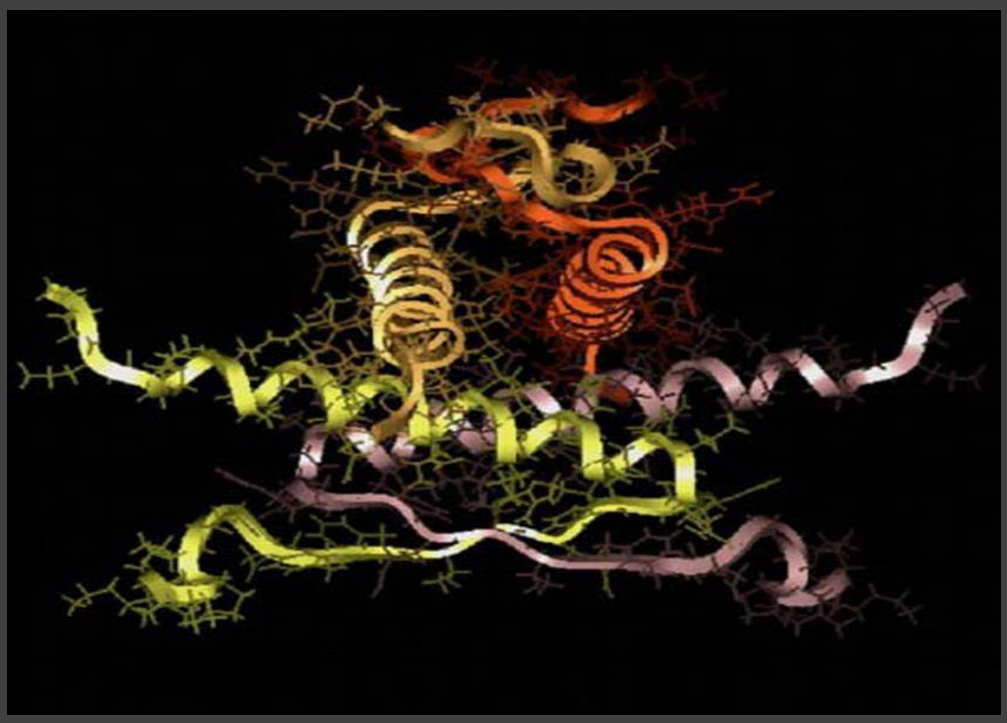
University of Insubria
Department of Biomedical Experimental and Clinical Sciences

"p53 family members regulate the Otx1 gene expression in differentiation of breast cancer stem cells and in mammary gland development"

Dr. Ilaria Stefania Pagani



Tutor: Prof. Giovanni Porta
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PhD School in Biological and Medical Sciences
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XXIII Cycle

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To my parents,

To Gian Paolo

..learn to fly....

ABSTRACT

"p53 family members regulate the Otx1 gene expression in differentiation of breast cancer stem cells and in mammary gland development".

Tp53, Tp63 and Tp73 family members encode for transcription factors which play a key role in control of the genome integrity inducing cell-cycle arrest, senescence, apoptosis or cell differentiation. They take a part in cell stress response and in tumor suppression (De Young MP and Ellisen LW, 2007).

Wild type p53 protein is a growth modulator and its inactivation is a critical event in malignant transformation (Gasco M, 2002).

It has been recently demonstrated that p53 has developmental and differentiation functions (Hu W, 2008). Indeed an over-expression of p53 in tumor cells induces asymmetrical division avoiding a self-renewal of cancer stem cells (CSCs) and promoting their differentiation (Cicalese A, 2009).

In this study 43 human ductal and lobular invasive breast carcinomas have been analyzed for the expression of p53, p63, p73 and a pool of non-clustered homeobox genes. The homeogenes play a crucial role in embryogenesis, regulating cell differentiation and proliferation (Pagani IS, 2010). They are expressed in adult mammary gland and when deregulated, are involved in breast cancer (Lewis MT, 2000).

We demonstrated that the Otx1 homeogene is transcribed in breast cancer, in CSCs differentiation and in adult mammary gland development.

We established that the p53 and p73 proteins directly induce the Otx1 expression by acting on its promoter. Otx1 has been described as a critical molecule for axon refinement in corticogenesis (Zhang YA, 2002), and its activity in breast cancer suggests a synergistic function with p53 and p73 in CSCs differentiation.

In adult mammary gland development the Otx1 expression is not regulated by p53, but is correlated with the expression of Tp73 in lactation and in regression. This suggests that in physiological conditions Otx1 is regulated by p73, while in the tumors p53 regulates its expression.

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ABBREVIATIONS

Acro-dermato-ungual-lacrimal-tooth malformations (ADULT)

adrenocorticotropic hormone (ACTH)

all-*trans* retinoic acid (ATRA)

alveolar precursor (AP)

days post coitum (d.p.c.)

DNA binding domain (DBD)

ductal precursor cell (DP)

Ectrodactyly Ectodermal dysplasia-Clefting syndrome (EEC)

epidermal growth factor (EGF)

epithelial precursor cells (EPCs)

estrogen (E)

estrogen receptor (ER)

extracellular matrix (ECM)

follicle-stimulating hormone (FSH)

full-length p53 (FLp53)

gonadotropin-releasing hormone (GnRH

gonadotropin-releasing hormone receptor (GnRHR)

growth hormone (GH)

growth hormone releasing hormone (GRH)

growth hormone releasing hormone receptor (GRHR)

herpes virus (HPV)

histone deacetylase (HDAC)

Homeobox (HB)

homeodomain (HD)

horthodenticle (*Otd)*

immunoprecipitation analysis (ChIP)

in situ ductal carcinoma (DCIS)

leukemia inhibitory factor (Lif)

Lymb-Mammary Syndrome (LMS)

luteinizing hormone (LH)

mammary luminal epithelial cell (MEC)

mammary stem cell (MASC)

metalloproteinase (MMP)

mouse double-minute 2 (MDM2)

Non-Hodgkin Lymphoma (NHL)

non obese diabetic-severe combined immunodeficiency (NOD-SCID)

nuclear export signal (NES)

nuclear localization signal (NLS)

oligomerization domain (OD)

orthodenticle homeobox 1 (Otx1)

orthodenticle homeobox 2 (Otx 2)

polymorphisms at single nucleotide (SNP)

poly-proline-rich site (PRD)

progesterone (P)

progesterone receptor (PR)

prolactin (PRL)

prolactin receptor (PRLR)

responsive element (RE)

retinoic acid (RA)

retinoic acid receptor (RAR)

Split-hand/foot malformations (SHFM);

stem cells (SCs)

sterile alpha motif (SAM

transactivation domain (TA)

TA inhibitory domain (TID)

TATA-binding protein-associated factor (TAF)

terminal ductal alveolar unit (TDLU)

terminal end bud (TEB)

thyroid-stimulating hormone (TSH)

transactivation domain (TA)

visceral endoderm (VE)

1 INTRODUCTION

1.1 The homeobox genes

Homeobox (HB) genes are a family of regulatory genes which encode for transcription factors, which play a crucial role in embryonic morphogenesis. Their functions are critical in specifying cell identity, in cell differentiation, and in the positioning of the bodily axis during embryo development. They were originally identified through mutations that cause segment transformation in *Drosophila melanogaster,* in which a body part or segment is converted to the likeness (identity) of another. Since their discovery, more than 200 homolog homeogenes have been identified from vertebrates to plants and fungi. In mammals, they play key roles in a variety of processes, including central nervous system and skeletal development, limb and digit specification, and organogenesis (Lewis MT, 2000).

HB genes contain a common 180-nucleotide sequence, termed "homeobox", that encodes for a 60-aminoacid domain, the "homeodomain (HD)", responsible for the recognition and binding of DNA-specific sequences on the target genes (Pagani IS, 2010). The HD recognizes DNA through a region of homology to the "helix-turn-helix" motif present in a group of prokaryotic transcription factors: the N-terminal two helices are antiparallel (helix 1 and helix 2), and the longer C-terminal helix (helix 3) is perpendicular to the axes established by the first two. It is this third helix, the "recognition helix", which interacts directly with the DNA major groove, forming hydrogen bonds (Fig. 1.1) (Wilson D, 1993). The HD mediates sequence-specific interactions with DNA responsive elements (REs), primarily those containing a TAAT, TGAT, TTAT or TTAC core motif. Many homeoproteins have been shown to function as transcriptional regulators, as activators or as repressors, regulating genes involved in embryonic stem cell differentiation (Chen H, 2003).

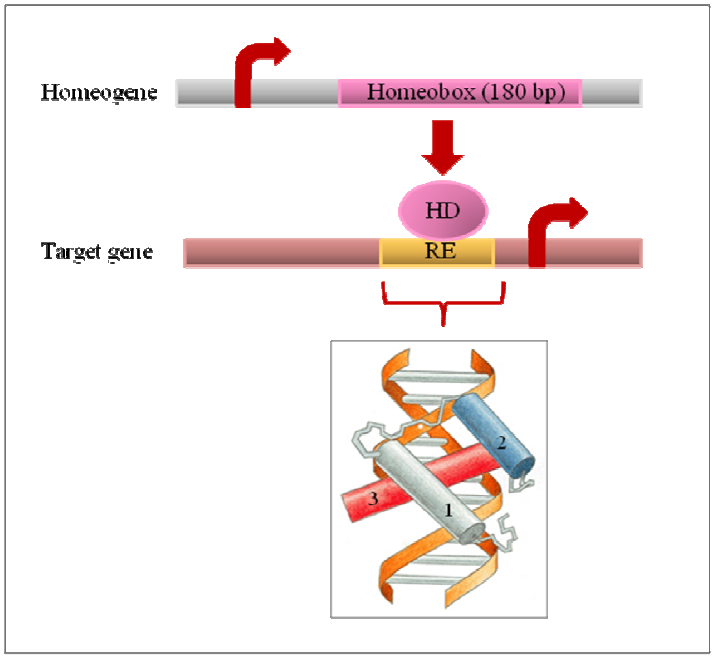


Figure 1.1. The homeoprotein and the homeodomain. The HB genes contain a 180 bp sequence, called "homeobox", which encodes for a 60 amino acid "homeodomain (HD)". The homeodomain recognizes and binds to specific "responsive elements (REs)" in the promoter of the target genes, activating or inhibiting their transcription. Despite its considerable variation in primary sequence, the three-deimensional structure of the HD has been conserved, and corresponds to three α-helices. The helix 3 is the "recognition helix" which interacts directly with the DNA major groove.

Based on sequence homology among their respective HDs, mammalian HB genes are classified into two large groups (Coletta RD, 2004).

The class I share high degrees of identity (>80%) in their HDs, with the first discovered HB gene of *Drosophila*, called *Antennapedia*. The most representative genes of this group are the HOX genes, organized in clusters of minimum nine genes with homologous HDs, aligned on the chromosome and arranged as 13 paralogous groups. Their order of expression along the anterior-posterior axis of the embryo, is collinear with 3' to 5' organization of HOX genes in the chromosomes, and is regulated in spatial-temporal manner (Coletta RD, 2004).

The class II comprises diverged HB genes, such as PAX, EMX, OTX or MSX, so called for their homologs in *Drosophila* (*paired*, *empty spiracles*, *orthodenticle* and *muscle segment homeobox* genes, respectively) (Coletta RD, 2004). New isolated and divergent HB genes are continually

being identified. Recent indications suggest that these genes constitute as much as 0.1-0.2% of the whole vertebrate genome (Abate-Shen C, 2002).

The two classes of HB genes differ for the sequence diversity within the HD, that contribute to their distinct functional properties, generating distinct DNA-binding specificities, and promoting unique protein-protein interactions. Functional specificity is also generated by additional conserved domains or motifs that occur in particular homeoprotein families (Fig. 1.2) (Abate-Shen C, 2002).

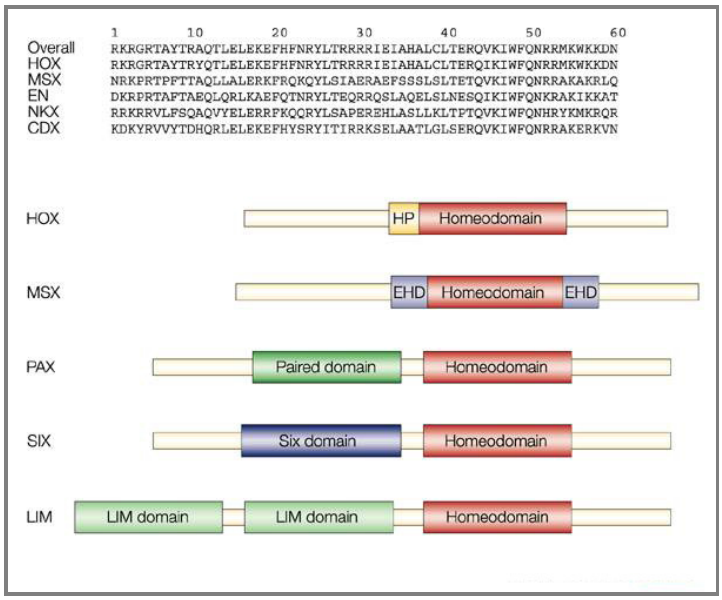


Figure 1.2. Homeoproteins diversity. The distinct classes of homeoproteins show diversity in the primary sequence of HD, responsible for specific DNA binding. Additional conserved domains or motif occur in particular homeoprotein families, and contribute to generate functional diversity. HOX proteins contain a hexapeptide (HP) motif, a short stretch of conserved residues that are responsible for mediating interactions with PBX homeoproteins. MSX proteins also have short stretches of conserved amino acids that flank the HD, called the extended HD (EHD), although their functions are unknown. PAX proteins contains an additional DNA binding domain, known as the paired box. Members of the SIX family have a conserved amino-terminal domain called the Six domain, and LIM homeoproteins have two LIM domains, the protein interaction motifs.

