.senologia.it/wp-content/uploads/2021/08/CIS-LG-Epidemiologia-del-carcinoma-mammario-2021.08.pdf*

1.3 ANATOMY OF THE BREAST

The breast is an even and symmetrical gland located above the pectoral muscle between the second and sixth ribs in the cranio-caudal direction, and from the sternum to the median axillary line in the latero-lateral direction [5,6].

In the deep part the mammary parenchyma borders the superficial pectoral fascia, in the upper portion borders the cervical fascia and in the lower portion borders the Cooper's abdominal fascia.

There are fibrous tralci that from the cutaneous dermis continue into the parenchyma of the gland, forming the Suspensory Cooper ligament whose function is to bind the gland to the portion of skin above it. The nipple is centrally positioned in the portion of the skin called the "areola-nipple" area [6,7].

The blood supply of the breast comes for about 60% from the internal mammary artery and for about 30% from the lateral thoracic artery and internal intercostal arteries. Blood outflow occurs through specific venous pathways that develop parallel to arterial vessels [5,6].

The lymphatic drainage takes place mostly through the axillary pathway. In fact, the lymphatic ducts and lymph nodes of the axillary portion drain more than 75% of the

lymphatic flow. Another lymphatic drainage route is the lymphatic ducts of the internal mammary chain; through these ducts about 25% of the lymph is drained by means of the internal thoracic mammary lymph nodes that are positioned along the sternal portion. Finally, a small amount of lymph is drained into the supraclavicular, infraclavicular and intramammary lymph nodes [5,7].

Clinically the udder can be schematized in quadrants numbered from 1 to 6. The supero-external quadrant Q1, the supero-internal quadrant Q2, the infero-external Q3, the infero-internal Q4 and the central retro-areolar region Q5, which concerns the nipple, can be identified; finally there is the axillary extension indicated with Q6 (**Figure 3**).

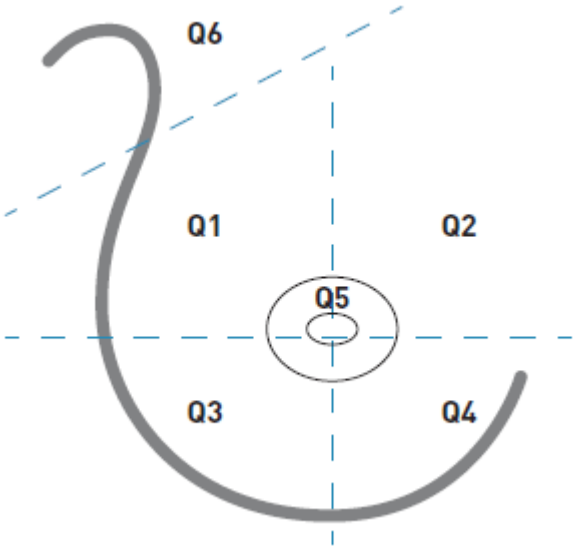

Figure 3 *Schematic representation of the clinical-functional subdivision into quadrants of the breast. AIOM (Associazione Italiana Oncologia Medica)*

Histologically, the breast is composed of ducts and berries located in the stroma, formed by fibrous and adipose tissue, which makes up most of the parenchyma of the gland. The glandular tissue proper has a branched structure where the major galactophore ducts, starting from the nipple, branch into gradually smaller ductal formations up to the ductile-lobular terminal units, which are the functional units of the mammary gland from which most carcinomas originates.These units are formed by an intralobular collector duct, an intralobular stroma and alveoli whose epithelium undergoes changes in the pregnancy and in the lactation phases. (**Figure 4**)

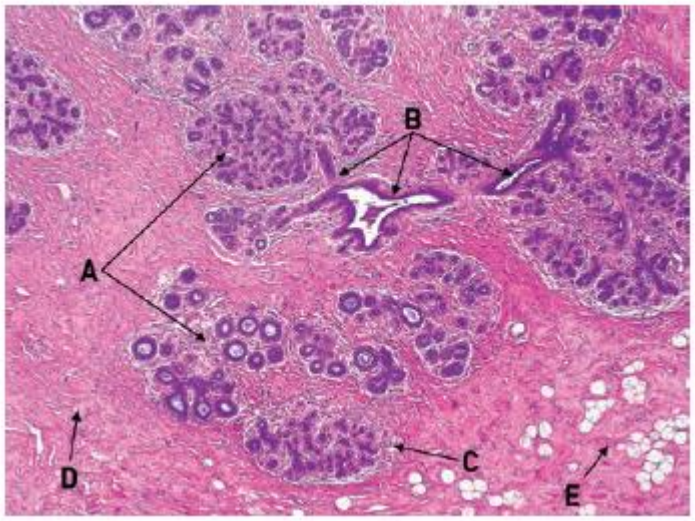

Figure 4 *Microscopic anatomy of the breast (hematoxylin-eosin). The section shows the normal architecture of ductural-lobular terminal units. A) lobules, B) terminal ducts with their main branches, C) specialized intra-lobular stroma and D) the extralobular non-specific stroma are observed. E) adipose tissue (10X magnification). AIOM (Associazione Italiana Oncologia Medica)*

The alveolar structures of the breast are made up, in the inner portion, of cuboid-shaped luminal cells that are epithelial cells facing the lumen of the alveoli; the external portion, on the other hand, is composed of myoepithelial cells.

The stromal part is divided into inter-lobular stroma which consists of abundant connective and fibro-adipose tissue, and intra-lobular stroma consisting of an abundant fibrovascular component and some types of cells, including immune cells such as lymphocytes, plasma cells and macrophages.

The stroma is affected by hormonal changes that have effects on the glandular component of the parenchyma: in fact, estrogen, progesterone and androgens induce the differentiation and proliferation of lumen cells, while oxytocin causes the contraction of myoepithelial cells. [6.7].

1.4 HISTO-PATHOLOGICAL FEATURES OF THE BREAST

Generally, breast cancers are classified into invasive and non-invasive neoplasms. Non-invasive breast cancers, on the other hand, are represented by in situ ductal carcinomas (**Figure 5A**). The most common invasive forms are ductal carcinomas (**Figure 5B**), which arise from an abnormal and uncontrolled proliferation of a cell of the milk ducts.

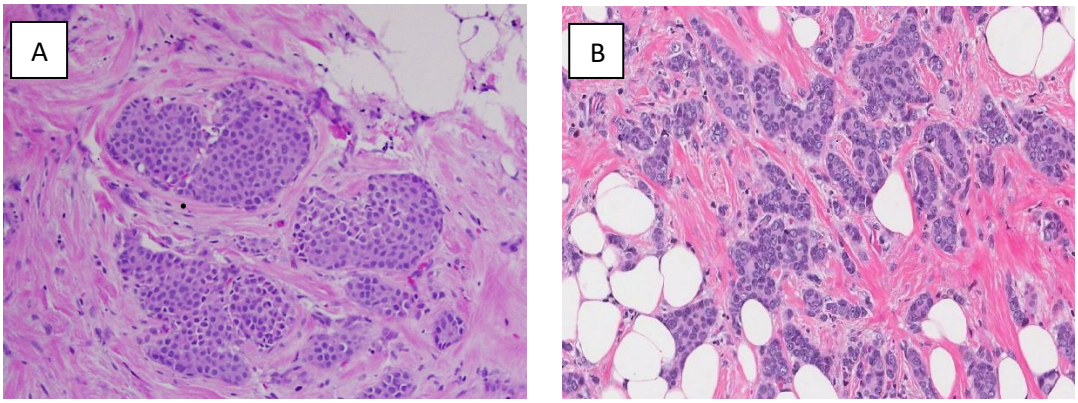

Figure 5 *A) In situ lobular carcinoma Hematoxylin and eosin staining, 20X*

https://en.wikipedia.org/wiki/Lobular_carcinoma_in_situ;

B) Invasive ductal carcinoma https://www.mypathologyreport.ca/it/breast-invasive-ductal-carcinoma

In situ lobular carcinoma, also known as intralobular, is recognizable by the presence of very expanded alveoli and is a precancerous lesion; in fact, cancer cells develop inside the lobules not invading the surrounding breast tissue. In situ ductal carcinoma, on the other hand, is morphologically more heterogeneous and four types are recognized: comedonic, cribriform, solid, micropapillary, papillary and mixed (**Figure 6 A-F**).

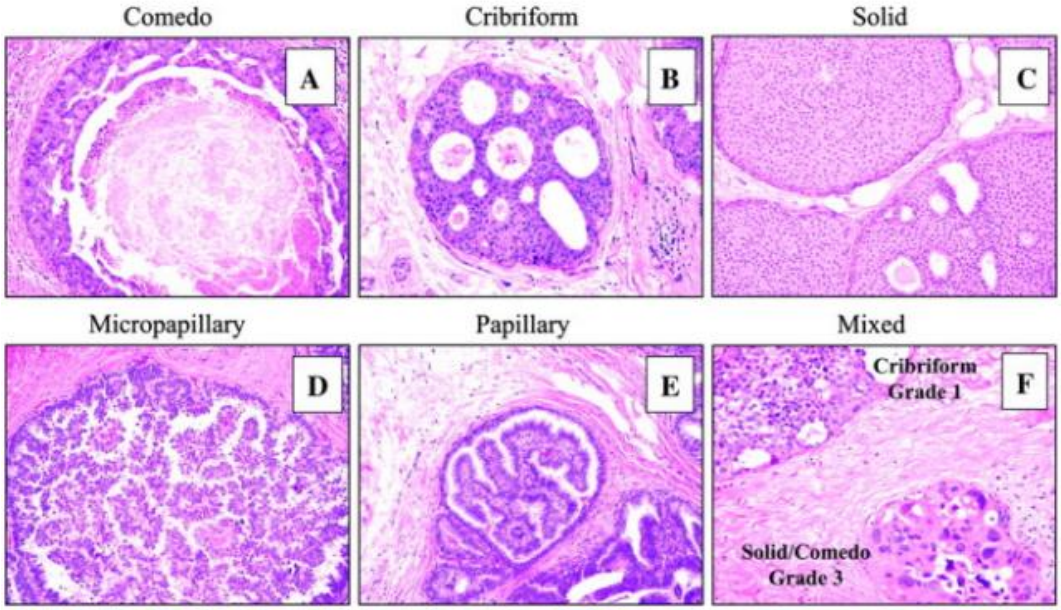

Figure 6 *A) In situ ductal comedonic, B) cribiform; C) solid; D) micropapillary; E) papillary and F) mixed carcinoma types.* *http://www.fertilitycenter.it/mammella/carcinoma-duttale-in-situ-dcis*

In situ ductal carcinoma is identifiable by the presence of areas rich in cancer cells, with a layer of normal myoepithelial cells. Typically, the papillary and cribriform types may take longer to degenerate into invasive forms as they are low-grade lesions as opposed to the solid and comedonic types that fall within the high-grade lesions.

Invasive breast cancers are characterized by the ability to infiltrate tissues randomly forming continuous layers of cells, and tend to grow as a cohesive, palpable mass similar to a lump. Typically, invasive lobular breast cancers are not easily diagnosed by mammography until the cancer reaches an advanced stage.

Invasive lobular carcinoma constitutes a low percentage of breast cancers; in fact, in pathology, mixed ductal and lobular tumors are more frequent. Invasive ductal carcinomas are typically identified based on the characteristics they exhibit. If the infiltration of cells generates structures coated with a single layer of the epithelium, it is called infiltrating tubular carcinoma. In addition, if infiltrating cells release an abundant amount of mucus, it is called mucous tumor. The latter two categories also fall under low-grade lesions and represent about 3% of invasive breast cancers.

Just 5% of breast cancers consist of medullary carcinomas, formed by highly proliferating invasive cells and layers of cells surrounded by an infiltrate of small lymphocytes [8-10].

1.5 DIAGNOSIS

In the absence of obvious symptoms breast neoplasms are diagnosed by mammography or ultrasound as potentially altered breast nodules.

Clinically evident breast cancers present with an accentuated tastable swelling associated with both skin invasion of cancer cells of the nipple areola complex and invasion of the loco-regional lymph nodes.

Typically, breast cancer presents with a hard lesion and irregular edges. The symptoms of an advanced loco-regional disease are axillary adenopathy and hardening or ripple of the portion of the skin with subsequent formation of ulcers on the affected part. The initial examination is an objective examination that consists of the palpation phase of the breast and is completed by making the patient stretch out with the chest upwards with the arm raised above the head in order to favor the sensation of all the glandular tissue [10-17].

Clinically, evaluation can also be done through palpation of the neck, supraclavicular lymph nodes and of course both breasts.

The possible identification of suspicious nodules leads to a biopsy by needle aspiration; generally, in case of presumed breast cancer, a biopsy of the sentinel lymph node located in the axillary portion above the mammary gland is carried out. Initially, palpation of the axillary lymph nodes is performed followed by a biopsy that will be evaluated by the pathologist.

Cytological and histological analyses are performed on the biopsies, evaluating the features of cells and tissues. For prognosis and treatment purposes, it is important to carry out molecular investigations evaluating some characteristics of the tumor, such as the expression of hormone receptors, the growth rate and the expression of the HER-2 oncoprotein.

Another method to diagnose breast cancer is the magnetic resonance imaging which is a non-invasive examination, followed by mammography and ultrasound. Magnetic resonance imaging is strongly indicated for patients with previous family cases and is also useful to evaluate both the extent of the pathology and other abnormalities that may have been found previously. Magnetic resonance imaging is therefore used to assess the nature of breast lesions on the basis of the absorption characteristics of the radiomagnetic contrast medium; for this reason, the faster the absorption of the contrast medium, the greater the swelling [18].