The role of HB genes in embryonic development has been extensively investigated since their discovery. Boncinelli asserts that the complexity of an organism is measured by the fact that genes expressed during embryogenesis are re-activated in the adult organism, with different

functions (Boncinelli, 1996). Recent evidences indicate that HB genes are involved in crucial biological processes of adult eukaryotic cells, such as control of cell identity, cell growth and differentiation, cell-cell and cell-extracellular matrix (ECM) interactions (Cillo C, 2001). The homeogenes regulate the proliferation, controlling the cell cycle. The majority stimulate the cell cycle, to expand progenitor populations prior to the differentiation, while a subset of HB genes, such as Gax1 or HoxA10, inhibits the cell cycle progression and promotes cellular differentiation (Fig. 1.3). Several HB genes are also associated with apoptosis during cell remodeling. The cell vitality and the phenotype are regulated by extracellular signals, such as cytokines, growth factors, hormones, adhesion molecules and ECM molecules, which activate an intracellular pathway resulting in the expression of the HB transcription factors. Variations in the acetylation state of histone proteins, covering the promoter regions, affect the transcriptional activity of specific genes. Interestingly, it has been recently proposed that acetylation/deacetylation may regulate transcription of HB genes, like Hox genes (Cillo C, 2001).

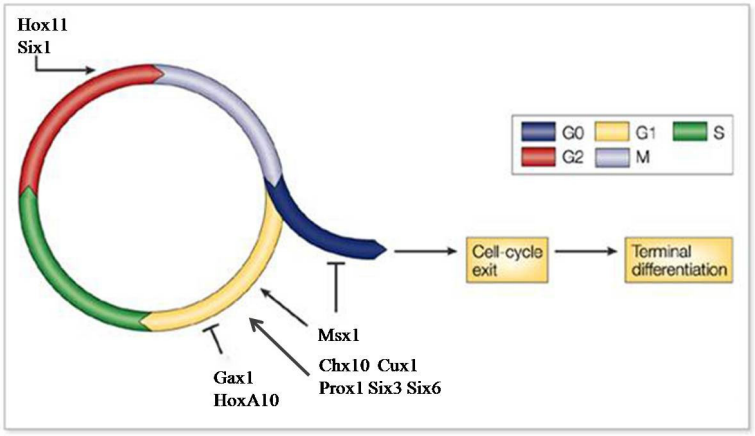


Figure 1.3. HB proteins have both a positive and negative effect on the cell cycle. HOX11 and SIX1 act at the G2-M transition by disrupting the G2 checkpoint, and causing cells to enter mitosis inappropriately. MSX1 up-regulates cyclin-D1, so preventing cells from leaving the cell cycle and undergoing differentiation. GAX1, on the other end, does not promote proliferation, but up-regulates the cyclin-dependent kinase inhibitor WAF1, which prevents entry into S phase.

Due to crucial role of the HB genes, mutations occurring in these genes are associated to human congenital, somatic or metabolic defects. For example, mutation of HoxA13 causes hand-foot-genital syndrome, and fusion between nucleoporin gene Nup98 and HoxA9 by chromosome

translocation is involved in myeloid leukemia (Cillo C, 2001). Deregulation of homeogene expression plays a key role during tumorigenesis. Normal development and cancer share many properties: both processes involve alterations in cell proliferation and differentiation, cell death, neovascularization, cell motility and invasion. Thus, genes involved in normal development are frequently utilized in neoplasia. The deregulated expression of HB genes has been described in many solid tumours, in leukemias and lymphomas (Coletta RD, 2004).

The involvement of the HB genes in carcinogenesis may be classified, based on their expression pattern (Fig. 1.4) (Abate-Shen C, 2002).

HB genes expressed in undifferentiated cells during development, may be re-expressed in tumorigenic cells, derived from the same cell lineage. There are many examples, including brain, mammary gland and kidney, in which tissue specific Hox genes are over-expressed in tumours derived from tissues in which they are normally expressed during development (Abate-Shen C, 2002).

On contrary, HB genes that are normally expressed in fully differentiated tissues, are down-regulated or lost in tumor. Recently, it has been reported that loss of expression of Cdx2 cause colon cancer, and that loss of expression of Nkx3.1 is linked to prostate cancer (Abate-Shen C, 2002).

Then, HB genes which are not normally expressed in a specific tissue during development, can be expressed in tumors derived from this cells. For example, Pax5 is expressed in medulloblastoma, but not in the cerebellum from which this tumor is derived (Abate-Shen C, 2002).

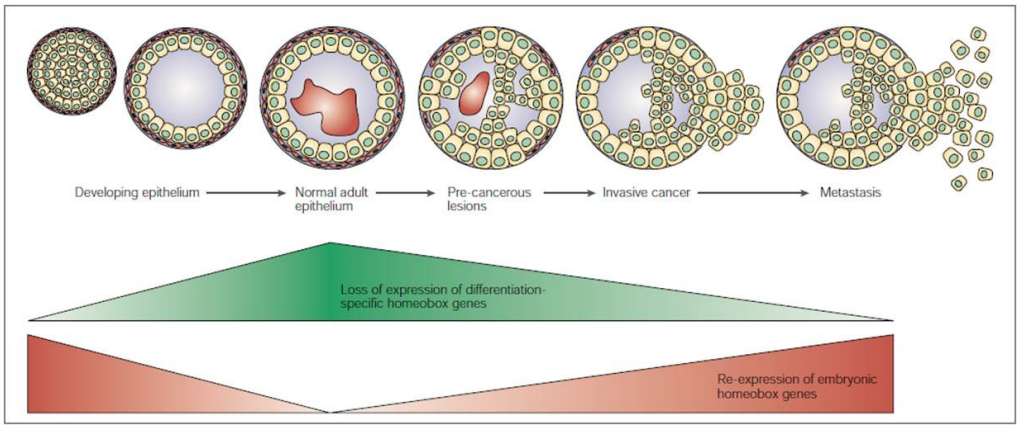


Figure 1.4. The relationship between HB gene expression and epithelial development and carcinogenesis. HB genes that are normally expressed in developing tissues and are down-regulated in differentiation, are re-expressed in cancer. Conversely, HB genes that are expressed in differentiated tissues are often down-regulated in cancer progression.

The differentiation status of a particular cell types can be considered the result of the balance of the expression of some HB genes and the silencing of the other. The gain or loss of HB genes promotes tumorigenesis as a consequence of their inappropriate effects on growth and differentiation. Although these genes are over-expressed or down-regulated in cancer, they are not considered "classic" oncogenes or tumor-suppressor, like p53, but they are defined as positive or negative "tumor modulators". While not necessarily proved, it has been presumed that oncogenic activities are produced by wild-type, rather than mutant, homeoproteins. The oncogenic potential is not due to the acquisition of new or altered activities, but reflects a wrong spatial-temporal extension of their normal functions. Their loss-of-function is not sufficient for tumorigenesis (Abate-Shen C, 2002).

Indeed, it is evidenced the HB genes involvement in tumorigenesis, further studies are required to clarified their role in the development and progression of cancer (Table 1.1).

Table 1.1. Deregulated homeobox genes in solid tumors

Genes	Deregulation in cancer	Normal expression	Functional insights
*HOX**	Gain of expression in primary tumours and cell lines from brain, breast, colon, lung and kidney	Expression patterns during embryogenesis reflect roles in patterning, segmentation and fate determination of many tissues	Overexpression promotes cellular transformation in culture
MSX (1,2)	Gain of expression in mammary, colon, stomach, kidney, thyroid and other carcinomas	Expression during embryogenesis associated with epithelial–mesenchymal interactions and inversely correlated with differentiation	Overexpression leads to inhibition of differentiation, correlated with upregulation of cyclin D1
HSIX1	Gain of expression in mammary and other carcinomas	Limited expression analyses available; homologues expressed in the developing brain, eye, muscle and other tissues	Overexpression abrogates the G2 cell-cycle checkpoint in response to X-ray irradiation
GBX2	Gain of expression in prostate carcinoma	Expressed in the developing nervous system; limited information concerning expression in prostate	Downregulation of *GBX2* via antisense correlated with reduced tumorigenicity
PAX (2,5,6,8)	Gain of expression in Wilms' tumour, brain and breast cancer; translocation of *PAX8* in thyroid carcinoma	Expression patterns during embryogenesis reflect roles in organogenesis of kidney and other tissues	Overexpression promotes cellular transformation in culture
CDX2	Loss of protein expression in colon carcinoma	Expressed during embryogenesis in extraembryomic and embryonic tissues; expression in older embryos and adults restricted to intestinal epithelium	Overexpression promotes differentiation of intestinal cells, while leading to reduced proliferation and tumorigenicity; heterozygous mutant mice predisposed to colon cancer
NKX3.1	Loss of protein expression in prostate cancer and pre-neoplastic (PIN) lesions	Expressed during embryogenesis in somites and other derivatives; expression in older embryos and adults is restricted to prostatic epithelium	Localized to 8p21, which is frequently deleted in prostate cancer; overexpression leads to reduced cell growth and tumorigenicity; homozygous and heterozygous mutant mice are predisposed to prostate cancer
BARX2	Loss of expression in ovary carcinoma	Expressed in normal ovarian surface epithelium; limited expression analyses available	Localized to 11q24, which is frequently deleted in ovarian cancer; displays tumour suppression and anti-metastastic activities in cell culture

1.2 Otx family

The OTX proteins, the vertebrate homologues of the *Drosophil*a gene *horthodenticle* (*Otd*), include an important class of HD-containing transcription factors involved in the induction and in the morphogenesis of the neuroectoderm, leading to the formation of the vertebrate central nervous system. OTX/*Otd* proteins, belong to the Paired-like HD family, are characterized by a lysine at position 9 of the recognition (third) helix of the HD. The lysine in this position is relatively unusual, and confers to the OTX proteins a high affinity and selective binding to TAATCC/T sites on DNA (Klein WH, 199).

This gene family underwent to gene duplication during vertebrate evolution and probably, like other HD protein families, contributed to increase the morphological and functional complexity of the vertebrates. Based on OTX-HD similarity, several other OTX-related proteins have been identified. Outside of the HD, homologies between OTX proteins may be found in the glutamine stretch. This domain is followed by the $KXRX_{1-2}KXK$ domain adjacent to the HD. After this there are the highly conserved WSP domain [(S/A)(I/L)WSPA], 45-50 amino acids C-terminal to the HD, and an imperfect repeat of an 18-25 amino acid region, containing the highly conserved Otx tail domain (D/E)CLDYK(D/E)(Q/P) located upstream to the C-terminal W(K/R)FQVL motif (Fig. 1.5) (Bellipanni G, 2009).

Based on sequence homologies within the C-terminal domain, Otx proteins can be classified into three groups: OTX1/OTX3, OTX2, and OTX5/CRX. In particular, OTX1 and OTX3 show a region containing three separate stretches of histidine (HRSs: histidine-rich sites) (Fig. 1.5) (Bellipanni G, 2009).