2. BONE

2.1 INTRODUCTION

Bone tissue is a connective tissue characterized by a mineralized extracellular matrix, which provides hardness and resistance to pressure and traction, while remaining a light structure. The association between maximum strength and minimum weight makes the bones light but mechanically competent. The bone performs important functions, supporting our body and representing the main deposit of calcium and phosphorus, which are continuously mobilized to maintain the ion homeostasis of the organism. Among other functions, it provides protection to the internal organs by constituting the cranium for the brain, the rib cage for almost the entire respiratory system, heart and large vessels, and the vertebral column for the spinal cord. Furthermore, bone is a very dynamic tissue continuously remodeled and renewed throughout life, a process that is useful not only for mechanical reasons but also for regulating blood calcium and phosphorus concentration. Like other connective tissues, the bone is made up of cells immersed in abundant extracellular matrix and is strongly vascularized and innervated. These structural components remain the same in prenatal and adult life but will greatly change the way they organize and combine. In the embryonic and fetal skeleton we speak of non-lamellar bone, in which the collagen fibers are organized in large intertwined bundles that make the bone mechanically incapable to support the body. With the development, this primary tissue undergoes its first remodeling and is replaced by lamellar bone with parallel fibers, in which cells and matrix are organized in distinct and parallel lamellae, giving the bone its particular resistance and robustness. This resistence is due to the peculiar feature of bone matrix mineralization[19-23] .

2.2 BONE STRUCTURE

2.2.1 Macroscopic structure

From a macroscopic point of view, bones are classified into long, short and flat (**Figure 7**). Orbserving with the naked eye a long bone cut longitudinally it is possible to distinguish two different forms of bone tissue: the spongy bone and the compact bone. The spongy bone is located at the level of the epiphysis and appears as an alveolar structure with trabeculae that anastomize to each other to form a three-dimensional network within which the bone

marrow is located. The more peripheral trabeculae are thicker and have a greater mechanical function while the more central ones are thinner and serve for metabolism. The diaphysis is formed by compact bone that appears as a solid and dense mass that circumscribes the central medullary cavity. There is a third zone called metaphysis, which includes the growth plate between the epiphysis and the diaphysis, formed by cartilage involved in the growth in height throughout the period of development. The outer surface of the long bones is covered by a sheath of very vascularized fibroelastic connective tissue, the periosteum, absent in correspondence of the articular cartilage and in the insertion areas of tendons and ligaments. The inner surfaces of the compact and spongy bone are instead lined with a membrane called endosteum, consisting of a monolayer of paving cells that continues with the vascular channels of the bone named Havers and Volkmann canals, containing nerves, blood vessels, lymphatic vessels and matrix.

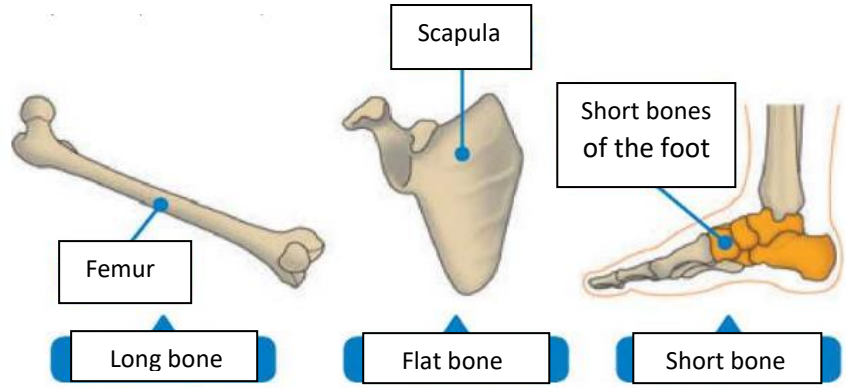

Figure 7: *Examples of long, flat and short bones*

https://digimparoprimaria.capitello.it/app/books/CPAC78_2613560A/html/293

2.2.2 Microscopic structure

The main feature of post-natal spongy and compact bone is their lamellar structure. Each lamella represents a lamina consisting of cells and intercellular substance with a thickness of 3 to 7 microns (**Figure 8**). The matrix is strongly mineralized, with type I collagen fibers parallel to each other. Between the lamellae there are cavities ordered one after the other along the parallel lines of the lamellae. Each has a series of canaliculi which anastomize with those of the adjacent lacunae, forming a network that joins with the longitudinal Havers canals and with the trasversal Volkmann canals, in which the vessels of the compact bone

flow. In the spongy bone the lamellae aggregate to form the trabeculae, arranged in an irregular network. In the compact bone, the lamellae are organized concentrically in the osteons, cylindrical structures formed by a central Havers channel oriented parallel to the major axis of the bone, and a series of concentric lamellae surrounding the canal (**Figure 8**). The spaces between three or more contiguous osteons are occupied by interstitial lamellae, while at the periphery of the bone there are systems of parallel lamellae disposed tangentially to the internal and external bone surfaces, forming the internal and external circumferential systems, respectively. This architectural design confers resistance not only to compressive forces but also to tensional and torsional stresses. Externally, the adjacent osteons are separated by an irregular demarcation line that under the microscope is intensely colored and indicates the point where the bone deposition took place after the resorption phase. In the formation of the osteone, the first lamella formed is the one near the demarcation line. The other lamellae progressively fill the osteon canal reducing its lumen. In each lamella the collagen fibers are oblique to the right or the left; if one lamella is right-handed, the next one is left-handed and so on alternately. This precise order makes the bone lamellae refracting under the polarized light microscope.

The periosteum consists of an external layer of compact connective tissue with few cells and several vessels, and an internal layer of alveolar connective tissue with many cells and a dense capillary network. From the outer layer large bundles of collagen fibers, called Sharpey fibers, penetrate perpendicularly in the inner layer of the periosteum and then into the external circumferential system, anchoring the periosteum to the bone (**Figure 8**). In the inner layer of the periosteum there is an epithelioid layer of osteogenic cells, which become fibroblastoid and quiescent when bone formation stops, for instance at the end of skeletal growth. In this state of quiescence, however, they maintain osteogenic potential and can reactivate to become osteoblasts.

The endosteum is a thin layer of cells that covers the spongy bone and the inner surface of the compact bone, including the Havers and Volkmann canals walls [24].

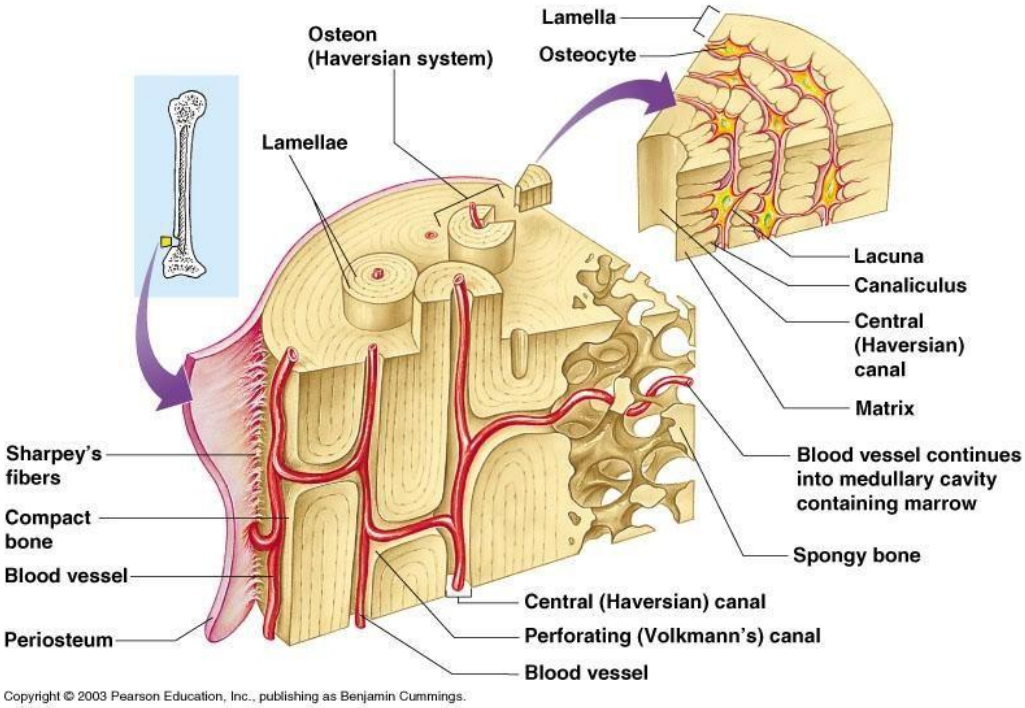

Figure 8: Microscopic *organization of bone*

https://www.pinterest.it/pin/491385009337385112/

2.3 BONE CELLS

2.3.1 Osteoblasts

Osteoblast are osteo-forming cells of cuboid shape (**Figure 9**) that derive from mesenchymal stem cells. They produce and secrete the components of the matrix and abound in the sites of active bone synthesis, where they form an epithelioid layer. Morphologically, they appear with clear nucleus, evident nucleolus and basophilic cytoplasm rich in rough endoplasmic reticulum, Golgi apparatus and mitochondria. In the apical cytoplasm facing the bone surface, there are membrane-delimited bodies called calcifying globules or matrix vesicles, which play an important role in the mineralization of the organic matrix. They are released by the cell by budding and are found in the bone matrix being mineralized. The deposition of the matrix takes place next to the apical membrane of osteoblasts according to a very precise orientation: initially the osteoblasts depose bone matrix from the side facing the pre-existing bone surface; subsequently deposition continues on each side around the osteoblast, which remains embedded in the new formed matrix. At this stage, the osteoblast slows down its metabolic activity and turns into an osteocyte, while new osteoblasts differentiate from the osteoprogenitor cells. When bone formation ends, the osteoblasts that remain close to the

bone surface, reduce their organelles and transform into a layer of flattened quiescent cells, the so-called lining cells. Osteoblasts also produce cytokines, such as insulin-like growth factor (IGF) I, II, transforming growth factor β (TGF-β), and osteocalcin, which regulate energy metabolism, brain development and muscle functions. Additionally, osteoblasts produce macrophage colony stimulating factor (M-CSF), receptor activator of NFKB ligand (RANKL) and osteoprotegerin (OPG), which regulate osteoclast formation and differentiation [25-47].

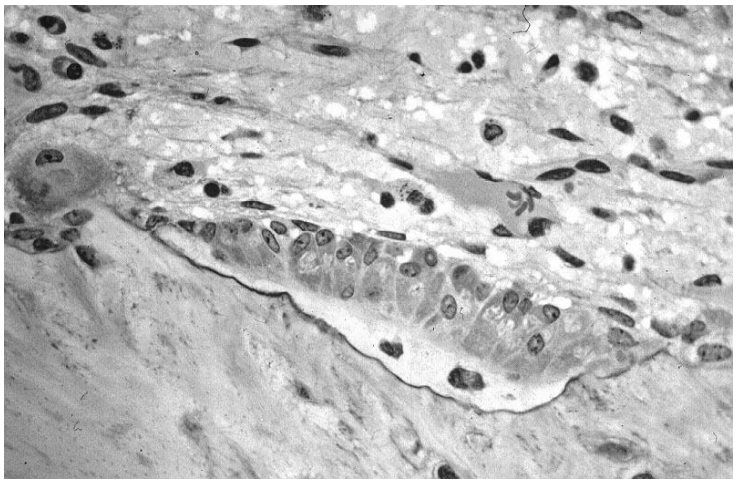

Figure 9: *Active osteoblasts producing bone matrix (Robert M. Hunt)*

https://it.wikipedia.org/wiki/Osteoblasto

2.3.2 Osteocytes

Osteocytes derive from osteoblasts and are found in the so-called osteocytic lacunae (**Figure 10**).

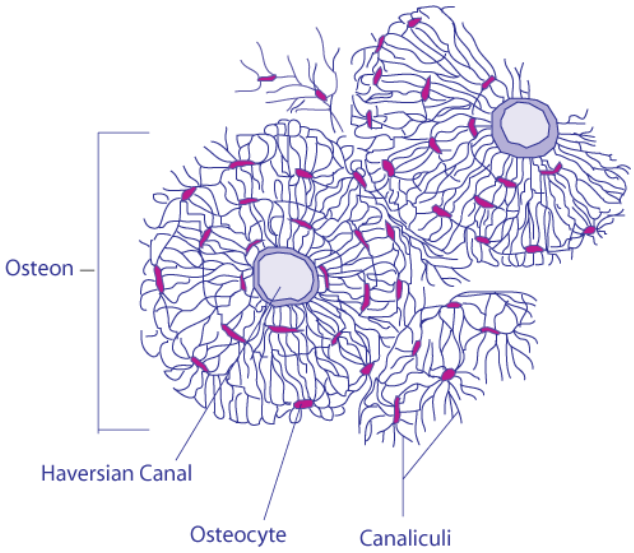

Figure 10: *Schematic representation of osteocytes.*

https://it.wikipedia.org/wiki/Osteocita#/media/File:Transverse_Section_Of_Bone.png

The cell body of osteocytes is spindle-shaped to adapt to the shape of its lacuna [53,57-61]. They have cytoplasmic extensions lodged in bone canaliculi that begin to emerge before the cells is trapped in the bone matrix.

These cytoplasmic extensions connect the osteocytes to each other, to the osteoblast and to the lining cells by means of gap junctions that favor the intercellular transport of small signaling molecules [61-67] .

Osteocytes are mechanosensor able to detect the mechanical stress caused by pressure and load, thus helping the bone to adapt to mechanical forces. Observing them under an electron microscope, they show a primary cilium, which is probably the sensor they use to detect the variations in the interstitial liquid flow[58]. In addition, osteocytes influence bone remodeling in response to mechanical forces and to changes in bone mineralization by secreting FGF-23 following the reduction of calcium and phosphate. Osteocytes also release RANKL and Sclerostin to regulate bone resorption and formation, respectively [55,68-70].

2.3.3 Osteoclasts

Osteoclasts are the cells responsible for bone resorption, a fundamental process for the modelling and remodeling of the bone tissue. They are multinucleated syncytia with a diameter up to 100 microns, which derive from the fusion of the mononuclear precursors of the monocyte-macrophage lineage. Morphologically they have well-defined nuclei separated from each other, weakly acidophilic cytoplasm, rich in mitochondria and lysosomes. They are found on the surface of resorbing bone at the level of their own erosion area, called Howship's lacuna. The membrane facing the resorbing bone presents a ruffled border with irregular membrane extensions that increase the membrane surface. The peripheral cellular region in contact with the bone is called clear zone because of the absence of organelles except ribosomes. The space below the ruffled border is separated from the rest of the extracellular space by the sealing membrane which, allows the adhesion of the osteoclast along the entire cell perimeter facing the bone surface. The sealing property of the membrane is due to specific adhesion structures, the podosomes, in which bundles of actin microfilaments are connected to the integrin αvβ3 that ensure dynamic interaction with the bone surface. Within the resorption lacuna the dissolution of the mineral matrix and the degradation of organic matrix take place through the acidification of the extracellular space followed by release of collagenolytic enzimes, respectively[71-75].

In the osteoclastogenesis process, the cytokine M-CSF stimulates the proliferation of macrophages and the expression of the receptor RANK. The interaction between RANK and its ligand RANKL activates the transcription factor NFkB, which translocates to the nucleus and activates the transcription of specific osteoclast differentiating genes.

The osteoclastogenesis process is modulated by osteoblasts through the secretion of the M-CFS, RANKL and the RANKL decoy receptor, OPG (**Figure 11**). Osteoclast activity is also regulated by different hormones. A hormone involved in the differentiation of osteoclasts is parathyroid hormone (PTH), produced by the parathyroid glands in response to changes of calcemia. If the blood calcium level is low, the PTH is released and stimulates the expression of RANKL, which induces osteoclastogenesis and therefore bone resorption. An important role in the development of osteoclasts is played by vitamin D. This is taken as an inactive precursor from the diet and its activation is indirectly regulated by the plasma calcium concentration and directly by PTH. If the calcemia is low, PTH acts on the renal tubule increasing the reabsorption of calcium and stimulating the 1-α-hydroxylase that by adding

the second hydroxyl group to 25-hydroxyvitaminD3, leads to the formation of the active 1-25- dihydroxyvitaminD3, which stimulates the gut absorption of calcium and phosphate. For this reason, vitamin D deficiency leads to poor calcification of the bones called rickets in children and osteomalacia in adults. [73,76-95].

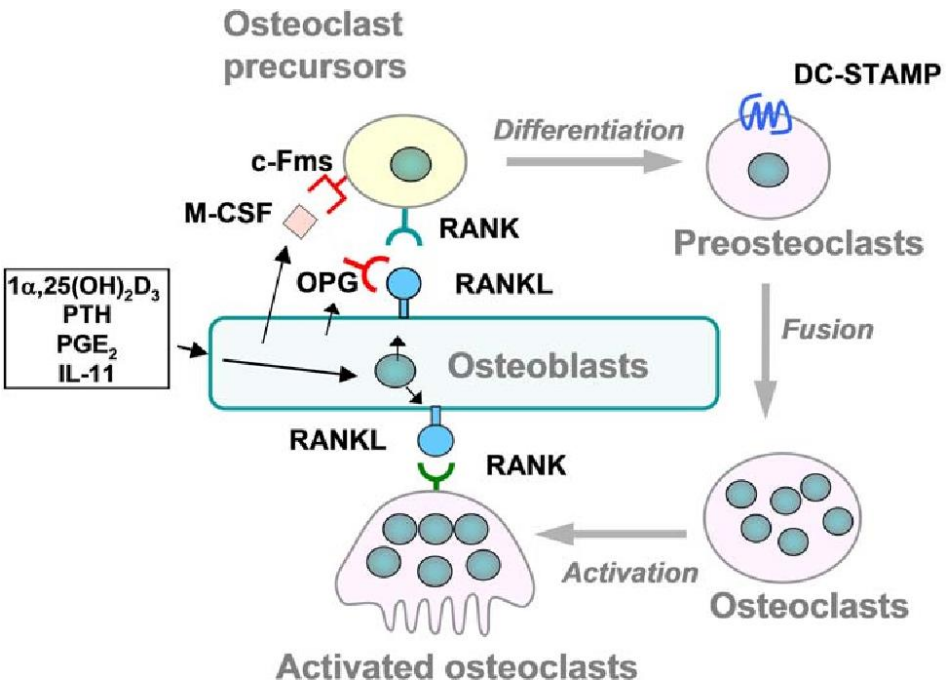

Figure 11. *Regulation of osteoclast differentiation and function by osteoblasts. Bone resorption-stimulating factors, such as 1α,25(OH)2D3, PTH, PGE2 and IL-11, act on osteoblasts to induce expression of RANKL. Osteoblasts constitutively produce MCSF. Osteoclast precursors of the monocyte-macrophage lineage express RANK and c-Fms. Osteoclast precursors recognize RANKL, expressed by osteoblasts in response to M-CSF, through cell-to-cell interaction, and differentiate into mononuclear preosteoclasts. Mononuclear preosteoclasts express DC-STAMP, which is essential for the fusion between preosteoclasts to form multinuclear osteoclasts. Mature osteoclasts also express RANK, which induces bone-resorption by mature osteoclasts binding RANKL.* *https://www.semanticscholar.org/paper/Regulatory-mechanism-of-osteoclastogenesis-by-RANKL-Takahashi-Maeda/33cdf06cc5a3b3bdbd772253077782669a5527db/figure/0*

2.3.4 The extracellular matrix

The bone extracellular matrix is similar to the other connectives but is mineralized. This matrix has an organic and an inorganic component.

The organic component is mainly composed of type I collagen and glycosaminoglycans, non-collagenic proteins and regulatory factors; the percentage of glycoproteins and proteoglycans does not exceed 5% while that of type I collagen reaches 95%, making the matrix acidophilic.

Among the glycoproteins there are osteopontin, osteonectin and sialoprotein 2, which are classified as adhesion proteins. Sialoproteins contain carbohydrate residues of sialic acid, and most adhesion proteins contain an amino acid sequence called RGD (Arg-Gly-Asp), which mediates the adhesion to the substrate of different cell types, including bone cells. The organic matrix is also rich in cytokines and growth factors, including BMPs (bone morphogenetic proteins), belonging to the TGF-β family. Most of these factors are produced by osteoblasts which release them in the form of inactive precursors, then activated by enzymatic modifications.

The inorganic component privides hardness and rigidity to the bone and is mainly made up of hydroxyapatite, with minor content of calcium carbonates and other minerals. Under the microscope, hydroxyapatite appears in the form of needle-like crystals deposited along the bundles of collagen fibrils. Hydroxyapatite crystals are very electrondense; they tend to arrange themselves parallel to each other and to the collagen microfibrils, of which they cover the surface. Observations conducted during the bone mineralization showed that calcium phosphate initially precipitates in the form of small amorphous aggregates. These initial structures are rapidly replaced by thin needle-like crystals arranged parallel to filamentous molecules of the fundamental substance called axial filaments (crystal ghost). These crystals grow assuming the typical appearance of hydroxyapatite crystals, occupying much of the space interposed between the collagen microfibrils and permeating the microfibrils themselves [28, 96-108].

Inside the matrix there are numerous secreted vesicles (matrix vesicles). These vesicles constitute the first mineralization sites of the matrix. Matrix vesicles are 100 nM diameter and are located at initial calcification sites in cartilage and bone. The biogenesis of these vesicles occurs by polarized budding from specific regions of the plasma membranes of chondrocytes and osteoblasts. The polarized release of the vesicles in specific areas of the

developing matrix induces the non-random distribution of mineralization. The first hydroxyapatite crystals form within the matrix vesicles near the inner surfaces of their lining membranes. The development of the mineral crystals is induced by the activity of alkaline phosphatase, adenosine triphosphatase and pyrophosphatase and by calcium-binding molecules including annexin I and phosphatidylserine. The second stage of biological mineralization begins with the release of crystals through the vesicle membrane, exposing the preformed hydroxyapatite crystals to the extracellular fluid. The extracellular fluid normally contains sufficient Ca2 + and PO4 (3-) to support continued crystal proliferation, with preformed crystals serving as templates for the formation of new crystals by a homologous nucleation process [167].

2.3.5 Ossification

There are two types of ossification: intramembranous or direct and endochondral or indirect. In both cases the bone originates from the mesenchyme but, while in the first case the bone is formed directly by differentiation of the pluripotent mesenchymal cells into osteoblasts, in the case of indirect ossification the bone is preceded by a cartilage rudiment which is then replaced by bone.

Intramembranous ossification occurs in the flat bones of the skull, most of the bones of the face and other flat bones. The process begins with the formation in the mesenchyme of ossification centers, areas where the tissue thickens, becomes more fibrous and vascularized and the cells actively proliferate. Some of them differentiate directly into osteoblasts and begin to secrete the osteoid, around which the cells are arranged in an epithelioid row to form the first primary trabeculae. The osteoid is initially non-mineralized, formed only by amorphous matrix and collagen. Subsequently it undergoes mineralization and further growth occurs by apposition: the osteoblasts around the primitive trabecula secrete new osteoid, which overlaps the previous one and the osteoblasts trapped in the matrix become osteocytes. As the bone grows, new osteoblasts differentiate from osteoprogenitor cells. The newly formed bone tissue is spongy and non-lamellar, with disordered collagen fibers oriented in all directions. Primary bone will then be resorbed and replaced by secondary lamellar bone during the subsequent remodeling phase. In regions where the bone will remain spongy, such as the diploe of the flat bones of the skull, the trabeculae gradually take on a lamellar organization and the surrounding connective tissue becomes hematopoietic. In

areas that will give rise to compact bone tissue, the initial trabeculae continue to thicken with a lamellar organization, entrapping vessels and nerves in the Havers and Volkmann canals. The surrounding connective tissue condenses to form the periosteum, which will continue to deposit new bone matrix for the entire duration of development.

The endochondral ossification concerns the bones at the base of the skull, spine, pelvis and limbs. During the embryonic stage a hyaline cartilage model is formed, which will be completely replaced by bone tissue with the exception of the articular cartilage. During pre-natal development, hyaline cartilage forms rudiments. At the level of the future diaphysis, within the third month of pregnancy, a first ossification center forms, in which the chondrocytes become hypertrophic and calcium deposition begins in the matrix; chondrocytes are no longer able to absorb nutrients due to the mineralization of the tissue and degenerate. The calcified cartilage matrix is subsequently partially resorbed by chondroclasts, multinuclear cells similar to osteoclasts. In the central part of the long bones the primitive medullary cavity forms, which is gradually invaded by blood vessels coming from the mesenchyme from which the osteoblastic cells derive. During bone growth, the remaning cartilaginous portions is resorbed by chondroclasts and osteoclasts, thus increasing the size of the primitive medullary cavity. Subsequently, two secondary ossification centers form at the level of the epiphyses, developing as described but with the medullary cavity containing primary trabeculae. The increase in length of long bones depends on the presence of the growth plate at the level of the metaphysis. Throughout the growth period, the epiphyseal disc continues to elongate due to growth on the side facing the epiphysis, while it is completely replaced by bone on the side facing the metaphysis. When the chondrocytes stop proliferating, ossification proceeds to completely replace the growth plate [166].

2.3.6 Bone remodeling

Bone remodeling requires the formation of anatomical structures called BMU (basic multicellular units). These units consist of a group of osteoclasts in the front, where a cutting cone is formed, and a group of osteoblasts in the back, where they form the closing cone[49,129].

Bone remodeling is regulated by various biochemical and mechanical factors and involves first bone resorption, carried out by osteoclasts, and then bone formation, carried out by osteoblasts. These cells cooperate closely in the BMUs. The bone remodeling cycle involves several sequential phases:

Resorption phase in which chemokines or chemotactic cytokines, including monocyte chemotactic protein-1 (MCP-1), are secreted by stromal cells or bone lining cells to stimulate the recruitment of osteoclast precursors. MCP-1 is produced in response to PTH or inflammatory cytokines, such as TNF-α. During resorption, enzymes released in the resorption lacuna digest the organic bone matrix releasing the breakdown products of type I collagen, including terminal peptide fragments of both ends of the collagen, called N-terminal (NTx), C-terminal (CTx) telopeptides, and a ring structure called deoxypyridinoline (DPD). During bone resorption, proteins released from the bone matrix, such as BMP, IGF and TGFβ, can be involved in the engagement of osteoblasts, which, in turn will perform bone formation.

Reversal phase: the regulatory mechanisms that stop osteoclastic activity are not well known but may be due to several events including osteoclast apoptosis. During the reversal phase, osteoclasts disappear and macrophage-like cells are seen on the bone surface, which may release factors that inhibit osteoclasts and stimulate osteoblasts.