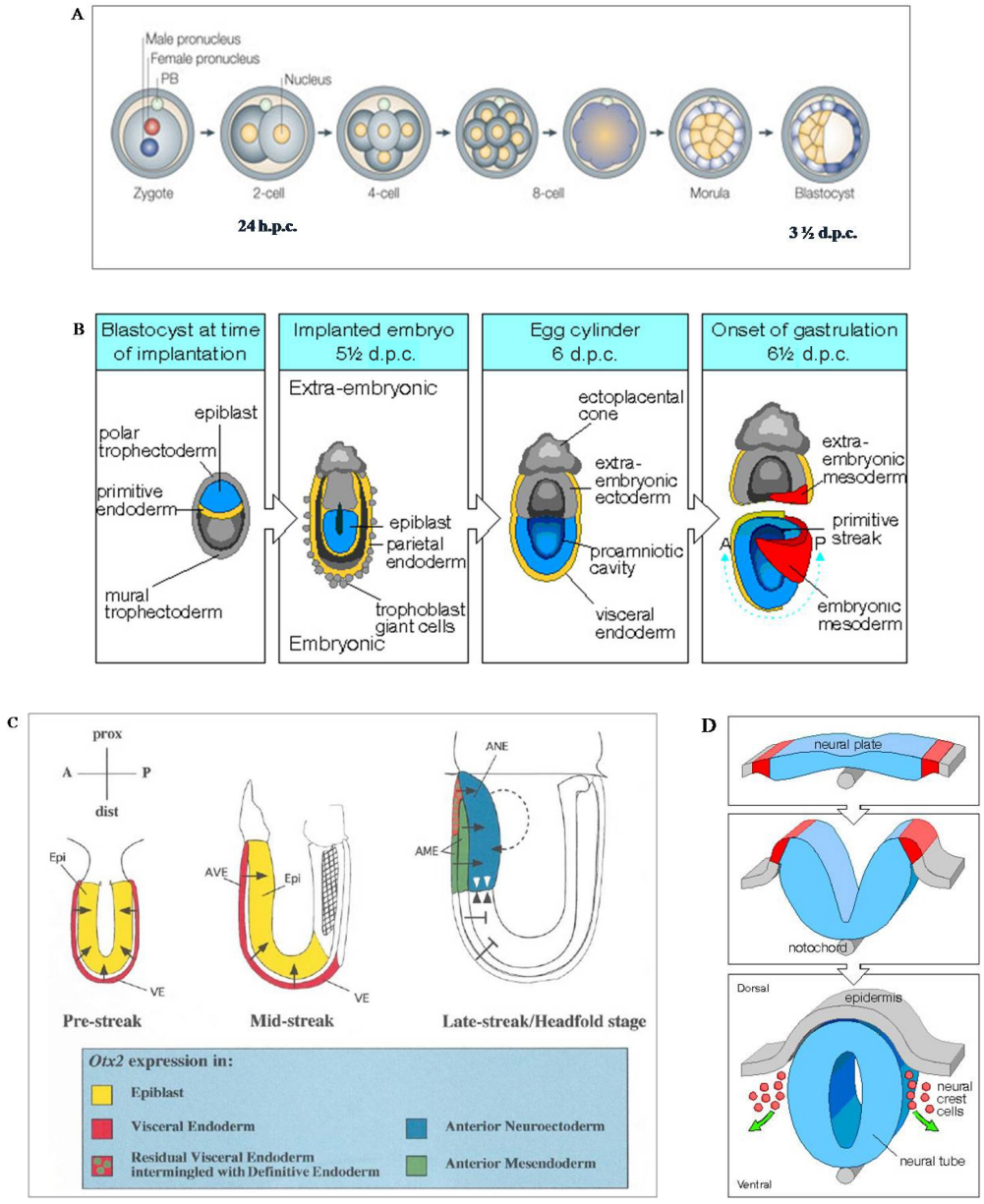
Figure 1.5. Primary structure of the OTX1 and OTX3 proteins.

Otx1 (2p13) and Otx2 (14q22.3) genes play a pivotal role during early and later brain development. During brain regionalization, these genes are regulated in spatial and temporal manner, and are sequentially expressed in overlapping domains (Larsen KB, 2010).

The Otx2 gene. Otx2 (orthodenticle homeobox 2) is required during gastrulation, for anterior neural plate specification (Acampora D, Gulisano M *et al*., 2000). In the mouse embryo, the Otx2 gene is transcribed in the epiblast (blastocyst at time of uterus implantation, 4½ days post coitum-d.p.c.) (Box 1B). At the onset of gastrulation (5½ d.p.c.), Otx2 is required in the visceral endoderm (VE) to maintain its transcription in the epiblast, and to mediate Otx2-dependent signals directed from the VE to epiblast (Box 1B-C). From early to late streak stage (7 ½ d.p.c.), this signal persists in the VE. At the end of gastrulation (7 ½ d.p.c.), a positive signal from the anterior mesendoderm is required for Otx2 transcription in the surrounding neuroectoderm, while a negative signal from the posterior mesendoderm, contributes to the positioning of its posterior border (Box 1C) (Acampora D, Gulisano M *et al*., 2000). The neuroectoderm of the gastrulating embryo forms the neural plate, that give rise to the entire central nervous system. Neural folds arise in the neural plate, appose and fuse to form the neural tube (Box 1D). The brain develops from the most anterior region of the neural tube and is divided into three primary vesicles: the hindbrain vesicle (rhombencephalon), the midbrain vesicle (mesencephalon) and the forebrain vesicle (prosencephalon). The forebrain becomes divided into the diencephalon caudally and telencephalon rostrally. Negative signals, contribute to distinguishing between fore-midbrain and hindbrain, inhibiting the Otx2 expression in the hindbrain. Otx2 is predominantly expressed in the telencephalon, diencephalon, mesencephalon, and archicortex, while it is not expressed in the adjacent neocortex. Therefore, it might be used as a hippocampal marker in the telencephalon (Simeone A, 1998).

In the adult, Otx2 expression is present exclusively in the choroid plexus and pineal gland (Larsen KB, 2010).

Box1.1. Mouse embryo development



Box 1.1.A. Stages from the fertilized egg to blastocyst during mouse embryo development. In zygote, the female and male pronuclei are labelled red and blue respectively and the second polar body is present at the animal pole. At the 4-cell stage the blastomeres are arranged in a tetrahedral form. At the 8-cell stage all blastomeres are initially round, but then they flatten at the process of compaction and undergo apical–basal polarization (shown by blue to yellow gradient). At the morula (32-cell stage), the embryo consists of both inside (light yellow) and outside (light blue) cells. At the blastocyst, the inner cells form the inner cell mass (yellow), which is surrounded by polar trophectoderm (light blue) whereas the mural trophectoderm (darker blue) surrounds the cavity. **1.1.B.** At 4 d.p.c the

blastocyst reaches the uterus. The inner cell mass forms the primitive endoderm and the epiblast (Epi) (primitive ectoderm), which alone gives rise to all cells of the embryo proper. The primitive endoderm forms the visceral endoderm (VE), which covers the epiblast and extra-embryonic ectoderm when they become elongated into a cylinder at 6 d.p.c. At 6 ½ d.p.c. the gastrulation begins with the formation of the primitive streak at the posterior end of the embryo. Mesoderm , definitive endoderm, and axial mesendoderm (AME) precursors arise in the streak by 7.0 d.p.c and undergo coordinated morphogenetic movements that leads to their eventual placement in anterior positions. **1.1.C.** At the pre-streak stage , Otx2 is transcribed in the entire VE and epiblast. As the primitive streak progresses, Otx2 expression gradually is restricted to the anterior third of the embryo, and at the late streak stage includes all three germ layers. A positive vertical signal (arrows) from the anterior mesendoderm (AME) maintains Otx2 expression in the surrounding neuroectoderm. Negative signals (T) deriving from posterior axial mesendoderm may contribute to defining the posterior border of Otx2 expression. **1.1.D.** The neural plate is formed from the ectodermal layer of the gastrulating embryo and gives rise to the entire central nervous system. Neural folds arise in the neural plate, appose and fuse to form the neural tube. The brain develops from the most anterior region of the neural tube and is divided into three primary vesicles: the hindbrain vesicle (rhombencephalon), the midbrain vesicle (mesencephalon) and the forebrain vesicle (prosencephalon). The forebrain becomes divided into the diencephalon caudally and telencephalon rostrally. The telencephalon eventually differentiates to become the olfactory bulbs anteriorly, the cerebral cortex dorsally and the basal ganglia ventrally.

In order to define the Otx2 role in brain development, the knock-out mouse has been generated. Otx2 null embryos die early in embryogenesis, lacking the rostral neuroectoderm, fated to become the forebrain, midbrain and rostral hindbrain, and show major abnormalities in their body plan (Simeone A, 2002).

Otx2 is an essential regulator of the identity, extent, and fate of the neuronal progenitors, and controls the proliferation and the differentiation of the dopaminergic progenitors. The dopaminergic neurons are involved in voluntary movements, cognition, and reward responses, and their degeneration is associated with Parkinson's disease. Therefore, Otx2 might be a potential target for cell replacement-based therapeutic approaches in Parkinson's disease. Otx2 also controls the identity and the fate of glutamatergic progenitors of the thalamus by repressing GABAergic differentiation. Any malfunction of this gene may cause neurogical and psychiatric disorders (Larsen KB, 2010).

Furthermore, Otx2 plays a role in the development and function of the retina, in which it is expressed at both prenatal and postnatal stages. It is necessary for the development and differentiation of the rods and cones via transactivation of another homeobox gene, the cone-rode gene (Crx). OTX2 protein was also recently detected in the fetal retina in the pigment

epithelium (Larsen KB, 2010). The subcellular localization of OTX2 is important to determine the fates of rod photoreceptor and bipolar cells, during their generation, and initial differentiation in the neonatal retina. The segregation of postmitotic cells into immature rods and bipolar cells, under the influence of various extrinsic factors, such as retinoic acid, is accompanied by the OTX2 localization into cytoplasm and nucleus, respectively (Baas D, 2000).

Recent studies have identified a significant number of target genes downstream of Otx2, up-regulated or down-regulated by this gene. Most Otx2 target genes fall into one of six functional categories: actin/myosin binding proteins; extracellular adhesion molecules; proteases and proteases inhibitors; RNA binding proteins; transcription factors; and secreted polypeptides, such as gonadotropin-releasing hormone (GnRH), released from a subset of hypothalamic neurons which control many aspects of reproduction (Boncinelli E and Morgan R, 2001).

The OTX2 protein acts as a morphogen, generating a gradient in which sequentially and in temporal and spatial manner, other genes are activated or repressed in distinct cells, contributing to cell proliferation and differentiation.

In the action space of morphogen OTX2, in the next time, the Otx1 gene is activated.

The Otx1 gene. Otx1 (orthodenticle homeobox 1) is required for corticogenesis and sense organ development. During murine embryogenesis, Otx1 expression is detected first at 8 d.p.c., throughout the forebrain and midbrain neuroepithelium. In particular, during cerebral cortex development, Otx1 initially is transcribed throughout the entire dorsal telencephalic neuroepithelium; subsequently, towards the stages corresponding to the generations of neurons belonging to the deep cortical layers, it is restricted to the ventricular zone. At the end of gestation Otx1 expression becomes prominent in the cortical plate, consisting of post-migratory neurons of layers 5 and 6. Postnatally, Otx1 is expressed prevalently in a subset of neurons in layers 5 and 6 (Simeone A, 1998).

The development of precisely wired neuronal circuits requires that axons grow to appropriate targets and form specific patterns of synaptic connections. In many animals, the emergence of the final adult pattern of connectivity is preceded by the formation of transient “exuberant” connections. A striking example of exuberance in development is seen in layer 5 neurons, of the

cerebral cortex, which extend axonal connections to multiple subcortical targets and then eliminate a subset of these projections during early postnatal life. Otx1 is required for the elimination of these exuberant projections. In the embryo, OTX1 protein is initially located to the cytoplasm of the progenitor cells in the ventricular zone, and remains cytoplasmic up to neurons migrate and begin start to differentiate. During the first week of the postnatal life when the neurons reach the layer 5 in the cortex, the OTX1 protein is actively translocated to the nucleus and is involved in the pruning of long distance axonal projections (Zhang YA, 2002).

The importance of Otx1 in the development of the neocortex is demonstrated in some human pathology. In focal cortical dysplasia, a developmental malformation of the cerebral cortex highly associated with epilepsy, the OTX1 protein is expressed in balloon cells. These balloon cells are derived from cortical radial glia and not from the ganglionic eminence (Larsen KB, 2010).

In the adult, Otx1 is expressed in the choroid plexus of the lateral ventricles, in the ventricular zone of the ganglionic eminence, in the hippocampus, and in the cerebellum. Furthermore, Otx1 is expressed in sense organs: is involved in the formation of the ciliar body, in the anterior part of the retina, in the inner ear morphogenesis, and is expressed in the olfactory bulb (Larsen KB, 2010).

Postnatally, Otx1 is also transcribed at relatively low levels in the anterior lobe of the pituitary gland, where it activates transcription of the pituitary hormones. The pituitary gland is an essential regulatory, that integrates signals from the periphery and brain to control the production and secretion of hormones involved in growth, reproduction, behavior and metabolism. The mature pituitary gland consists of five distinct cell types, each defined by the specific hormone(s) production. The cell types found in the anterior lobe are thyrotropes, somatotropes, corticotropes, lactotropes and gonadotropes. They produce the thyroid-stimulating hormone (TSH), growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin (PRL), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The OTX1 protein directly binds to the promoter of GH, βFSH and βLH genes, activating the production of the respective hormones. The pituitary gland and the hypothalamus constitute the main axis of the neuroendocrine system and exhibit a remarkable coordination in temporal and spatial events regulating their development and differentiation (Acampora D, 1998).

Most of what is known about the functions of Otx1 comes from Otx1 null mice. Otx1-/- mice exhibit both spontaneous high speed turning and epileptic behavior. The adult Otx1-/- brain is reduced in weight and size, and histological analysis revealed that the dorsal telencephalic cortex is reduced in thickness, mainly at the level of the temporal and perirhinal areas, where a 40% reduction in cell number and a disorganization of cortical layers are detected. An abnormal apoptosis is not observed, but a reduction of proliferating cells (about 25%) in the dorsal telencephalic neuroepithelium suggests that an impaired proliferation may contribute to the cortical abnormalities. Further morphological defects are detected in the eye and inner ear. Lachrymal and Harderian glands are absent. The inner ear abnormalities in the Otx1-/- mice reflect the Otx1 expression pattern in the lateral canal ampulla, and in a part of the utricle as well as in the saccule and cochlea. Interestingly, Otx2 is coexpressed with Otx1 in the saccule and cochlea, but not in the components of the superior pars. Lack of Otx1 results in the absence of the semicircular canal. In the eye and annexed structures of the Otx1-/- mice, the ciliary process is absent, and the iris is thinner. At the prepubescent stage the null mice exhibit transient dwarfism and hypogonadism due to low levels of pituitary GH, FSH and LH hormones. Nevertheless, Otx1-/- mice gradually recover from most of these abnormalities, showing normal levels of GH, FSH and LH pituitary hormones, with restored growth and gonadal function at 4 months of age. Expression patterns of corresponding hypothalamic hormones, such as the growth hormone releasing hormone (GRH), GnRH, and their pituitary receptors (GRHR and GnRHR) suggest that, in Otx1-/- mice, hypothalamic and pituitary cells of the somatotropic and gonadotropic lineages appear unaltered and that the ability to synthesize GH, FSL and LH, rather than the number of cells producing these hormones, is affected (Acampora D, 2000).

The Otx1-/- null mice are also anaemic. Recently, Levantini and coworkers reported that Otx1 is expressed in hematopoietic pluripotent and erythroid progenitor cells, playing a role during haematopoiesis, enhancing stem cell (SC) differentiation. Otx1 is present also in mature cells of the erythroid and megakaryocytic pathway in bone marrow (Levantini E, 2003). Mice lacking Otx1 exhibit decreased levels of erythroid genes, such as Scl, essential for erythroid differentiation, and Gata-1 transcription factors. Scl is a direct transcriptional target of OTX1, and OTX1 interacts with GATA-1 to activate the transcription of SCL proximal promoter (Martin R, 2004).

The Otx1 gene is expressed in proliferating cells in embryo and in adult tissues, and it is over-expressed in tumors. Both Otx1 and Otx2 genes are involved in the pathology of the

medulloblastoma, which is the most common malignant in children brain tumors. Their expression correlates with clinicopathologic classification. Several studies reported that Otx2 has an important role in the tumor development, it is over-expressed in a high percentage (20%) of primary anaplastic medulloblastomas suggesting its oncogenetic role. (De Haas T, 2006). Similarly, Otx1 but not Otx2, is over-expressed in the nodular/desmoplastic sub-type of medulloblastomas and in B-cell Non-Hodgkin Lymphoma (NHL). The NHL represent an heterogeneous group of malignancies arising from mature B-cells, recruited in germinal centers of secondary lymphoid organs during the T-cell dependent immune response. In lymphoma cells OTX1 protein exhibits nuclear distribution, whereas in non malignant B-cells it is prevalently localized to the cytoplasm. The over-expression of Otx1 in tumor cells may alter the gene expression profile and cause abnormalities in cell-identity and/or proliferation (Omodei D, 2009).

1.3 The mammary gland

The mammary gland, unlike most mammalian organs which develop embryonically with linear progression until the maturity, is an organ that primarily forms at puberty, rather than during embryogenesis. This growth is characterized by a linear phase (encompassing puberty) and by a cyclical phase (encompassing pregnancy), both of which are regulated by reproductive hormones. Mammary glands are epidermal appendages, derived from the ectoderm, probably evolved from ancient apocrine glands associated with the skin. Their primary function is to provide nutrition for the young in the form of milk protein, and fat. However, there are other benefits provided by lactation, such as the provision of immune factors that are secreted into the milk, which confer protection from infection (Watson CJ and Khaled WT, 2008).

The adult mammary gland is a complex secretory organ whit a lobulo-alveolar structure, composed of two tissue compartments: the epithelium, which consist of ducts and milk-producing alveolar cells; and the stroma, or connective tissue, which is also called the mammary fat pad. The mammary luminal epithelial cells (MECs) are classified in ductal epithelial cells, that form the ducts, epithelial structures that transport the milk until the nipple, and in alveolar epithelial cells, that compose alveoli, ball-shaped structures with a central lumen that opens to the body surface, by the ducts. The alveolar epithelial cells are secretory cells, which undergo

functional differentiation in pregnancy to produce milk. The MECs are encased by a mesh-line system of basal myoepithelial cells, which are contractile and participate in the delivery of the milk. The extensive system of ducts and alveoli, forms a bi-layered structure of simple epithelium embedded in the stroma, the main components of which are adipocytes, but fibroblasts, cells of the hemopoietic system, immune cells, blood vessels and neurons are also present (Fig. 1.6.A) (Hennighausen L and Robinson GW, 2005) (Watson C J and Khaled WT, 2008).

The existence of distinct epithelial cell lineages, that are derived from an elusive multipotent mammary stem cell (MASC), with capacity of self-renewal and ability to generate differentiated cells, has been proposed. Models of how these lineages develop have been defined. It has been proposed that the cells forming the epithelial compartment of the mammary gland are derived from the MASCs, which have the capacity to self-renewal and give rise to committed epithelial precursor cells (EPCs). The progeny of EPCs then becomes restricted to a ductal or alveolar fate. The ductal precursor cells (DPs) form myoepithelial cells and luminal cells, the two cell types that constitute the ducts. During pregnancy, alveoli are generated from alveolar precursors (APs), which give rise to myoepithelial and luminal cells, that differentiate in milk producing cells. The alveolar epithelium expands during pregnancy, secrets milk during lactation and undergoes apoptosis and remodeling during involution. The epithelial remodeling of the ducts and alveoli is due to the presence of the multipotent MASCs and progenitor cells, that are located in the ducts (Fig. 1.6.B) (Polkyak K, 2007).

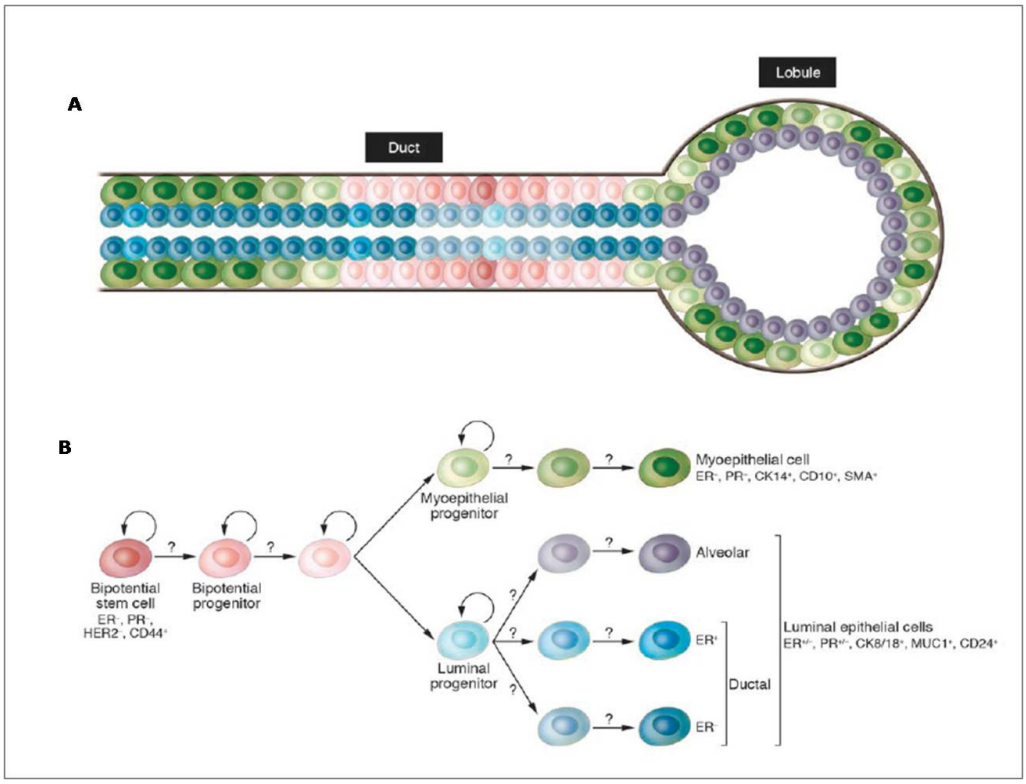


Figure 1.6. Hypothetical model of human MASC hierarchy and differentiation. A. Schematic picture of a normal terminal duct lobular unit with the putative location of the various stem and differentiated cells. Gray line denotes the basement membrane; color of the cell types correlates with than in B. **B.** Hypothetical depiction of MASCs and their various progeny. A bipotential SC gives rise to luminal and myoepithelial cells. The intermediary steps and their regulation are largely unknown. ER, estrogen receptor; PR, progesterone receptor; CK14, cytokeratin 14; MUC1, mucin 1.

In the mouse, numerous experiments demonstrated the existence of progenitor cells, in all stage of mammary gland development, that can regenerate a functional mammary gland when transplantated into the cleared fat pad of a recipient mouse (Vaillant F, 2007). In the human breast, putative mammary epithelial progenitors have been identified using in vitro clonogenicity assays as a measure of "stemness". Specifically, several studies have shown that a subset of MECs are able to form colonies in vitro and give rise to both luminal epithelial and myoepithelial cells. Similar to other tissue types, such as neural stem cells that in nonadherent culture forms neurospheres, cultured MECs creates mammospheres enriched from stem/progenitors cells. The mammospheres are able to differentiate along all three mammary lineages and to clonally generate N-generations of mammospheres (Fig. 1.7) (Dontu G, 2003).

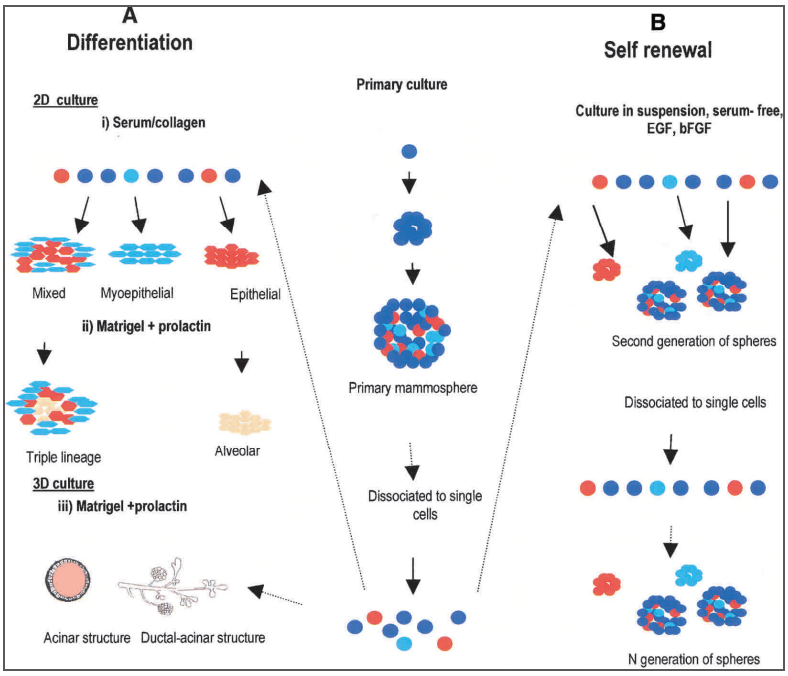


Figure 1.7. Mammosphere ability to differentiate and self-renew.

During the development, the mammary gland progresses through distinct stages: in the embryonic and fetal stage, the mammary anlage (the embryonic primordium of an organ) develops; in the neonatal and prepubertal stage, there is an isometric growth; in the peripubertal stage, the gland grows allometrically and ducts elongate and branch; in sexual maturity branching continues and alveolar bud form. The final stage involves the process of functional differentiation. During pregnancy the lobulo-alveolar structures develops to support lactation, and, when nursing ceases, there is an involution of these structures. All the processes are hormonally regulated throughout development. Ductal elongation is directed by estrogen (E), growth hormone (GH), insulin-like growth factor-I, and epidermal growth factor (EGF), whereas ductal branching and alveolar budding is influenced by additional factors such as progesterone (P), prolactin (PRL), and thyroid hormone. The effect of these hormones and growth factors is influenced by ductal epithelium and epithelial-stromal interactions (Hovey R, 2002).