Formation phase: the formation phase is characterized by the differentiation of osteoblast precursors, activated by clastokine molecules secreted by osteoclasts, including sphingosine-1-phosphate (S1P), and the polypeptide B homodimer of the growth factor derived from platelets (PDGF-BB). After the activation of the osteoblasts, these cells lay new bone matrix until the resorbed bone is completely replaced. At the end of the bone remodeling process, the osteoblasts trapped in the new bone matrix become osteocytes, while the others become lining cells on the bone surface, until a new remodeling cycle is activated. [48-52,80,114-117,130-157].

2.3.7 Metabolic and endocrine aspects of bone

The cooperation of various factors, mainly of endocrine and metabolic nature, is at the basis of the control of bone function [56,79].

These factors are:

PTH, produced by the parathyroid glands, which acts on osteoblasts by stimulating their proliferation and promoting their differentiation. In addition it also stimulates bone resorption inducing the production of RANKL by osteoblasts;

Calcitonin, produced by the C or parafollicular cells of the thyroid, acts on osteoclast inhibiting their function.

Growth hormone, produced by the pituitary gland, acts on the liver inducing the production of somatomedins, which stimulates the growth and metabolism of the chondrocytes of the growth plate proliferating layer, promoting the longitudinal growth of the long bones. Congenital defects in the production of growth hormone cause pituitary dwarfism, while its excess production induces gigantism during development and acromegalia in adults.

Thyroid hormones (Triiodothyronine and Tetraiodothyronine), produced by the follicular cells of the thyroid, promote cellular metabolism and therefore stimulate bone deposition and maturation. Abnormalities in the production of thyroid hormones during development lead to bone malformations of various degrees, up to thyroid dwarfism.

Sex hormones (estrogens and androgens) have a positive action on the differentiation and functional activity of osteoblasts, promoting bone formation. At the end of the growth, they also exert an inhibitory action on the proliferation of the chondrocytes of the growth cartilage, promoting the closure of the epiphyses and arresting bone growth. In particular, estrogens are involved in the processes of bone deposition: recent in vitro studies show that estrogens stimulate the proliferation of osteoblasts and the apoptosis of osteoclasts. This could explain why after the menopause there is a progressive reduction in bone mass with the possible development of osteoporosis.

Vitamin D promotes the differentiation of osteoblasts, stimulating the production of bone matrix and the deposition of calcium in the bones. In the intestine it promotes the absorption of calcium, while in the kidney it inhibits the excretion of this ion. A deficiency in vitamin D leads to defective mineralization of the bone called rickets if it arises during growth, and osteomalacia if occurs in adulthood.

Vitamin C is a water-soluble protein that acts as a coenzyme for collagen synthesis. It is a cofactor for osteoblasts engaged in the biosynthesis of matrix collagen. Vitamin C deficiencies lead to insufficient collagen production resulting in retarded growth and fracture repair.

Vitamin A is a fat-soluble protein acting on osteoblasts by reducing their proliferation and increasing the expression of vitamin D receptors. The deficiency of this vitamin causes delay in bone growth, while an excess causes the closure of the epiphyses with premature growth arrest.

Molecular oxygen plays an important role in bone formation not only because it is essential for oxidative phosphorylation but also as a stimulating factor of bone cells. In fact, in any type of ossification, the differentiation of mesenchymal cells into osteoprogenitor cells and then into osteoblasts occurs in conjunction with the genesis of new blood vessels, which can ensure a high partial pressure of oxygen in the sites where bone formation occurs.

Nitric oxide (NO) is a gaseous radical produced by many cells, including endothelial cells. NO is capable of inducing differentiation of osteoblasts. Therefore the role of the vascular endothelium in the osteogenesis processes is likely to be at least in part mediated through the release of NO [54,79,109-127].

Osteocytes are also involved in endocrine functions as they regulate the metabolism of phosphates through the production of FGF23, which acts on other organs, including the parathyroid gland and kidneys, to reduce the circulating levels of phosphates [158-165].

FGF23 is one of the most important endocrine factors secreted by osteocytes. Initially identified in the ventrolateral thalamic nucleus of the brain[166], FGF23 is mostly expressed in bone, predominantly by the osteocytes and highly elevated under hypophosphatemia. FGF23 is a 32 kDa member of the FGF protein family that binds FGF receptors (FGFR)[167-170]. Furthermore, FGF23 has effects on the musculoskeletal system and its increase in the circulation causes hypophosphatemic disorders.

The kidney is the main target of FGF23. In fact, signaling between bone and kidney plays a fundamental role in the maintenance of serum phosphate levels, which depends on the circulating levels of FGF23. FGF23 decreases the expression of sodium / phosphate cotransporters NaPi-IIa and NaPi-IIc in the kidney which serve for phosphate reabsorption. This causes an increase in urine phosphate. High levels of FGF23 regulate the expression of 1-α hydroxylase, which is required for the conversion of 25-hydroxyvitamin D to the active

metabolite of vitamin D, 1,25-dihydroxyvitamin D [1,25 (OH) 2D]. Reduction in the 1,25 (OH) 2D level causes decreased NaPi-IIb expression in the intestine, thereby reducing phosphate uptake and leading to hypophosphatemia [171,172].

2.3.8 Breast cancer bone metastases

Bone metastases are frequent complications of advanced malignancies. Along with lung and liver, bone is one of the most common sites of metastasis for breast cancers. Bone metastases can develop anywhere in the skeleton, but most commonly involve pelvis, spine, skull, legs and arms. The impact of skeletal involvement is variable and depends on several factors, including the number and localization of lesions. Metastases form when cancer cells migrate, through the blood or lymphatic flow, from the site of the primary tumor to other organs or tissues, giving rise to new neoplastic formations. According to the mechanism by which bone metastases interfere with normal bone resorption, they are classified into:

Osteolytic, characterized by the destruction of bone. This is the most common outcome in cancers that have spread to bone from breast [173,174].

Osteoblastic (or sclerotic), characterized by the deposition of new bone in response to the spread of cancer. This type of metastasis is not typical of breast cancer, most often occurring in prostate[175].

Mixed, characterized by both osteolytic and osteoblastic lesions [176].

Bone metastases mainly cause severe pain, reduced mobility, pathological fractures, spinal cord compression, medullary aplasia and hypercalmeia. Patients with localized spinal bone metastases may develop nerve damage, which can lead to paralysis of the legs and arms.

In some cases, bone metastasis are discovered before the primary tumor. In this circunstance it is possible to determine the site of origin of the disease by characterizing the type of cancer cells that make-up the metastasis. Importantly, many cancer patients present with bone and joint pain also as a side effect of some chemotherapy regimens.

Bone metastases from breast cancer negatively affect survival, mobility and general quality of life of patients and the molecular mechanisms involved in breast cancer cell metastasis colonization and proliferation in bone are complex and involve crosstalk between breast cancer cells and the bone microenvironment[177].

Breast cancer cells that extravasate from the circulation are recruited to the bone by the C-X-C motif chemokine 12 (CXCL12), which binds the CXCR4 receptor expressed by the tumor

cells. Here they establish a deleterious loop with the bone microenvironment called "vicious cycle" by which cancer cells and bone resident cells stimulate each other (**Figure 12**). Specifically, cancer cells interact with the bone marrow ECM and with the endosteal microenvironment, where they can become dormant. Outgrow of tumor cells is induced by many factors, both intrinsic and extrinsic. The former requires the expression of tumor cell specific genes and small non-coding RNAs, especially miRNAs, which induce their grow in the bone marrow permissive microenvironment. Among the genes highly expressed by tumor cells, some are osteoblast-specific (e.g. Osterix) and confer osteomimetic features to the tumor cells, facilitating their adaptation to the bone microenvironment.

An important trait of cancer cells seeding the bone microenvironment is their ability to activate osteoclastogenesis through the release of paracrine factors, including the Parathyroid Hormone-like Hormone (PTHLH) that induces upregulation of the pro-osteoclastogenic cytokine, RANKL, and downregulation of the anti-osteoclastogenic cytokine OPG. This leads to an increase of osteoclast number and exacerbation of bone resorption. Excess bone resorption releases large amounts of pro-tumoral factors from the bone matrix, including TGF-β, various EGFs and others, which perpetuate the vicious cycle further stimulating metastatic tumor growth (**Figure 12**) [178].

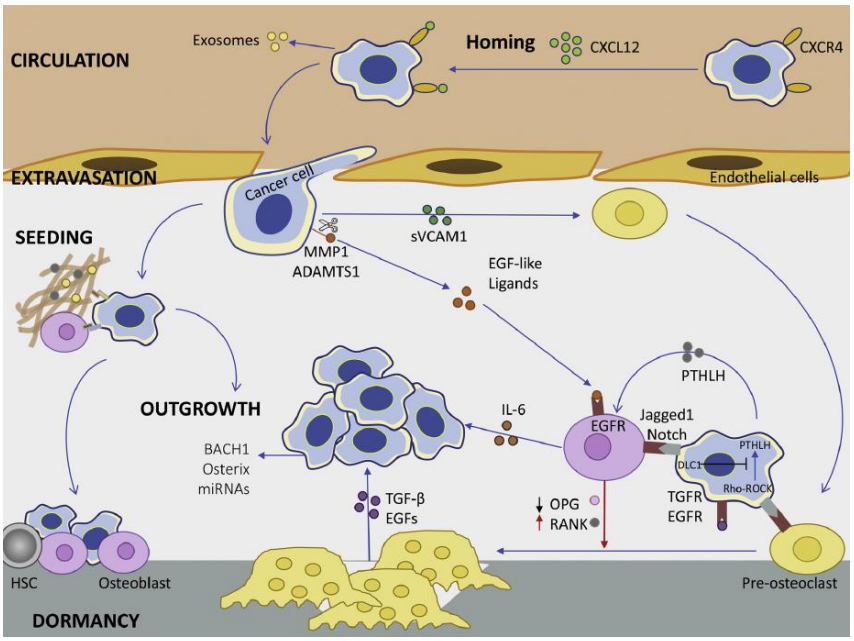

Figure 12. ***The molecular mechanism of breast cancer bone metastasis.*** *Bone cells recruit circulating CXCR4-positive cancer cell to the bone via the release of CXCL12. Tumor cells then seed into bone parenchyma by adhesion to the ECM and to the endosteal microenvironment. Metastasis expansion is then stimulated by the vicious cycle between the tumor cells and the bone microenvironment. This includes the release of tumor cell*

stimulating cytokines (e.g. PTHLH and IL-6) and pro-osteoclastogenic factors (e.g. RANKL). These latter promote bone resorption, enhancing the release and the activation of bone matrix-stored pro-tumoral factors (e.g TGF-β and EGFs) further stimulating metastasis outgrow. Liang Y, Zhang H, Song X, Yang Q. Metastatic heterogeneity of breast cancer: Molecular mechanism and potential therapeutic targets. Semin Cancer Biol. 2020 Feb;60:14-27. doi: 10.1016/j.semcancer.2019.08.012. Epub 2019 Aug 14. PMID: 31421262.

2.3.9 Breast cancer cell stemness and dormancy in the bone microenviroment

Breast cancer (BrCa) is the most common malignancy in women and has a 5-year survival rate of around 90%. About 20-45% develop tumor recurrence between 7 to 25 years after mastectomy and this correlates with the ability of a subpopulation of BrCa cells to survive in the host in a dormant state. Dormant cells are able to resist to conventional therapies and are difficult to detect, making their eradication difficult [119-183].

Cell dormancy is asymptomatic until dormant cells are reactivated, generating tumor relapse. Recent studies have shown that dormant breast cancer cells that develop new tumors, show cellular and molecular characteristics typical of stem cells [184,185].

Dormant BrCa cells colonize the bone marrow microenvironment and are held there in a quiescent status by specific cellular interactions with the endosteal niche, in which there is a specialized subtype of osteoblasts called SNO (Spindle-shape N-cadherin positive Osteblasts) expressing high level of N-cadherin [186-188].

According to recent studies, the endosteal niche is implicated in BrCa cell dormancy and studies conducted in our laboratory have shown that dormant BrCa cells expressing a high level of N-cadherin and Notch2 lodge in proximity to the endosteal niche enriched in SNOs. This work suggested that SNOs keep cancer cells dormant through a mechanism similar to long term Hematopietic Stem Cell (HSC) quiescence. Furthermore, this BrCa subpopulation exhibits haematopoietic stem cell-like characteristics suggesting HSC mimicry [189,190].

N-cadherin is an adhesion protein belonging to the cadherin class encoded by the *Cdh2* gene and mediating cell-cell adhesion. Generally, high expression of N-cadherin is associated with a reduction in cell proliferation that in osteoblasts is mediated by the activation of the Wnt3 signal, which inhibits the expression of cyclin D1 [191-197].

In cancer, N-cadherin plays different roles depending on the cellular context. In fact, in osteosarcoma, it acts as a tumor suppressor, while in other tumors it promotes invasion. At the level of epithelial breast cells, it interacts with the fibroblast growth factor receptor

(FGFR) and Rho GTPase, activating ERK signaling pathways and MMP9 expression, ultimately promoting cell motility [194, 198-201] .

In BrCa cells, N-cadherin is not regulated properly and Rho GTPase-induced cell motility increases. It also promotes the interaction between BrCa and endothelial cells to migrate and form metastsases. Furthermore, recent works have shown that N-cadherin, interacting with connexin 43 expressed by BrCa stem cells, mediates communication between the latter and bone marrow cells [202-204]. Although N-cadherin is expressed by HSCs and promotes LT-HSC engraftment and bone marrow quiescence [197,205-207], the role of N-cadherin in mediating BrCa-induced cell dormancy is not yet fully understood.

Based on these observation in our laboratory, we hypothesized that N-cadherin may play an important role in BrCa dormancy and HSC-like stemness, cooperating with Notch2 in the process of dormancy and subsequent ability to develop tumor. To demonstrate our hypothesis, for the part of the work discussed in this thesis, we used in vitro and in vivo models of murine BrCa cells, noting a negative role of N-cadherin in 4T1 cell stemness and HSC mimicry.

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CHAPTER 2

2 MATERIALS AND METHODS

2.1 Materials

Dulbecco's Modified Eagles's Medium (DMEM) (cat: ECB7501L) penicillin-streptomycin (cat: ECB3001D), Dulbecco phosphate buffered saline solution (DPBS) (cat: ECB4004L), Hanks balanced saline solution (cat: ECB4007L) and disposable plastic come from Euroclone (Milan, Italy). Bovine fetal serum (FBS) (cat:26140-079), ethylenediaminetetraacetic acid (EDTA) (cat: 15576-028), TRIzol® (Life Technologies, cat:15596018), N2 and B27 supplements (cat: 17502048, 17504044) and primers synthesis come from Invitrogen (Carlsbad, CA). cDNA Synthesis Kit (cat: K1622) comes from ThermoFisher. OneTaq® Hot Start 2X Master Mix (cat: M0484S) and Luna® Universal qPCR Master Mix (cat: M3003) come from New England BioLabs (Massachusetts, USA). Osteodec (cat: 05-03005E) and all reagents for histology come from BioOptica (Milan, Italy). SignalStain® Boost IHC Detection Reagent (cat:8125S (anti-mouse) 8114S (anti-rabies)) comes from Cell Signaling (Massachusetts, USA). All regents for Magnetic-Activated Cell Sorting (MACS) come from Miltenyi Biotec (Germany). The mouse plasmid Cdh2 CRISPR/Cas9 (cat: KN503008) comes from Origen (Rockville MD, USA).

2.2 4T1 Breast Cancer Cell Lines

For all experiments, we used 4T1 BrCa cell lines, parental or transfected with turboGFP. The cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM) with the addition of 1% glutamine, 1% penicillin-streptomycin and 10% FBS.

2.3 Magnetic-Activated Cell Sorting (MACS)

4T1 cells were sorted using the MACS method. The cells were detached with sorting buffer containing DPBS, 5% BSA and 0.5 M EDTA. The resuspended cells were incubated for 20 minutes at 4°C using an N-Cadherin biotinylated primary antibody. Subsequently, the cells

were incubated under the same conditions using streptavidin-conjugated magnetic microbeads and were eluted through the magnetic column to separate the antigen-depleted and antigen-enriched cell populations. The cells obtained from this procedure were used for RNA isolation, in vitro assays and in vivo experiments.

2.4 4T1-SNOs and NON SNOs co-culture system

Mouse primary calvarial osteoblasts were MACS-sorted into SNOs or NON-SNOs using anti-N-Cadherin-biotin antibody and Streptavidin-conjugated magnetic microbeads. $4T1^{Cdh2\text{-}KO\text{-}GFP}$ and $4T1^{Cdh2\text{-}Res\text{-}GFP}$ cells were seeded onto MACS-sorted SNOs and NON-SNOs and allowed to attach for 1 h at 37 °C, followed by extensive washing. The number of $4T1^{Cdh2\text{-}KO\text{-}GFP}$ and $4T1^{Cdh2\text{-}Res\text{-}GFP}$ cells was assessed after 1h of adhesion and after 24–48 h of co-culture. Subsequently $4T1^{Cdh2\text{-}KO\text{-}GFP}$ and $4T1^{Cdh2\text{-}Res\text{-}GFP}$ cells were sorted by MACS into $Notch2^{High}$ and $Notch2^{Low}$ were co-plated with SNOs or NON-SNOs. The number of $4T1^{Cdh2\text{-}KO\text{-}GFP}$ – $Notch2^{High}$, $4T1^{Cdh2\text{-}KO\text{-}GFP}$- $Notch2^{Low}$, $4T1^{Cdh2\text{-}Res\text{-}GFP}$- $Notch2^{High}$ and $4T1^{Cdh2\text{-}Res\text{-}GFP}$-$Notch2^{Low}$ cells was assessed after 1h of adhesion and after 24–48 h of co-culture.

2.5 RNA extraction and gene expression analysis

RNA extraction was performed using TRIzol® according to the manufacturer's directions. Subsequently, the quality of RNA was verified by electrophoretic stroke on the 1% agarose gel and quantified at the Nanodrop® using an absorbance of 260 nm wavelength. The purity of the RNA was assessed by measuring the 260/280 nm ratio for protein presence and the 260/230 ratio for phenols.

For gene expression analysis, 1µg of RNA was reverse-transcribed using the Revertaid First Strand cDNA kit. Semiquantitative PCR was then performed using the OneTaq® Hot Start 2X Master Mix, while Real-time PCR was performed using the Luna® Universal qPCR Master. The primer sequences used for gene expression analysis are listed in **Table 1**.

Gapdh	Fw: TGTGAGGGAGATGCTCAGTG
	Rv: TGTTCCTACCCCCAATGTGT
Cdh2	Fw: ATGACGTCCACCCTGTTCTC
	Rv: CTGGGACGTATGTGATGACG

Notch2	Fw: CCAGGTTATTGCACGTTCCT
	Rv: ACCCTTGTATGCACGGAGTC
Cxcr4	Fw: TTGCCGACTATGCCAGTCAAG
	Rv: TCCAACAAGGAACCCTGCTTC
CyclinD1	Fw: TCAAGTGTGACCCGGACTG
	Rv: ATGTCCACATCTCGCACGTC

Tabel 1: *Primer sequences*

2.6 Flow cytometry

Cells were detached with sorting buffer containing DPBS, 5% BSA and 0.5 M of EDTA. Resuspended cells were incubated with primary antibody against Notch2 and N-Cadherin for 1h at 4°C. Secondary incubation was performed using fluorochrome-conjugated secondary antibodies, then cells were analysed by the FACS Melody® (BD) and FlowJO software. Unmarked cells were used to set the laser for the fluorescence threshold.

For the analysis of cells sorted from genetically modified 4T1 ($4T1^{Cdh2KO\text{-}GFP}$ and the $4T1^{Cdh2Res\text{-}GFP}$), the cell gating was performed using the GFP fluorescence.

2.7 Animals

For the in vivo experiment we used 4-week-old immunocompetent Balb/c female mice. Procedures involving animals and their care were conducted in accordance with national and international laws and policies (Council Directive of the European Economic Community 86/609, OJ L 358, 1, 12 December 1987; Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana 40, 18 February 1992, National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health, 8th edition, 2011). The procedures have been approved by the Institutional Ethical College of the University of L'Aquila and the Ministry of Health (Authorizations No. 270/2018-PR and 1151/2020-PR). The study was conducted according to the requirements of Animal Research Reporting In Vivo Experiments (ARRIVE).

2.8 Intratibial injection of 4T1$^{N\text{-}CadherinHigh}$ and 4T1$^{N\text{-}CadherinLow}$

The 4T1 cells sorted for N-Cadherin (4T1$^{N\text{-}CadherinHigh}$ and 4T1$^{N\text{-}CadherinLow}$) were injected into the left tibia of 4-week-old immunocompetent female Balb/C mice (1×10^4 cells/0.01 ml PBS) anesthetizing them by intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. Animals were monitored daily for food intake, behavior and survival. Mice were subsequently sacrificed according to the timing of the experimental design.

2.9 Micro-computed tomography (µCT) analysis

Left tibias harvested from the tumour cell-injected mice were fixed in 4% formaldehyde for 48 hours and then scanned by the µCT SkyScan 1174. The scan was performed with a 9.80 µm resolution using the X-ray voltage of 50 kV. The Skyscan NRecon software was used to reconstruct the images using a modified Feldkamp algorithm. Three-dimensional (3D) analysis was carried out employing a Marching Cubes type model with a rendered surface. The cortical bone parameters were calculated on 300 consecutive slides starting from 100 µm below the growth plate, where the osteolytic lesions were located. Pratt's algorithm was adopted to take 2D measurements. Threshold values were applied for segmenting trabecular bone. Bone trabecular and cortical variables were measured according to Bouxsein et al [1].

2.10 Histology

Left tibias were decalcified for 48 hours in Osteodec and then embedded in paraffin. Livers were fixed in 4% paraformaldehyde and embedded in paraffin. Microtome sectioning was used to obtain tissue slices of 5-µm thickness. Liver sections were stained with hematoxylin and eosin while tibia sections were also processed for immunohistochemistry or immunofluorescence staining.

2.11 Immunohistochemistry and immunofluorescence

For immunohistochemistry, mouse tibia sections were deparaffined and incubated with 0.07M citrate buffer (pH 6) for 30 min at 96°C and for 10 minutes at room temperature. The blocking was made with 3% H_2O_2 and 5% BSA. Then samples were incubated overnight at 4 °C with primary antibodies against N-Cadherin and pan-Cytokeratin AE1/AE3. The staining signals were revealed using the SignalStain® Boost IHC Detection Reagent (HPR rabbit or

mouse). Sections were counterstained using Gill's No.3 hematoxylin for 10 seconds. Positive and negative controls were performed in parallel.

For immunofluorescence, tissue sections or fixed cells (4% paraformaldehyde) were incubated with primary antibodies against pan-Cytokeratin AE1/AE3, N-Cadherin or Ki67, either singularly or in combinations. Primary antibody incubations were carried out at room temperature for 1 h, then overnight at 4 °C. Then, incubations with secondary antibodies conjugated with AlexaFluor 488 or 594 were performed for 1 h at room temperature. Nuc-Spot® or DAPI were used to stain the nuclei. The supplier, product code and dilution of the primary and secondary antibodies used for the analyzes are listed in **Table 2.**

Antibody	Diluition	Species	Cat #	Company
N-Cadherin	(IF)(IHC)1:100	Mouse	NBP1-48309	Novus
N-Cadherin-PE	(FACS)1:50	Mouse	NBP1-48309PE	Novus
Pan-Cytokeratin (AE1/AE3)	(IHC)(IF)1:100	Mouse	sc-81714	Santa Cruz Biotechnology
Ki67	(IF)1:500	Rabbit	MA5-14520	ThermoFisher
Notch2	(FACS)1:50	Rabbit	sc5545	Santa Cruz Biotechnology
AlexaFluor488 anti-mouse	(IF)1:500	Goat	A11001	Invitrogen
AlexaFluor488 anti-rabbit	(FACS)(IF)1:500	Goat	A11008	Invitrogen
AlexaFluor594 anti-mouse	(FACS)1:500	Goat	A11005	Invitrogen
AlexaFluor594 anti-rabbit	(IF)1:500	Goat	A11037	Invitrogen

Tabel 2*: Antibody information*

2.12 Histomorphometry

Endosteal niche colonization analysis was performed counting the number of cytokeratin positive cells in proximity of the endosteum (4mm^2 in area, 50 µm away from the growth plate and 20 µm away from the endocortical surface ^{2}), and their distance from the endosteal surface was measured. All histomorphometric analyses were performed using the software Fiji® by ImageJ.

2.13 Primary and secondary mammospheres

Primary mammosphere assays were performed using $8X10^3$ suspended cells seeded in non-adhesive Petri dishes with serum-free DMEM, supplemented with 1% N2, 1% B27, 1% penicillin/streptomycin and 1% L-glutamine. They were incubated for 6 days in a humidified CO2 incubator. For secondary mammospheres, the primary mammospheres were disaggregated using trypsin to obtain single-cell suspensions and cultured again under the same conditions. Imaging for the analysis was performed using the SXView Software. Mammosphere's volume was calculated using the formula V=3/4πr3.

2.14 TaqMan™ Array Mouse Stem Cell Pluripotency

Two micrograms of total RNA isolated from $4T1^{Cdh2KO\text{-}GFP}$ and the $4T1^{Cdh2Res\text{-}GFP}$ cells was retrotranscribed into cDNA using Revertaid First Strand cDNA Synthesis. Then 200 ng of cDNA per condition were loaded in the TaqMan™ Array Mouse Stem Cell Pluripotency plates (Applied Biosystem cat: 4414080) containing specific Fluorescein Amidite (FAM)-tagged probes for mRNAs involved in mouse stemness and pluripotency. Real time PCR was performed using the TaqMan™ Fast Advanced Master Mix (Applied Biosystem cat: 4444556). Gene expression was normalized by mouse *Gapdh* and expressed as fold *vs*. the 4T1*Cdh2KO-GFP*. The real time RT-PCR arrays were run in triplicate.

2.15 Statistical analysis

Results are expressed as mean ± Standard Deviation (SD). Sample size is indicated in the figure legends. Groups' comparisons were performed carrying out independent samples Student's *t* tests and non-linear regression, fitting with F-test when dealing with continuous parameters. Data from RNA dSeq were analysed using an False Discovery Rate (FDR)-adjusted p-value. To assess distributional pattern of the BrCa cells in the bone marrow in relation to the endosteal surface we used a cumulative frequency distribution with Gaussian regression and F-test.