Human embryonic development and morphogenesis. In all mammals the mammary gland arise from a localized thickening of the ectoderm or epidermis. In the human fetus, MECs arise from

an ectodermal bud to form clusters, that later represent the areolus of each breast. The milk streak is first observed during the 4th week of embryonic life and becomes the milk line during the 5th and 6th week. During the 7th and 8th week, the mammary parenchyma begins to invade the underlying stroma and the mammary disc appear. Further inward growth of the mammary parenchyma commences at the 9th week, concomitant with regression of the overlying skin. Between the 10th and 12th week, epithelial buds sprout from the invading parenchyma, followed by indentation during the 12th and 13th week that results in the formation of epithelial buds with notches at the epithelial stromal border. Branching of the parenchyma during the 13th-20th week results in 15-25 epithelial strips or solid cords, that eventually give rise to the multiple galactophores at each nipple. During the branching process, and up to the 32nd week, the solid cords become canalized by apoptosis of the central epithelial cells. Finally, between the 32nd and 40th weeks of gestation, limited lobulo-alveolar development occurs in association with the development of end vescicles composed of an epithelial monolayer. In the 32-week-old fetus the periductal stroma has a loose appearance, while in full-term infants the rudimentary lobular structures are surrounded by a dense stroma (Fig. 1.8) (Hovey R, 2002).

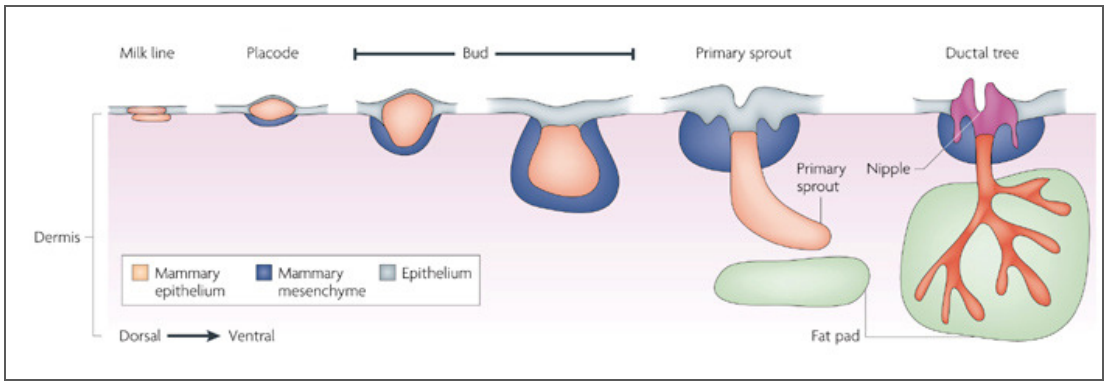


Figure 1.8. Embryonic development stages of the human mammary gland.

Neonatal and prepubertal development and morphogenesis. The mammary gland of newborns contain only rudimentary ducts with small club-like ends that regress within a short time after birth (Fig. 1.10.A.a). In neonates this gland grows isometrically before the onset of puberty at 8-12 years. At this stage the female breast begins to show growth activity both in the epithelium and the surrounding stroma (Hovey R, 2002).

Peripubertal period. From birth to puberty, the gland remains rudimentary and relatively growth quiescent. Several hormonal changes in the body precede the puberty. The female releases FSH and LH hormones, induced by OTX1 protein, in a cyclic pattern from the anterior pituitary gland. These hormones stimulates the ovaries to synthesize and release female sex steroid hormones, estrogen and progesterone. The part of the ovarian cycle characterized by follicular growth is dominated by estrogen, while the corpus luteum develops, is dominated by progesterone. These ovarian hormones stimulate rapid and invasive allometric ductal elongation, driven by growth of structures called "terminal end buds (TEBs)", a club-shaped terminal structures that form at distal ends of the rudimentary ducts, and serve as the foci for the cellular proliferation, differentiation and apoptosis. These structures are composed of 4-6 layers of cuboidal epithelial cells, and are the sites at which the cells divide at a high rate, advancing the progression of the ducts into the fat pad (Fig. 1.9). Both estrogen and progesterone have pleiotropic actions in the uterus and ovaries, regulating sexual development. In the mammary gland they control ductal outgrowth and alveolar expansion, regulating cell cycle, cell proliferation and cellular turnover (Fig. 1.10. b) (Lewis MT, 2000).

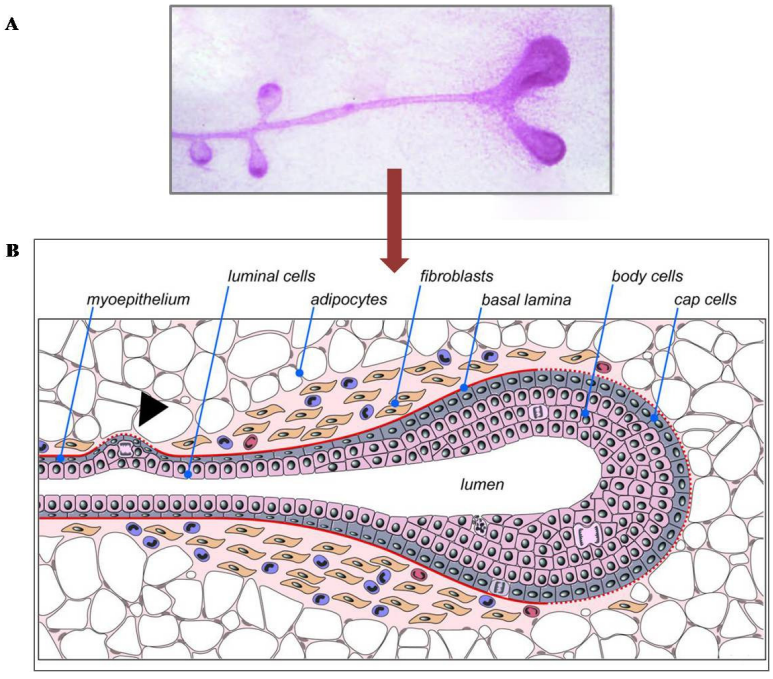


Figure 1.9. Terminal end buds. A. High-magnification carmine alum-stained wholemount of a primary duct. The bifurcating TEB is in the final stages of forming two new primary ducts with independent TEBs. Three newly formed lateral (secondary) side-branches are also present along the trailing duct. **B.** Schematic diagram depicting the salient architectural features of TEBs and their subtending ducts, including their fibroblast-rich stromal collar and high mitotic index. The arrow indicates the formation of a new lateral duct.

The estrogens (Es) bind to two distinct estrogen receptors (ERs), ERα and ERβ, which are encoded by two different genes. Like other steroid receptor, these are members of a large family of nuclear receptors, which function as transcription factors when activated by the binding of the steroid hormones. Whereas deletion of ERβ has not unfavorable effects on ductal and alveolar development, ERα is required for normal ductal elongation and outgrowth during puberty, and for pregnancy-mediated alveolar expansion. Instead, progesterone (P) bind to two isoforms of the progesterone receptors (PRs), A and B, which display differential activities. These isoforms are encoded by two transcript derived from the same gene by different start codons (Hennighausen L and Robinson GW, 2005). E and P stimulate cellular proliferation, but cells that express ER and PR are not proliferative, suggesting these two hormones stimulate the proliferation in an indirect manner. The ER+/PR+ cells, through the bond of estrogen and/or progesterone, activate the proliferation in adjacent cells ER-/PR- by a paracrine mechanism (Polyak). Analysis of the hormone-receptor status of the MASCs-enriched subset (CD44+ CD24- PROCR+ CD10-) revealed that they do not express ERα and PR. Expression of ERα on MECs inversely correlates with proliferative activity, invoking a model in which ERα+ cells secrete paracrine factors, in response to estrogen stimulation, that modulate the proliferation of adjacent ERα- cells. Furthermore, similar studies on the progenitors cells found that a subset of luminal progenitors cells express ERα. It is plausible that a hierarchy of stem cells reside within mammary tissue and that an immediate descendant of the MASCs express ERα and PR (Vaillant).

Ductal elongation is due also at interaction between MEC and the stroma. Fibroblastic stromal cells flank the neck on the TEBs, where there is the pronounced synthesis of extracellular matrix (ECM) molecules such as chondroitin sulphate or heparan sulphate. The TEB formation depends on the interaction of MECs with proteoglycans, adipocytes, collagen (Hovey R, 2002).

Pregnancy. The greatest portion of mammary growth occurs during pregnancy and is also controlled by hormones. During the pregnancy progress, the adipose cells of the pad are gradually replaced by ducts, alveoli (not formed before pregnancy), blood vessels, and connective tissue (Hennighausen L and Robinson GW, 2005). Proliferation and maturation of the side branches occurs to form alveoli which are lined by functionally differentiated secretory luminal epithelial cells responsible for milk production. These milk secretory alveoli are localized in clusters which resemble bunches of grapes and are called “terminal ductal alveolar units” (TDLUs). The TDLUs contain smaller ducts which link the alveoli and are themselves

linked by a network of larger ducts. At least some TDLUs are already present prior to pregnancy, although they are not as complex as during pregnancy. The epithelial expansion during pregnancy probably consists in a combination between existing TDLUs and the *de novo* ductal and lobular growth, although this is not entirely clarified (Molyneux G, 2007). The mammary glands growth until the peak of lactation. Proliferation, differentiation and survival of alveolar epithelium, depend on the lactotrophs, as prolactin (PRL), produced by the anterior pituitary gland (Fig. 1.10.c, .d and .e). Prolactin mediates its action through PRL receptor (PRLR), a trans-membrane protein of the class I cytokine receptor family. When PRL binds to its receptor, the conformation of the PRLR dimer change activating the associate Janus kinase-2 (JAK2). The activated JAK2 phosphorylates the tyrosine residue on STAT5, which become able to bind to specific site in the promoter of the genes involved in the milk protein production (Hennighausen L and Robinson GW, 2005).

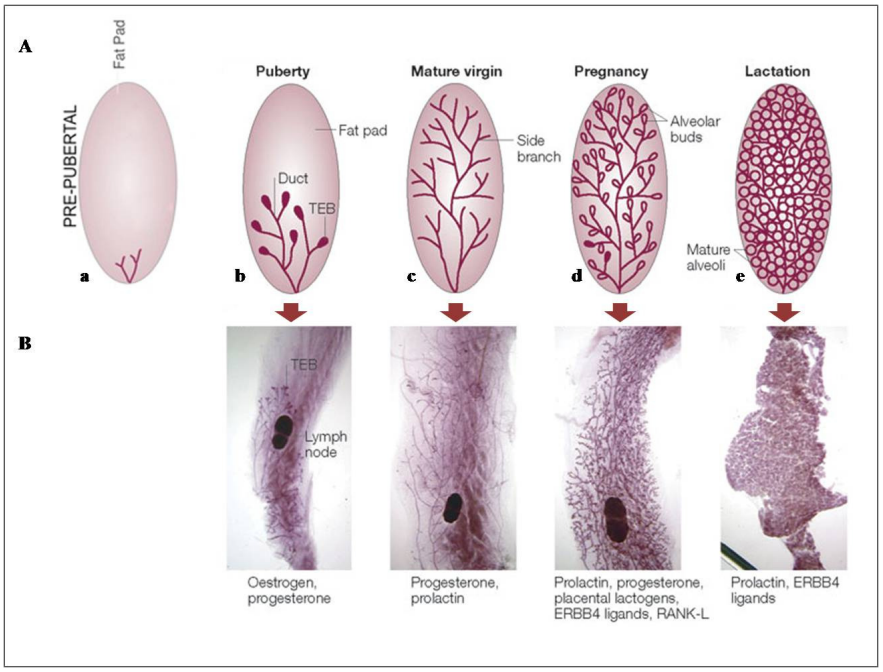


Figure 1.10. Adult mammary gland development. Schematic (Aa-e) and wholemount (Bb-e) presentation of the different stages and the principal hormones that control development. **A.a.** A rudimentary ductal design within the mammary fat pad is visible at birth, which grows at the same rate as the animal until the onset of puberty. **b.** During puberty, the cyclical production of ovarian estrogen and progesterone promotes and accelerates the ductal outgrowth. At this stage, TEBs appear. **c.** In the mature virgin, the entire fat pad is filled with a regular spaced system of primary and secondary ducts. **d**. Hormonal changes that occur when pregnancy begins increase cell proliferation and the formation of the alveolar buds. **e.** During lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components into the lumina.

Involution. The last stage of the mammary life cycle involves the removal of the differentiated MECs, and the remodeling of the gland to a duct system similar to that in the mature virgin. The involution of the mammary gland is triggered by the combination of milk stasis and the fall in prolactin levels. Lack of suckling and milk stasis results in a rapid, but reversible induction of apoptosis within the differentiated populations of MECs. If the lack of suckling is prolonged, PRL levels decline below a threshold level, and apoptosis is accompanied by a tissue-remodeling phase involving the induction of matrix-degrading enzymes and inflammatory cell infiltration. Once the transition to the alveolar remodeling phase begins, the process of involution cannot be reversed. The result of this process is the elimination of all lobulo-alveolar structures leaving behind a simple ductal tree (Hovey R, 2002).