CHAPTER 3

3 RESULTS

3.1 Preliminary data: genetically modified 4T1 breast cancer cells.

We evaluated the expression of N-cadherin on parental 4T1 cells by cytofluorimetry, revealing the presence of a subpopulation of 4T1 N-cadherin^{High} (**Figure 1a**). N-cadherin expression was knocked-out by CRISPR / Cas9 technique through the use of plasmids carrying two gRNAs targeting the N-cadherin gene co-transfected with a linear donor containing genes encoding GFP and resistance tags to puromycin (**Figure 1b**). Through this system we obtained two populations of 4T1 knock-out cells for N-cadherin (4T1$^{Cdh2KO1\text{-}GFP}$ and 4T1$^{Cdh2KO2\text{-}GFP}$) and using a pair of puromycin specific primers we confirmed the success of the transfection protocol by RT-PCR and subsequent electrophoretic run (**Figure 1c**). Untransfected 4T1 cells were used as controls for our analyses. Furthermore, cytofluorimetric analyses also confirmed the absence of N-cadherin expression in GFP-positive cells (knock-out cells) (**Figure 1d-g**). After transfection, the 4T1$^{Cdh2KO1\text{-}GFP}$ and the 4T1$^{Cdh2KO2\text{-}GFP}$ were sorted by FACS using the GFP tag and subsequently, to generate a rescued model, the FACS-sorted 4T1$^{Cdh2KO1\text{-}GFP}$ and the FACS-sorted 4T1$^{Cdh2KO2\text{-}GFP}$ were trasfected by Cdh2-turboGFP expression vector obtaining the 4T1$^{Cdh2Res1\text{-}GFP}$ and the 4T1$^{Cdh2Res2\text{-}GFP}$, where the N-cadherin expression was restored (**Figure 1h**). Cells transfected with the empty turboGFP vector (4T1$^{Cdh2KO1\text{-}GFP}$ and 4T1$^{Cdh2KO2\text{-}GFP}$) were used as controls. Finally, 4T1$^{Cdh2Res2\text{-}GFP}$ showing unstable Cdh2 overexpression and related control knock-out cells (4T1$^{Cdh2KO2\text{-}GFP}$) were excluded from the analysis (**Figure 1i**). Conversely, 4T1$^{Cdh2KO1\text{-}GFP}$ and 4T1$^{Cdh2Res1\text{-}GFP}$ were used for the experiments and were identified as 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{Cdh2Res\text{-}GFP}$

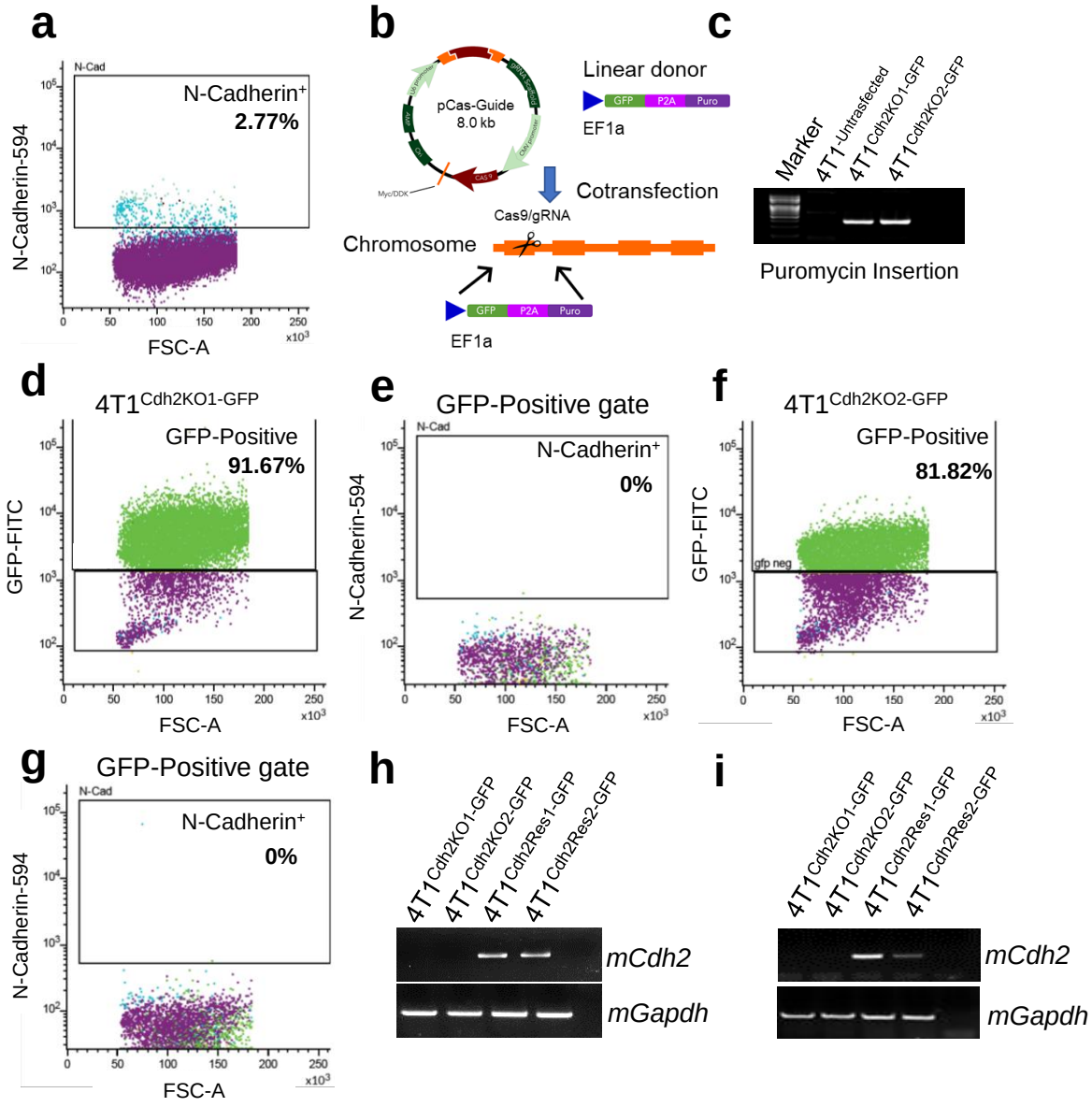
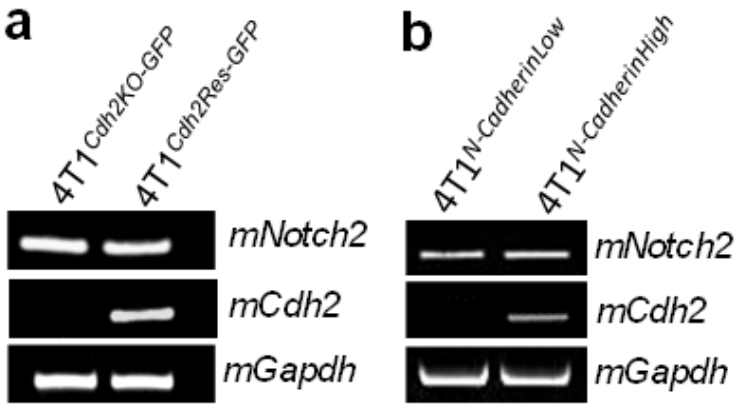


Figure 1 Genetically modified 4T1: Gene editing using CRISPR / Cas9: *(**a**) Intact 4T1 mouse BrCa cells were stained using a specific antibody for the N-Cadherin and the percentage of positive cells was assessed by flow cytometry. (**b**) Schematic representation of the CRISPR/Cas9 strategy used to generate the N-Cadherin Knock-Out (KO) 4T1 cells. (**c**) The integration of linear donor was evaluated in the 4T1$^{Cdh2KO1-GFP}$ and 4T1$^{Cdh2KO2-GFP}$ by RT-PCR using a specific primer pair for puromycin. (**d-g**) 4T1$^{Cdh2KO1-GFP}$ and 4T1$^{Cdh2KO2-GFP}$ were stained with a specific antibody for N-Cadherin and the percentage of the positive cells was measured in the GFP-positive gate to validate the success of the CRISPR/cas9 gene editing. (**h-i**) 4T1$^{Cdh2KO1-GFP}$ and $^{4T1Cdh2KO2-GFP}$ were stably transfected using Cdh2-turboGFP expression vector (4T1^{Cdh2Res1tGFP} and $^{4T1Cdh2Res2-GFP}$) or with the empty-turboGFP vector (4T1$^{Cdh2KO1-GFP}$ and 4T1$^{Cdh2KO2-GFP}$). The restored N-Cadherin expression was analysed by RT-PCR using specific primers for the mouse Cdh2. Gene expression was normalized by mouse Gapdh. Pictures are representative of 3 experiment or 3 cell preparations.*

3.2 Co-expression of N-Cadherin and Notch2 in 4T1

Our previous work has shown that dormant breast cancer cells express high levels of the Notch2 protein (Notch2^{HIGH}) which mediates the interaction of cancer cells with the endosteal niche in the bone microenvironment [2]. In addition, the Notch2^{HIGH} cells presented in the endosteal niche were also positive for the expression of N-Cadherin. Therefore, analyses were performed by semi-quantitative RT-PCR to evaluate the expression of Notch2 in both 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{Cdh2Res\text{-}GFP}$ cells (**Figure 2a**) and 4T1$^{N\text{-}CadherinLow}$ and 4T1$^{N\text{-}CadherinHigh}$ MACS-sorted cells (**Figure 2b**), finding that Notch2 expression is similar in the presence or absence of N-Cadherin. Densitometric analysis of mNotch2 and mCdh2 expression, confirmed the results obtained by semi-quantitative RT-PCR and electrophoretic run (**Figure 2c,d**).



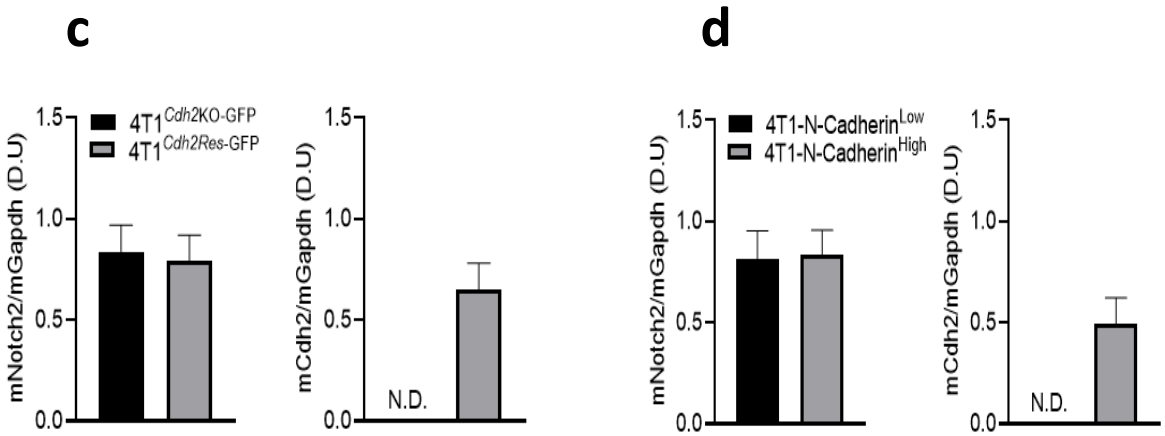


Figure 2 Notch2 and N-Cadherin co-expression analysis in 4T1 cells*. Semiquantitative RT- PCR to assess the expression of the indicated genes in (**a**) 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{Cdh2Res\text{-}GFP}$ or (**b**) N-Cadherin^{Low} and N-Cadherin^{High} MACS-sorted 4T1 cells. Mouse Gapdh was used to normalise gene expression. Pictures are representative of 3 independent cell preparations or cell sorting. Densitometries of mNotch2 and mCdh2 DNA bands in (**c**) 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{Cdh2Res\text{-}GFP}$ or (**d**) N-Cadherin^{Low} and N-Cadherin^{High} MACS-sorted 4T1 cells, normalized against the densitometry of the mGapdh.*

3.3 N-Cadherin mediates BrCa cell adhesion on SNOs in vitro

The role of N-cadherin in SNO-induced BrCa dormancy was evaluated using 4T1$^{Cdh2\text{-}KO}$ and 4T1$^{Cdh2\text{-}Res}$ cells. SNO cells were shown to induce cell dormancy in unsorted parental 4T1 cells. No significant differences were instead observed between the number of 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ cells co-cultured with SNOs or NON-SNOs after one hour (**Figure 3a**). Furthermore no differences were found in the number of 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ cells co-cultured with SNOs or NON-SNOs for 48 hours (**Figure 3b**). The number of 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ cells plated on SNO was instead greater after 1 hour of co-plating (**Figure 3c**), but their number increased less over the next 48 hours compared to 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ (**Figure 3d**).

According to the observation that the Notch2 expression level was independent of N-cadherin expression, when 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ and 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ cells sorted by MACS into Notch2^{High} and Notch2^{Low} were co-plated with SNOs or NON-SNOs, we confirmed that the slower growth rate of 4T1 cells was related to the presence of N-Cadherin and not to the Notch2 status. In fact, in all tested conditions, the 4T1$^{Cdh2Res\text{-}GFP}$-Notch2^{High} and 4T1$^{Cdh2Res\text{-}GFP}$-Notch2^{Low} cultures showed the lowest number of cells per plate compared to 4T1$^{Cdh2KO\text{-}GFP}$-Notch2^{High} and 4T1$^{Cdh2KO\text{-}GFP}$ Notch2^{Low} cells plated on SNOs on NON-SNOs for 48 h (**Figure 3e, f**). These results indicate that the Notch2 / N-Cadherin axis is not involved in SNO-mediated cell dormancy, while N-Cadherin strongly reduces the proliferation capacity of 4T1 cells on both SNOs and NON-SNOs.