1.4 Homeobox genes and mammary gland

The mammary gland is an excellent model for study the gene expression and their function during the development, because it maintains the plasticity to repeated cycles of growth, morphogenesis, and functional differentiation at the onset of each pregnancy. Specific regulatory molecules able to integrate a variety of signals from the cell's microenvironment and allow the MASCs to progress in a coordinated manner, to ensure the maintenance of cell fate and tissue identity, have been identified (Chen H and Sukumar S, 2003). The microenvironment is composed of myoepithelial and endothelial cells, fibroblasts, myofibroblasts, leukocytes, other cell types, and ECM molecules, which support stem cells (SCs) functions. In the mammary gland the SC niche modulates the tissue specificity of the normal breast, such as the growth, survival and polarity, and the role of the MASCs, by paracrine signals (Pagani IS, 2010).

The homeobox (HB) genes appear to be good candidates for the orchestration of this complex developmental phenomenon, in which form and function are dynamically intertwined.

In the normal mammary gland HB genes are involved in ductal formation, epithelial branching and lobulo-alveolar development, regulating epithelial proliferation and differentiation, through positive or negative cell cycle control (Fig. 1.11). They are controlled in a spatial and temporal manner in both stromal and epithelial cells, and they are coordinately regulated by hormones and ECM, and by other unknown factors, suggesting that many signaling pathways are involved in the HB gene functions. Mammary development is driven by a complex network of hormones

acting systemically, and is regulated locally by ECM molecules. If HB genes play a role in mammary gland development, it would be very reasonable to propose that HB gene expression is linked to primary endocrine regulation and is responsive to ECM changes. Many signaling pathways, including those involving the hormones and the growth factors, may be integrate into the HB genes networks to systematically regulate mammary gland development. The expression of HB genes may regulate the ECM and hormone molecules directly or indirectly, and the hormones and the ECM/growth factors may control the HB gene expression, with feed-back mechanism. These suggestion have been supported by the presence of potential estrogen responsive element (ERE) binding site (5'-GGTCAGCTGAC-3') in the proximal promoter of Hoxc6 gene, by in silico analysis. In addition has been reported that Otx1 and Otx2 bind to the responsive element in the promoter of LH, FSH, GH and GnRH genes, and regulate their expression in the pituitary gland (Chen H and Sukumar S, 2003).

The accurate spatial and temporal regulation of HB genes during mammary gland cycle are required to ensure proper differentiated state. Misexpression of these genes in mammary cells may led to failure of differentiation or uncontrolled proliferation, and therefore contribute to cancer initiation or progress. Aberrant expression of the HB genes are found in breast cancer cells (see table 1.1) and primary carcinomas (Chen H and Sukumar S, 2003).

Functional analysis indicate that the HB genes play an important role in cell cycle, proliferation, angiogenesis, and metastasis (Chen H and Sukumar S, 2003).

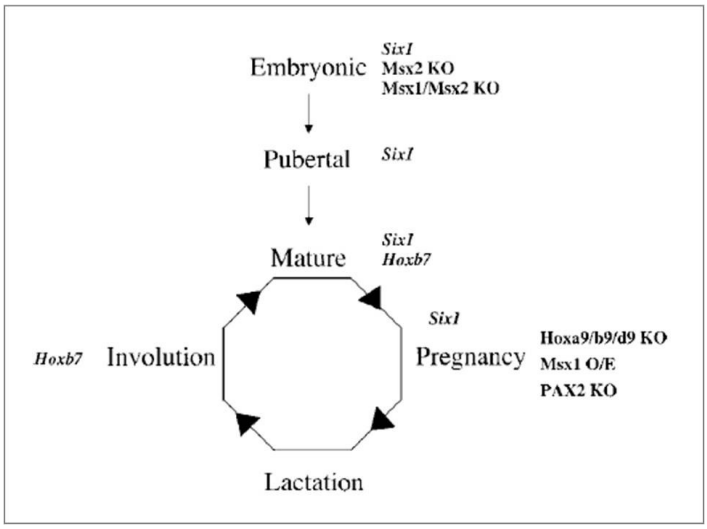


Figure 1.11. Homeobox genes implicated in proliferation in the developing mammary gland. The various stages of mammary gland development are depicted along with HB genes implicated .

1.5 Breast cancer and cancer stem cells

Breast cancer is the leading cause of cancer related death in women in the United States and Europe. Despite significant advances in the diagnosis and in the treatment of breast cancer, several unresolved clinical and scientific problems remain. They are related to prevention (who needs it and when), diagnosis (more specific and sensitive methods are necessary), tumor progression and recurrence (what causes it and how to predict it), treatment (who should be treated and how), and therapeutic resistance (how to predict, prevent, and overcome it). Resolving all these problems is complicated by the fact that breast cancer is not a single disease but is highly heterogeneous at both the molecular and clinical levels (Polyak K, 2007).

It is now known that breast cancers can be clustered on the basis of gene expression patterns into at least five, and probably more, molecular subtypes (Molyneux G, 2007).

Luminal A and B tumors have a gene expression pattern resembling normal luminal breast cells. Luminal A tumors tend to have higher levels of ERα expression and better prognosis, whereas Luminal B tumors have lower levels, or are ERα -, and have a poor prognosis. In the normal mammary gland the ER+ PR+ cells are not proliferative, while in the breast cancer this correlation is lost. The HER2 tumor group over-express HER2, a member of the epidermal growth factor receptor family of receptor tyrosine kinases. The normal breast-like tumor group shows a gene expression pattern like the normal breast. This group includes non-epithelial breast lesions. Finally, the ERα- PR- HER2- (”triple negative”) basal-like group expresses the basal cytoskeletal markers and it is find in younger women and associated with BRCA1 disease (Molyneux G, 2007).

Cancer stem cells and breast cancer heterogeneity. The “cancer stem cells (CSCs) hypothesis” posits that tumors are derived from mutated stem cells that have retained, or progenitors and differentiated cells that have regained, the stem cell properties of cell-renewal (Fig. 1.12). Only SCs have the capacity to indefinite self-renewal, whereas lineage-committed progenitor cells have lost this capacity, but still show extensive proliferation and differentiation potential (Zucchi I, 2008). The initiation of breast cancer is due to genetic and epigenetic transforming events in the SCs. Subsequent tumor progression is driven by the accumulation of additional genetic changes combined with clonal expansion and selection (Polyak K, 2007). The longevity of

normal SCs increases the risk that these cells, over a lifetime, acquire multiple genetic mutations necessary for tumorigenesis. The SCs hypothesis of cancer predicts that tumors contain a small number of CSCs, which drive tumor growth, and populations of more differentiated non-tumorigenic daughter cells, analogous to the transit amplifying and differentiated cells of the normal tissue (Molyneux G, 2007).

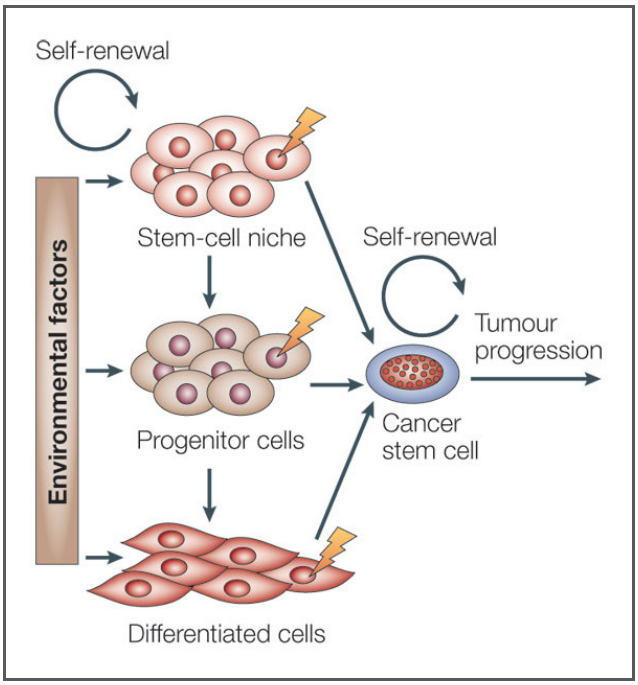


Figure 1.12. The cancer stem cell hypothesis. The cancer stem cell might appear after mutations in specific stem cells or early stem cell progenitors. It is also possible that cancer stem cells can be derived from differentiated cells. There might be numerous factors in the host microenvironment that trigger the initial steps of tumor formation.

Molyneux proposed a model that could explain the diversity of breast cancer, on the basis of the cell type mutation. The normal breast epithelium is composed of myoepithelial, ER+ luminal and ER- luminal cells, as well as stem/progenitor compartment. This model suggested that if mutations occur in the ERα+ luminal cells, the ERα+ tumor type may be generated, while mutations in the ERα- luminal cells may give rise to the ERα- tumor type. If mutations occur in progenitor able to differentiate into either ERα+ or – luminal cells, they could generate tumors with a mix of ERα+ and ERα – cells. Alternatively, basal-type tumors may arise by the

transformation of stem/progenitor cell as the result of the accumulation of genetic damage. In Furthermore, mutations that occur in differentiated cells can confer SC properties of self-renewal (Fig. 1.13) (Molyneux G, 2007).

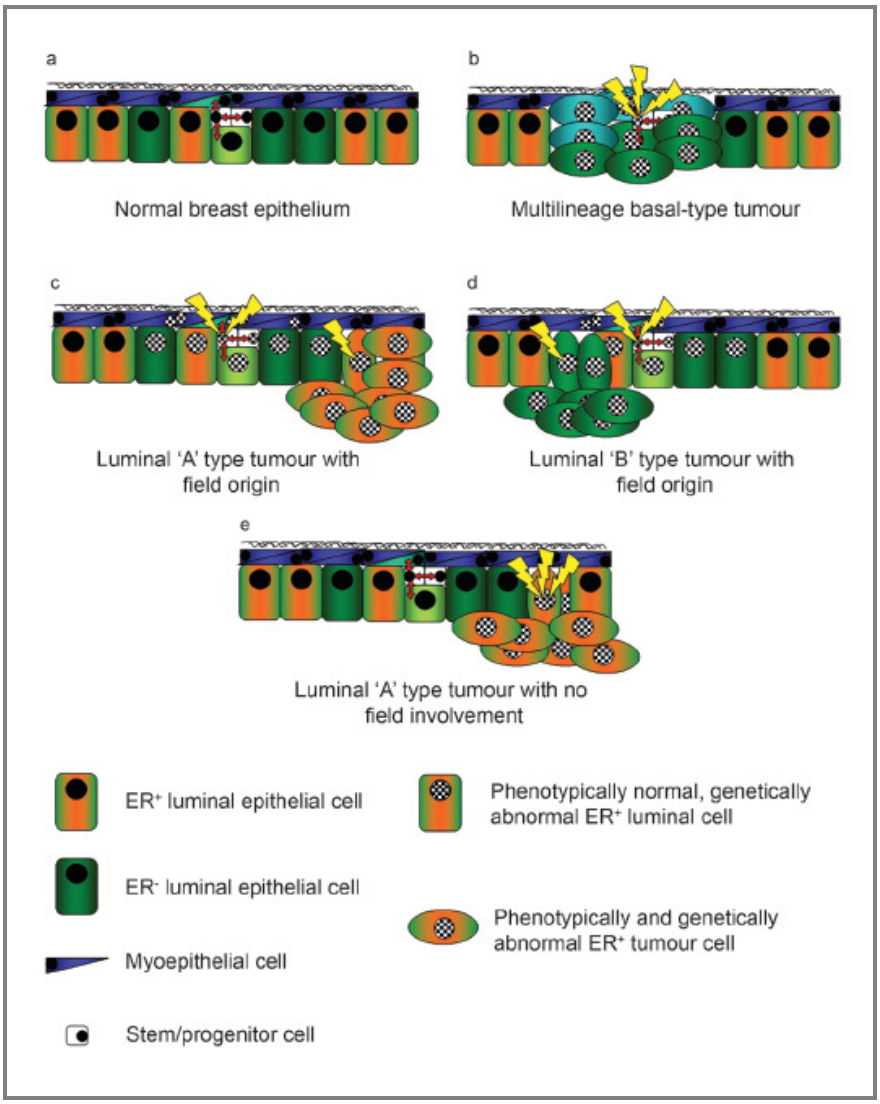


Figure 1.13. A model of the origins of breast cancer subtypes. a. The normal breast epithelium is composed of myoepithelial (blue), ER+ luminal (orange/green) and ER- luminal (green) cells as well as a stem/progenitor compartment (white). **b.** Basal like tumors may arise following transformation of a stem/progenitor cell as a result of accumulation of genetic damage (hatched nuclei). Luminal tumors may arise from a field of abnormal cells generated when stem cells acquire genetic damage without actually transforming. One final genetic lesion in an ER+ luminal cell **(c)** or ER- luminal cell **(d)** within the field generates a tumor. **e.** Alternatively, if the first genetic lesion in a non-stem cell promotes its self-renewal, mutations could accumulate in a differentiated cell to generate a tumor.

Breast cancer progression. The breast cancer involves progression through defined pathological and clinical stages. It starts with the ductal hyper-proliferation, subsequent evolution into *in situ* or invasive carcinomas, that may be cause in a metastatic disease (Fig. 1.14) (Polyak K, 2007).