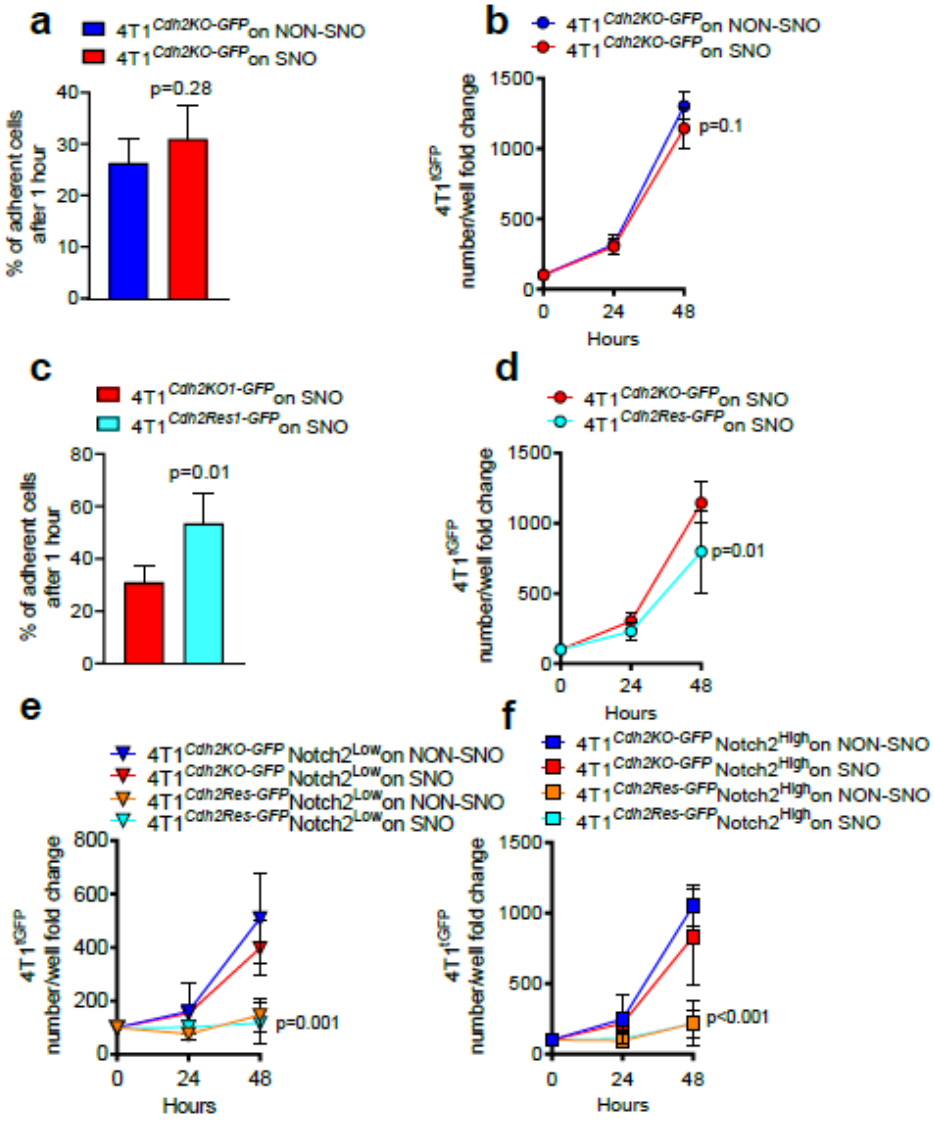
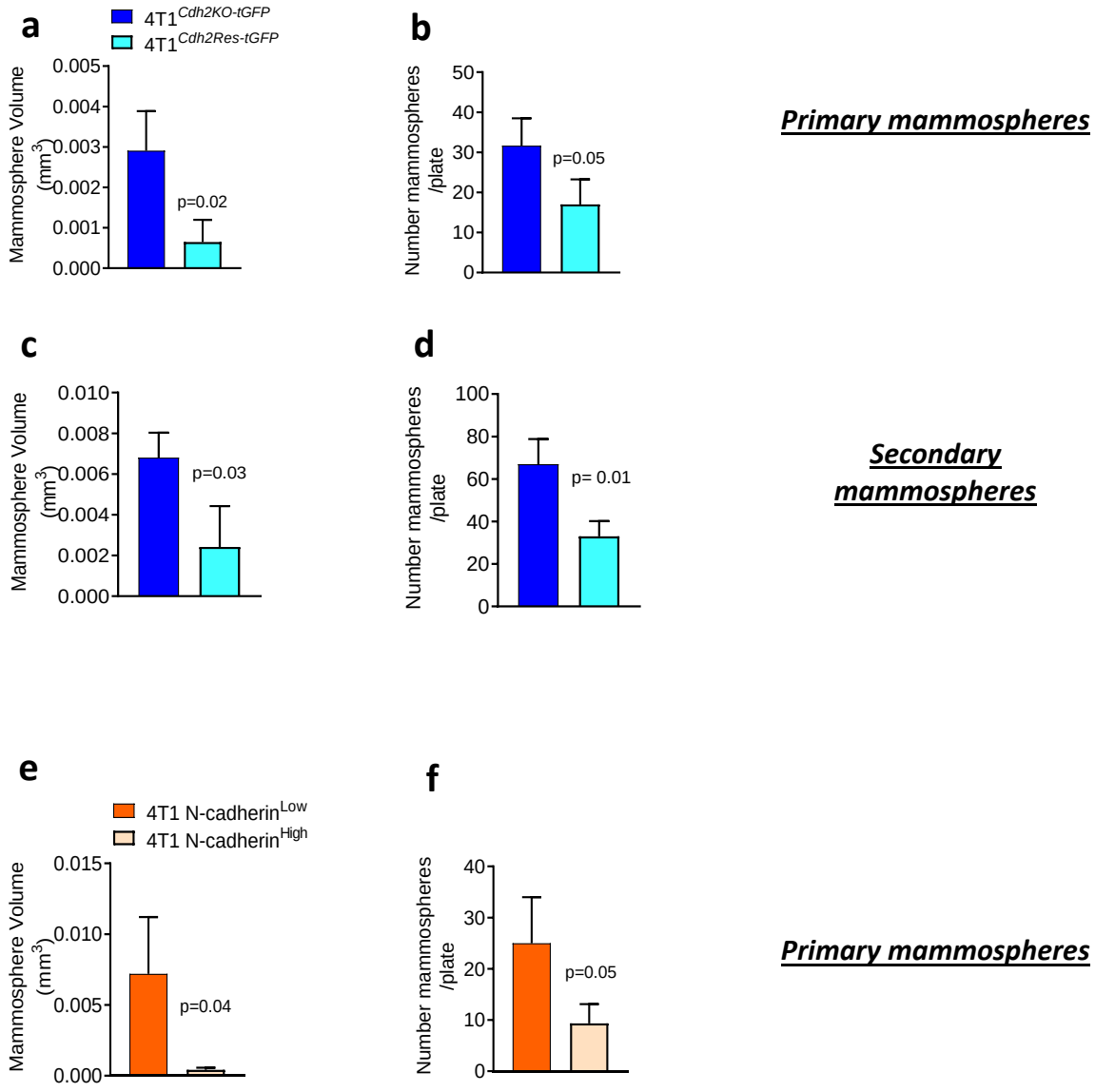


Figure 3 Role of N-Cadherin and Notch2 in 4T1-SNO interaction in vitro. *(**a**) 4T1GFP cells knock-out for N-Cadherin expression (4T1$^{Cdh2\text{-}KO\text{-}GFP}$) were seeded onto MACS-sorted SNOs and NON-SNOs and allowed to attach for 1 h at 37 °C, followed by extensive washing. The number of 4T1GFP cells was assessed after 1h of adhesion and (**b**) after 24–48 h of co-culture. (**c**) 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ cells in which the N-Cadherin expression was rescued (4T1$^{Cdh2\text{-}Res\text{-}GFP}$) were seeded onto MACS-sorted SNOs and allowed to attach for 1 h at 37 °C, followed by extensive washing. The number of 4T1GFP cells was assessed after 1h of adhesion and (**d**) after 24–48 h of co-culture. (**e**) Notch2^{High} and (**f**) Notch2^{Low} 4T1$^{Cdh2\text{-}KO\text{-}GFP}$ and 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ cells were seeded onto MACS-sorted SNOs and NON-SNOs cultured for 24 h and allowed to attach for 1 h at 37°C, followed by extensive washing. The number of 4T1GFP cells was assessed after 24–48 h of co-culture. (**a,d-f**) Cell number per well was normalized for time 0 (number of cells after 1h of adhesion). Data are the mean±SD of 4-5 independent cell preparations. Statistical analysis: (**a,c**) Student's t test, (**b,d-f**) Non-linear regression fitting and F-test.*

3.4 The role of N-Cadherin in HSC mimicry and stemness

Both 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ and 4T1$^{N\text{-}CadherinHigh}$ showed a lower ability to form primary and secondary mammospheres than 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{N\text{-}CadherinLow}$. In addition, the primary and secondary mammospheres of the 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ and 4T1$^{N\text{-}CadherinHigh}$ cells were smaller and less numerous than primary and secondary mammospheres generated by the 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{N\text{-}CadherinLow}$ cells (**Figure 4 a-h**). In line with this finding, Cxcr4 expression was also lower in 4T1$^{Cdh2Res\text{-}GFP}$ cells than in 4T1$^{Cdh2KO\text{-}GFP}$ cells (**Figure 5a**) while no significant differences were found in 4T1$^{N\text{-}CadherinHigh}$ cells compared to 4T1$^{N\text{-}CadherinLow}$ cells (**Figure 5b**). Finally, cell proliferation remained unchanged in both 4T1$^{Cdh2\text{-}Res\text{-}GFP}$ and 4T1$^{N\text{-}CadherinHigh}$ cells compared to 4T1$^{Cdh2KO\text{-}GFP}$ and 4T1$^{N\text{-}CadherinLow}$ cells (**Figure 5 c-e**).



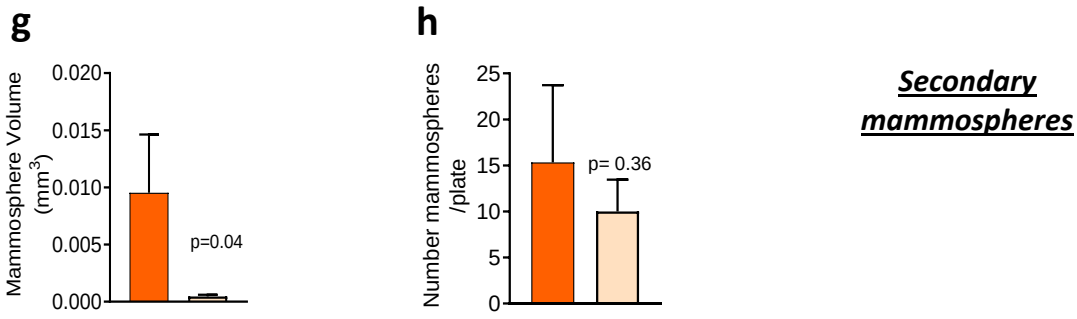


Figure 4 Effect of N-Cadherin expression of stemness of 4T1 BrCa cells. *(**a**) Volume and (**b**) number of primary mammospheres obtained from 4T1$^{Cdh2\text{-}KO}$ and 4T1$^{Cdh2\text{-}Res}$ GFP cells. (**c**) Volume and (**d**) number of secondary mammospheres obtained after trypsinization and re-plating of single cells harvested from primary 4T1 mammospheres. (**e**) Volume and (**f**) number of primary mammospheres obtained from MACS-sorted N-Cadherin^{High} and N-Cadherin^{Low} 4T1 cells. (**g**) Volume and (**h**) number of secondary mammospheres obtained after trypsinization and re-plating of single cells harvested from primary 4T1 mammospheres. Data are the mean±SD of 3 independent cell preparations. Statistical analysis: Student's t test.*