Normal breast ducts are composed by the basement membrane and a layer of luminal epithelial and myoepithelial cells. Cells composing the stroma include various leukocytes, fibroblasts, myofibroblasts, endothelial cells and ECM molecules. In *the in situ* carcinoma the myoepithelial cells are epigenetically and phenotypically altered and their number decreases, potentially due to the degradation of the basement membrane. At the same time, the number of stroma cells increase. Loss of myoepithelial cells and basement membrane result in invasive carcinoma, in which tumor cells invade surrounding tissues and migrate to distant organs, eventually leading to metastases. Molecular studies revealed that myoepithelial cells associated with *in situ* ductal carcinoma (DCIS) showed the lost of differentiation markers and the up-regulation of the genes, that promote angiogenesis and invasion (Polyak K, 2007).

DCIS-associated with myoepithelial cells have increased levels of ECM-degrading enzymes, such as several metalloproteinases (MMPs), that cause the ductal basal-membrane and ECM degradation, correlated to the occurrence of invasive tumors (Polyak K, 2007).

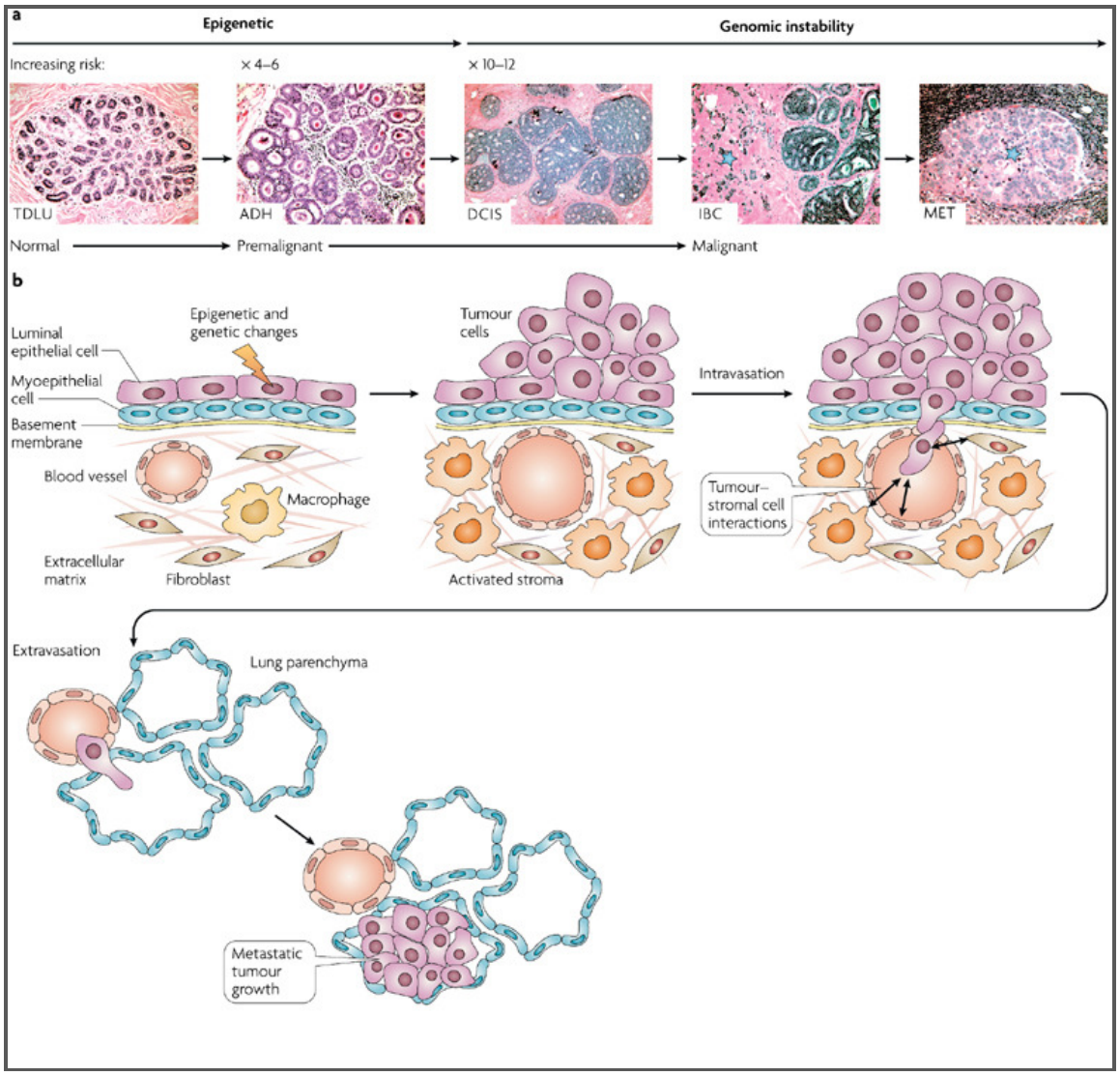


Figure 1.14. The breast cancer progression. a. Breast cancer is a genetically heterogeneous disease that develops along a continuum. The normal breast terminal ductal and lobular unit (TDLU) contains lobules and ducts. Atypical ductal hyperplasia (ADH) is a premalignant lesion characterized by abnormal cell layers within the duct or lobule. ADH is thought to be the precursor of ductal carcinoma *in situ* (DCIS), which is a non-invasive lesion that contains abnormal cells. With each stage, the risk of developing malignant or invasive breast cancer (IBC) increases. Once cells have invaded the stroma, the risk for developing metastasis significantly increases. The lymph nodes are the primary site for breast cancer metastasis (MET; indicated by a blue star). **b.** Schematic representation of breast cancer progression.

Microenvironmental factors may play a key role in tumorigenesis. Epithelial-mesenchymal interactions are known to be important for the normal development of the mammary gland and for the breast tumorigenesis. *In vivo* and *in vitro* studies demonstrated that the cells composing the microenvironment and the ECM molecules, modulate tissue specificity of the normal breast as well as the growth, the survival, the polarity, and the invasive capacity of the breast cancer cells (Polyak K, 2007). The microenvironment affects the efficiency of tumor formation, the rate of tumor growth, the extent of invasiveness, and the ability of cancer cells to metastasize. In carcinomas, the influences of the microenvironment are mediated, in large part, by paracrine signaling between epithelial tumor cells and neighboring stromal fibroblasts. Carcinoma formation from its earliest stages seems to depend on the ability of tumor cells to recruit and then subvert a variety of stromal cells originating from adjacent normal tissue. The most common stromal response in carcinoma is termed "desmoplasia", and it is characterized by pronounced changes in the ECM, including increased amounts of collagens, fibronectin, proteoglycans, and glycosaminoglycans. The desmoplastic stroma becomes highly vascularized as new blood vessels are recruited into the developing tumor mass. Fibroblasts in the stroma can become activated by the tumour cells and may secrete angiogenic and invasion-promoting factors (Elenbaas B and Weinberg RA, 2001).

1.6 The p53 family

The p53 protein, discovered in 1979 as the protein interacting with the oncogenic T antigen from SV40 virus, is a transcription factor product of a pivotal tumor-suppressor gene Tp53, "the guardian of the genome". The active form of p53 is a tetramer of four identical subunits, constitutes by the assemble of two dimers. Due to its tetrameric conformation, p53 is able to bind the DNA site containing four repeats of the pentameric sequence motif, called p53-responsive element (p53RE), in the promoter of the target genes (Chan WM, 2004) (Bourdon JC, 1997). The p53RE sequence is represented by 5'-PuPuPuCA/TT/AGPyPyPy-3', where Pu is purine and Py is pyrimidine.

p53 activity is promoted by a wide range of stress signals potentially affecting genome integrity and proper cell proliferation. When activated, p53 is able to coordinate complex cellular responses that lead to cell-cycle arrest, DNA repair, senescence, or programmed cell death (apoptosis) (Collavin L, 2010). These effects are predominantly due to p53's ability to bind

DNA through the p53REs that regulate the transcription of genes involved in these process, such as p21 (cell cycle arrest), Puma or Scotin (apoptosis). Therefore, p53 prevents the proliferation of the genetically abnormal cells and the cancer formation. Mutations in the Tp53 gene has been frequently associated with human cancer due to its loss of function (Murray-Zmijewski F, 2006).

Two Tp53-related genes, the Tp63 and the Tp73, were identified in 1997. The high level in sequence similarity between p53, p63 and p73 proteins, particularly in the DNA binding domain, allows to p63 and p73 to transactivate p53-responsive genes causing cell cycle arrest and apoptosis. However, they are not functionally redundant, and each protein has unique functions as determined by the generation of transgenic knockout mice (Murray-Zmijewski F, 2006).

In addition, p53 gene family members express multiple transcripts variants due to alternative splicing and use of different promoters. These isoforms code for several proteins, containing different domain, and show tissue-specific expression (Murray-Zmijewski F, 2006). Depending on the different regulation of these isoforms, the proteins may play complex roles in the control of cell growth, differentiation and development. In particular, the generation of a gradient of expression of the different isoforms within the tissue may represent an important signal for the spatial and temporal regulation of differentiation (Courtois S, 2004).

p53. The human Tp53 gene consists of 11 exons spanning over 19 Kb (GenBank Accession Number: NC_000017) on chromosome 17p13.1 (Murray-Zmijewski F, 2006). Human p53 protein, a monomer of 53 KDa, can be divided into five domains, each corresponding to specific functions. The amino-terminal region contain the acid transactivation domain, which is divided into two subdomains (TA1 and TA2) with complementary functions. While the main domain (TA1) provides to generic transactivation functions, the second one (TA2) may play a role in the selection of specific transactivation targets. The first domain also contains the binding site for the mouse double-minute 2 (MDM2), the main regulator of the p53 protein stability. These two domains are followed by poly-proline-rich site (PRD) (PXXP) (where P indicates proline and X indicates any amino acid), with complex roles of protein-binding site and regulator of apoptosis. The central part of the protein contains the DNA binding domain (DBD), in which about 90% of the p53 mutations have been found in human cancer. This region is followed by the oligomerization domain (OD) consisting of a beta-strand and alpha-helix necessary for dimerization. A nuclear export signal (NES) is localized in this domain. The carboxy-terminus

domain contains three nuclear localization signals (NLS) and a non-specific DBD that binds to damaged DNA, and is involved in the down-regulation of the DB of the central domain (Courtois S, 2004).

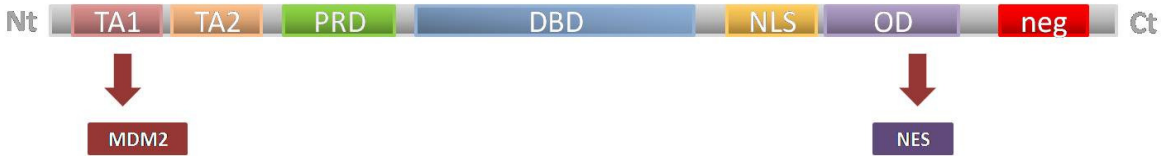


Figure 1.15. Primary structure of the p53 protein.

The p53 pathway. In normal unstressed cells, the half-life of the p53 protein varies between 6 and 20 min (Riley T, 2008). The levels of p53 protein are down-regulated by the binding of proteins, such as MDM2, COP1, PIRH2 or JNK, that promote p53 degradation via the ubiquitin/proteasome pathway (Harris SL and Levine AJ, 2005). A key negative regulator of p53 is MDM2, which binds to the TA domain of p53, and ubiquitylates the protein, targeting it for degradation in the proteasome. Because p53 transcriptionally activates MDM2, the expression levels of p53 and MDM2 are balanced through a negative feed-back loop, which is altered by an increase in p53 levels following stresses, such as DNA damage. MDM2 also binds to Tp53 mRNA, controlling the rate of translation (Whibley C, 2009).

In response to a stress signal, the p53 protein is rapidly activated in a specific manner by post-translational modifications, form a tetramer, and HDM2 polyubiquitylates itself, which results in the degradation of HDM2 (Riley T, 2008). The half-life of the protein in the cell increases, from 6-20 min to hours, and this results in 3-10 fold increase of the p53 protein concentration in the nuclei (Harris SL and Levine AJ, 2005).

The p53 pathway responds to a wide variety of stress signals. These include several types of DNA damage: telomere shortening, hypoxia, mitotic spindle damage, heat or cold shock, unfolded proteins, improper ribosomal biogenesis, nutritional deprivation, and even the activation of some oncogenes by mutations (Riley T, 2008).

These stress signals are detected by various “signal mediator proteins”, such as protein kinases, histone acetyl-transferases, methylases, ubiquitin and sumo ligases, that activate the p53 protein by modifications at different amino-acid residues. Different stress signals can induce phosphorylation at the serine and threonine residues, acetylation, methylation, ubiquitination or sumolation in the p53 protein. The nature of the stress signal determines the type of protein modification and, therefore, the transcriptional programme of p53. For example, gamma-radiation activates the ATM kinase and the CHK-2 kinase, both of which can phosphorylate the p53 protein, while UV-radiation activates ATR, CHK-1 and casein kinase-2, which results in the modification of different amino-acid residues on the p53 protein (Harris SL and Levine AJ, 2005).