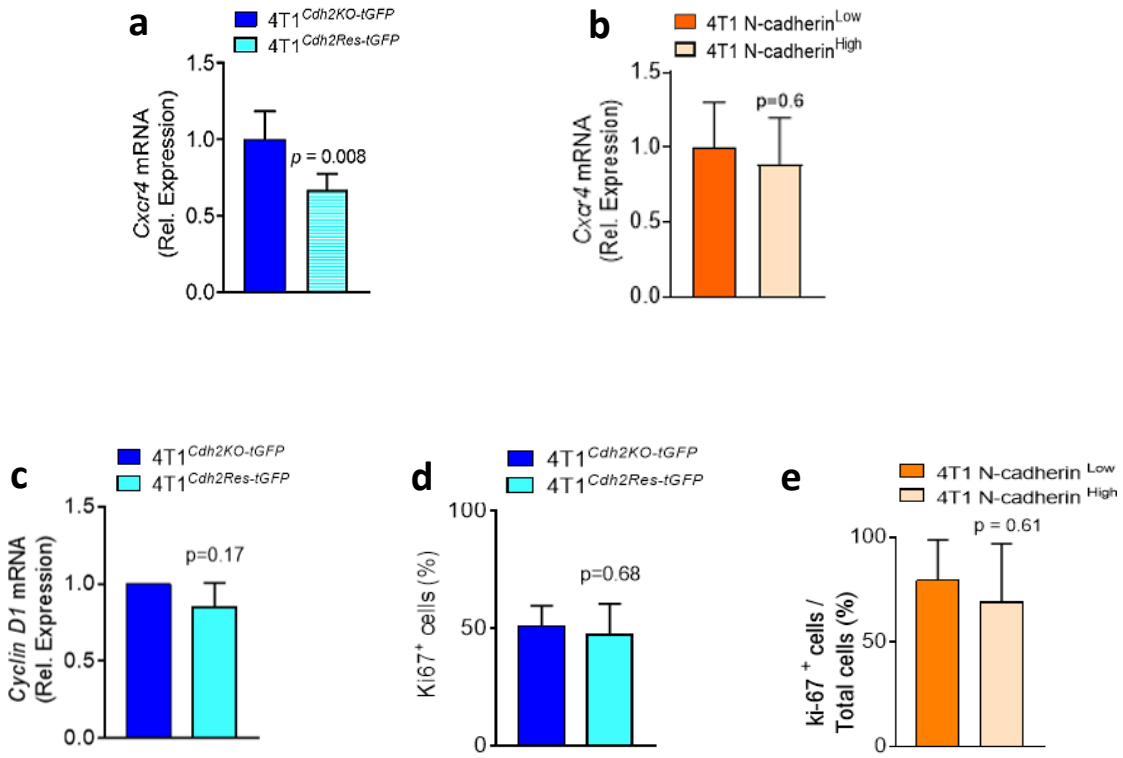
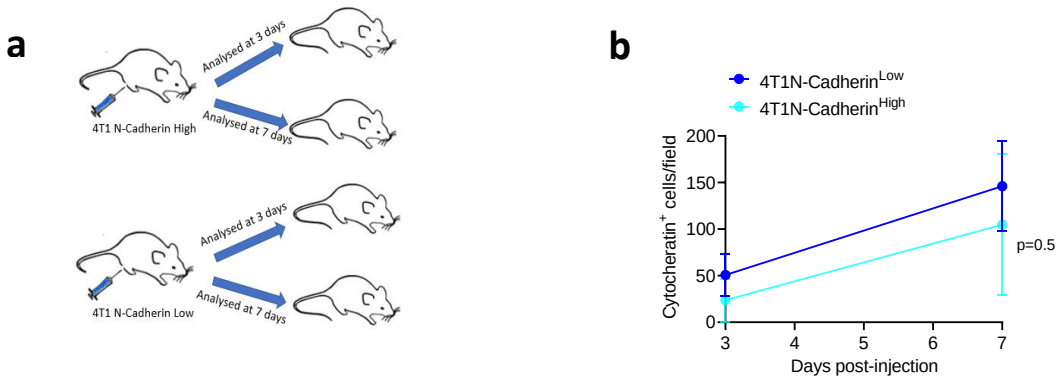


Figure 5 HSC mimicry and proliferation of 4T1 BrCa cells. *(**a**) Expression of the HSC marker Cxcr4 in 4T1$^{Cdh2\text{-}KO}$ and 4T1$^{Cdh2\text{-}Res}$ cells. Gene expression was normalised by mouse Gapdh. (**b**) Expression of the HSC marker Cxcr4 in 4T1$^{N\text{-}CadherinHigh}$ and 4T1$^{N\text{-}CadherinLow}$ cells. Gene expression was normalised by mouse Gapdh. (**c**) Cells*

*proliferation assessed in the 4T1$^{Cdh2\text{-}KO}$ and 4T1$^{Cdh2\text{-}Res}$ cells by Real time RT-PCR to evaluate the expression of Cyclin D1 and (**d**) by immunofluorescence using a specific antibody for the Ki-67. (**e**) Cells proliferation analyses on 4T1$^{N\text{-}CadherinHigh}$ e 4T1$^{N\text{-}CadherinLow}$ using a specific antibody for Ki-67. Data are the mean±SD of 3 independent cell preparations. Statistical analysis: Student's t test.*

3.5 Role of N-Cadherin in in vivo tumor growth and endosteal niche colonization

To evaluate the effect of N-Cadherin expression on 4T1 cells in vivo, we analyzed the effect of N-cadherin on tumor growth in the bone microenvironment and on the ability of cancer cells to colonize the endosteal niche. Four-week-old immunocompetent Balb/c femal mice were intratibially injected with 1x10^4 4T1$^{\text{N-Cadherin High}}$ and 4T1$^{\text{N-Cadherin Low}}$ cells (**Figure 6a**). Mice were sacrificed after three and seven days and tibias were subjected to immunohistochemical analysis to detect cancer cells using cytokeratins as marker (**Figure 6 e,f**). There were no significant differences in the number of cells in the tibias of mice injected with 4T1$^{\text{N-Caderin High}}$ and 4T1$^{\text{N-Cadherin Low}}$ (**Figure 6b**). Similarly, the analysis showed the absence of differences between 4T1$^{\text{N-Caderin High}}$ and 4T1$^{\text{N-Caderin Low}}$ cells in terms of endosteal surface colonization (**Figure 6 c,d**). Thus it can be concluded that the expression of N-Cadherin in 4T1 cells does not affect the proliferation and aggressiveness of these cells in vivo and has no effect on their ability to colonize the endosteal niche.



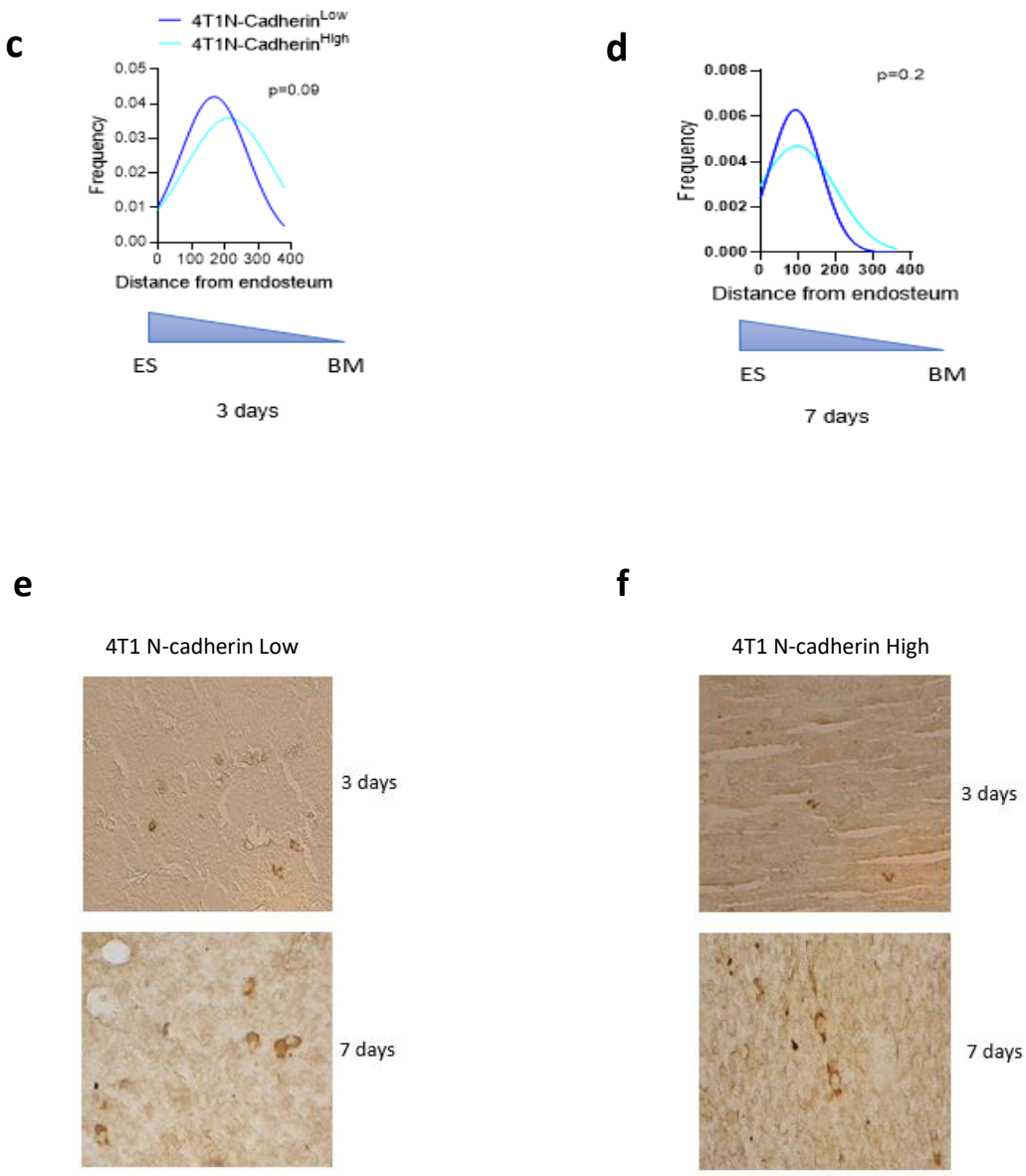


Figure 6 Role of N-Cadherin in in vivo tumour growth and endosteal niche colonization. *(**a**)Four weeks old Balb-c immunocompetent female mice were intratibially injected with 1x10^4 4T1 cells MACS-sorted into N-Cadherin^{High} and N-Cadherin^{Low} subpopulations and sacrificed after 3 and 7 days post-injection. Paraffin-embedded tibias harvested from the injected mice were analysed by immunohistochemistry using cytokeratins as marker to visualise the tumour cells in the bone tissue (**b**). Total number of cytokeratin positive 4T1 cells. (**c**) Cell distribution in relation to the endosteum measured after 3 and (**d**) 7 days. (**e,f**) Pictures are representative of 3 independent immunohistochemestry experiments. Data are the mean±SD of 3 mice per group. Statistical analysis: (b) Student's t test; (c-d) Gaussian curve regression fitting and F-test.*

CHAPTER 4

DISCUSSION

Tumor dormancy is a complex process involving several molecular pathways and cell-cell interactions according to cancer type and microenvironmental signaling[3]. Previously, in our laboratory, we have shown that BrCa dormant cells interact with a specific subpopulation of osteoblasts called SNOs (Spindle-shaped N-CadherinHigh osteoblasts) which induce cell cycle arrest of dormant BrCa cells through the Notch2 pathway. In previous work we have also shown that dormant BrCa cells compete with HSCs for bone marrow engraftment and intraosseous niche colonization due to their HSC mimicry characteristics.

The role of N-cadherin in tumors is very complex and depends on the cellular context and type[4]. In fact, some studies have shown that N-cadherin can induce or suppress development depending on the type of tumor [5-7]. In the context of BrCa, the role of N-Cadherin is still poorly understood, probably because its function is closely related to cellular and microenvironmental conditions. According to some data in the literature it has been shown that the expression of N-cadherin stimulates motility by improving their metastatic spread[8,9,10,11]. In contrast, a recent article has shown that N-cadherin determines the dormancy of human BrCa in the bone marrow in association with connexin 43 [10].

Our work was carried out using BrCa mouse cells called 4T1. We have used this cell line because they are able to establish syngenic interactions with mouse bone cells in vitro and in vivo and require N-cadherin interaction with SNOs in vitro. In fact, the lack of N-cadherin restored the 4T1 cell proliferation induced by SNOs while the re-expression of N-cadherin enhanced the adhesion of 4T1 cells to the SNOs, slowing down their proliferation.

Furthermore, by analyzing the stem characteristics of 4T1, we observed that the high expression of N-Cadherin was associated with a low ability to form mammospheres and a low expression of Cxcr4. These analyses were conducted using both the genetically modified N-cadherin KO and Rescued 4T1 cells and the 4T1 sorted cells for N-Cadherin High and Low. Other HSC markers including Tie-2 and Sca-1 were not expressed by 4T1 cells in our experimental conditions.

In this part of the work, carried out on the mouse cell line 4T1, we have defined the role of N-cadherin in the dormancy and in the stemness of BrCa, noting differences between human and mouse cell lines. For example, the results obtained from the in vitro characterization related to mammospheres showed that, in contrast with the mouse cell line, in the human cell line MDA-MB-231 high expression of N-Cadherin was clearly associated with cellular dormancy in partnership with Notch2 [2].

Overall, we can conclude that N-cadherin could play a role in inducing and maintaining tumor cell dormancy in a BrCa cell-type dipendent manner. Furthermore, we have shown that N-cadherin is mainly involved in anchoring tumor cells in the endosteal niche, rather than inhibiting cell proliferation. Therefore, we can hypothesize that N-cadherin targeting could be a potential co-adjuvant therapy to be administered in combination with anticancer drugs to prevent engraftment and dormancy of BrCa cells deposited in the endosteal niche. With this work we have added a new piece to this complex puzzle that shows a potential role of N-cadherin in SNO-mediated BrCa cell dormancy and cell stemness.

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CHAPTER 6

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5) QUALIFIED TO PRACTICE AS A BIOLOGIST.

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- 1 ORAL PRESENTATION OF THE PHD PROJECT AT THE ECTS VIRTUAL MASTERCLASS 2020
- PARTECIPATION AT 48th EUROPEAN CALCIFIED TISSUE SOCIETY CONGRESS 6-8 MAGGIO 2021
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- LABORATORY WASTE MANAGEMENT ACCORDING TO CURRENT REGULATION
- DUCUMENTATION AND DATA PROCESSING
- NUCLEIC ACID ISOLATION, PCR, NESTED-PCR, REAL TIME PCR AND ELECTROPHORETIC RUN ON AGAROSE GEL
- GENETIC ENGINEERING AND BIOMOLECULAR TECHNOLOGIES
- CELL SORTING: MACS
- MOLECULAR IMAGING
- OPTICAL AND CONFOCAL MICROSCOPY
- IMMUNOFLUORESCENCE ON CELLS AND TISSUES
- IMMUNOHISTOCHEMESTRY
- CELL LINE AND PRIMARY CULTURES
- GENOTYPING
- ANIMAL FACILITY MANAGEMENT

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