As p53 is activated by these protein modifications, it can be inactivated by phosphatases, histone deacetylases, ubiquitinases or even inhibitors of ubiquitin ligases (Harris SL and Levine AJ, 2005).

The activated p53 tetramer binds to specific p53REs in the promoter of the down-stream target genes, mediating their transactivation. Through protein-protein interactions, p53 can bind to and then recruit general transcription proteins, such as TATA-binding protein-associated factors (TAFs), to the promoter-enhancer region of p53-regulated genes. Recent experiments have shown that p53 can also recruit the histone acetyltransferase (HATs) CBP, p300 and PCAF, that acetylate Lys residues of histones in the chromatin of target genes, increasing transcriptional activity (Riley T, 2008).

In some genes, the binding of p53 to its RE results in direct repression of that gene. The p53 protein recruits histone deacetylases (HDACs), that deacetylates Lys residues of histones in chromatin , thereby repressing gene transcription (Riley T, 2008).

In response to specific signals, p53 activates different pathways that lead to cell cycle arrest, DNA repair, differentiation, senescence or apoptosis. In contrast to senescence and apoptosis, DNA repair and differentiation do not eliminate cells from the organism, but permit to reverse the damage and remove cells from the proliferative compartment. The choice between these outcomes in a stressed cell depends of a different transduction pathways (Fig. 1.16) (Riley T, 2008).

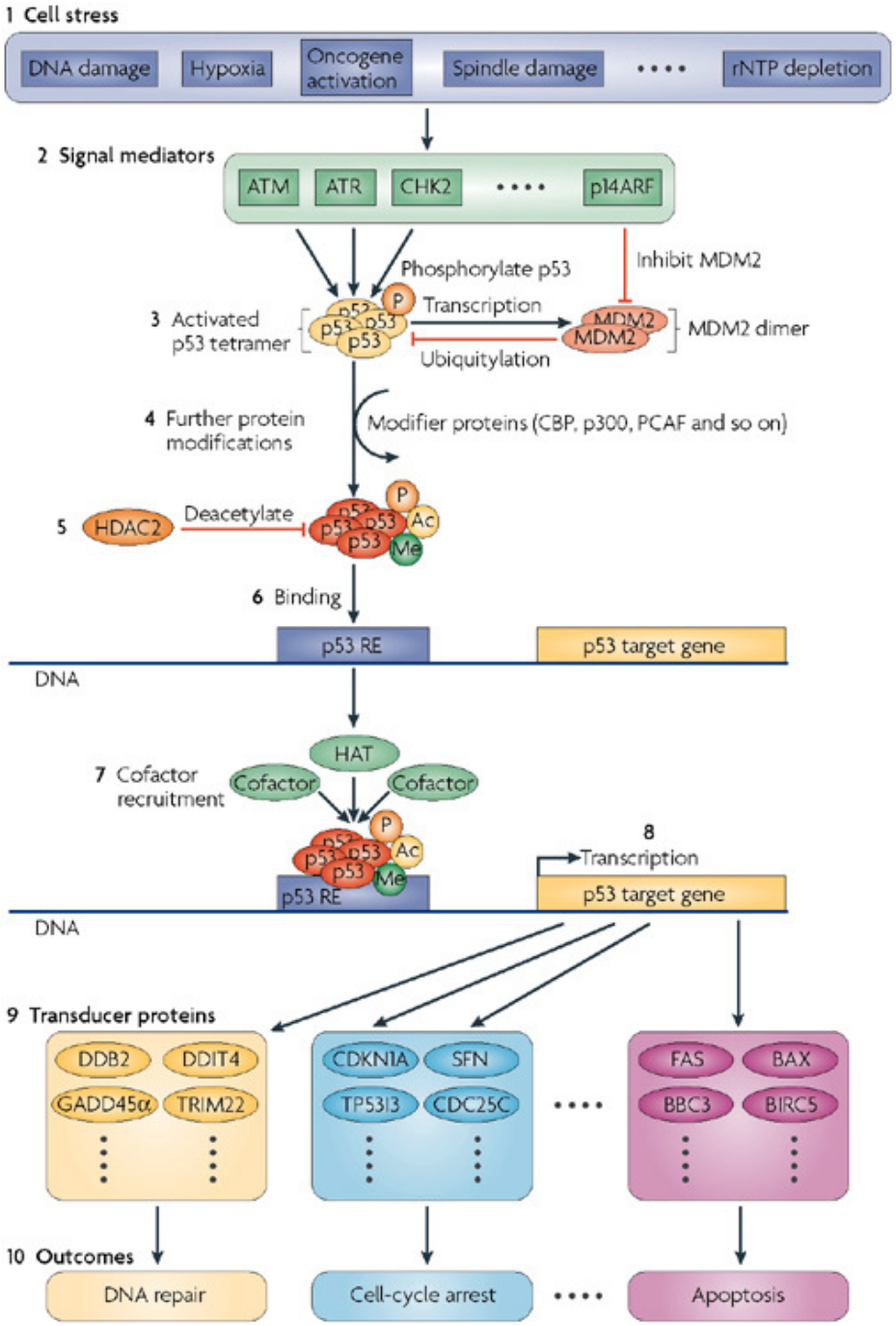


Figure 1.16. Mechanisms of p53 activation and regulation of downstream target genes.

p53 and tumors. In the tumors has been found the loss of function of p53, due to direct or indirect mechanisms. The p53 protein can be inactivated by mutations, or by nuclear exclusion or protein degradation (Gasco M, 2002).

In about 50% of common human cancers, Tp53 remains the most commonly mutated gene, with mutations, principally missense, in the DBD (Gasco M, 2002). The p53 mutated protein exert a dominant-negative effect toward the wild-type p53, through the formation of heterotetramer (Whibley C, 2009). Breast carcinomas, associated with germ-line mutations of Brca1 and/or Brca2 genes, and expressing the mutant Tp53, correlate with more aggressive disease and worse overall survival (Gasco M, 2002).

Germ-line mutations in Tp53 occur in a high proportion of individuals with the Li-Fraumeni cancer susceptibility syndrome, which confers an increased risk of breast cancer. This implies an important role for p53 inactivation in mammary carcinogenesis (Gasco M, 2002).

In the cancers lacking Tp53 mutations, p53 function is compromised by other mechanism, such as protein degradation or nuclear exclusion. For example, in virus-associated cancers, this may occur by direct interaction with proteins encoded by virus, resulting in the retaining of p53 protein in the cytoplasm, enhancing p53 degradation. Various DNA viruses, such as SV40, HPV or adenovirus, encode for proteins that target p53 protein. The E6 viral protein expressed by HPV specifically binds to p53 and induces its degradation. This observation explain the rarity of p53 mutations in HPV-related cervical cancer (Tp53 website). Nuclear exclusion may be exercised by an over-expression of HDM2. This protein is able to bind to the p53 protein and to promote the ubiquitination of the C-terminus of the p53 that causes the p53 degradation (Gasco M, 2002).

Other indirect mechanisms can contribute to p53 inactivation. Mutations have been identified in up-stream regulators of p53, in its co-activators and in transcriptional target genes of p53. For example, ATM kinase, activated by gamma-radiations, phosphorylates p53 on Ser 15, to protect DNA from radiation damage. Mutations in ATM, found in ataxia-telangectasia or in T-cell leukemia, impair the p53 response to gamma-radiations (Tp53 website). A co-activator of p53 is the BRCA1 protein. BRCA1 is phosphorylated, after DNA damage, by ATM, ATR and CHK2 proteins. Activated BRCA1 associates with the C-terminus of p53 and stimulates transcription from p53-responsive promoters. Tumors with mutants BRCA1 are deficient in co-activating activity (Gasco M, 2002).

p53 isoforms. The different functions of the p53 protein in cell growth, differentiation and development are due to the presence of tissue-specific p53 isoforms, able to response to stress (Fig. 1.17) (Courtois S, 2004).

The Tp53 gene is characterized by one promoters upstream to the exon1 (P1), and by an internal promoter located in intron 4 (P2). The usage of the distal promoter P1 leads to the production of p53 and Δ40p53 isoforms, while the internal promoter P2 regulates the expression of Δ133p53 isoforms. p53 is the full-length protein (FLp53), while Δ40p53 and Δ133p53 are N-terminal p53 isoforms (Bourdon JC, 2005).

Δ40p53 isoform is produced by alternative splicing of the intron 2, or by internal initiation of translation using ATG +40 of p53 transcript, that results in the lack of the first TA domain (Bourdon JC, 2005).

Δ133p53 isoform is encoded by the P2 promoter in intron 4. It lacks the TA domains, the poly-proline-rich site domain, and part of the DNA-binding domain (Bourdon JC, 2005).

In all the transcripts, the intron 9 can be alternatively spliced to produce three isoform, α, β and γ, where β and γ isoforms lack the oligomerization domain (Bourdon JC, 2005).

In summary the Tp53 gene encodes at least for nine different proteins: p53(α) full-length (the wild-type, FLp53), p53β, p53γ, Δ40p53(α), Δ40p53β, Δ40p53γ, Δ133p53(α), Δ133p53β and Δ133p53γ.

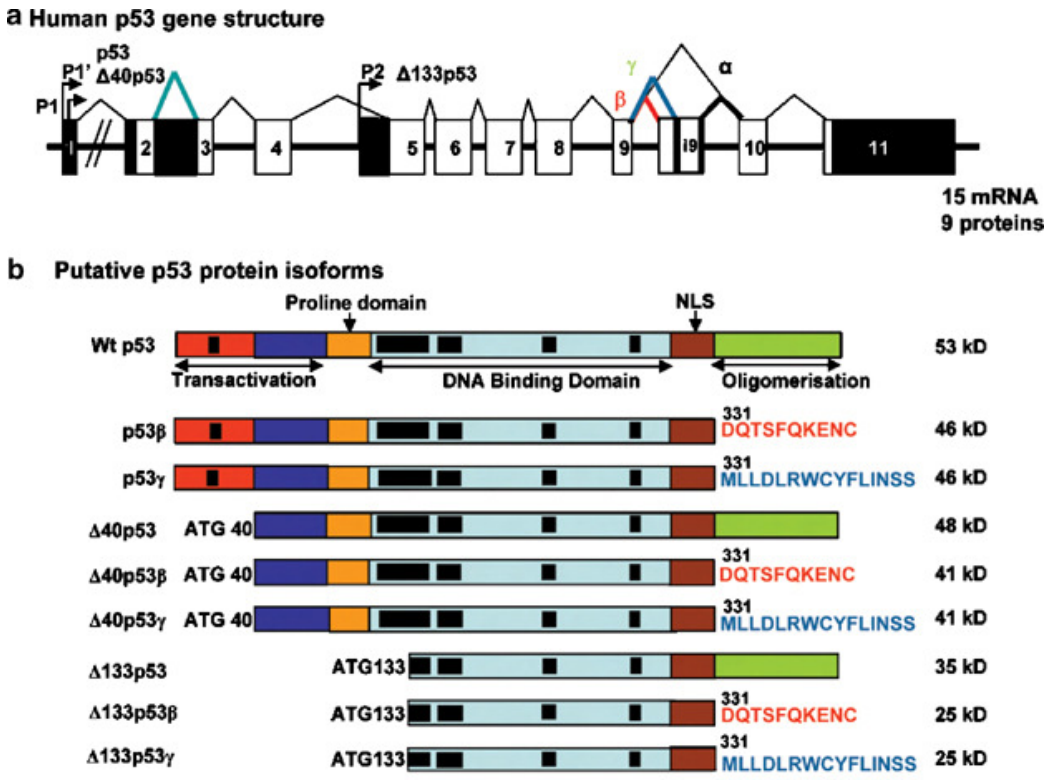


Figure 1.17. Schematic representation of the human Tp53 gene (a) and the human p53 protein isoforms (b).

FLp53 and its isoforms have distinct biochemical activities, and the complex balance between FLp53 and the isoforms regulates the cellular fate in response to p53 activation. The N-terminal isoforms can have a dominant-negative function on the p53 wild-type and inhibit its function (Levrero M, 2000).

Δ40p53 (also named p47 or ΔNp53) contains a part of the p53 TA domain and it can activate gene expression through the second TA domain located between amino acids 43 and 63. Δ40p53 can act in a dominant-negative manner toward FLp53, inhibiting both p53 transcriptional and the p53-mediated apoptotic activity, and can modify the p53 cell localization and inhibit p53 degradation acts by the MDM2 (Bourdon JC, 2005).

Recently it has been demonstrated that p53 regulates directly the transcription of its Δ133p53 isoform through specific RE contained within the TP53 P2 internal promoter, and Δ133p53 protein may participate in a negative feedback loop controlling and inhibiting the p53 functions, through formation of heterotetramers (Marcel). In zebrafish the human homolog of Δ133p53,

prevents the p53-mediated apoptosis in response to embryonic defects or DNA damage, and antagonizes p53-mediated apoptosis and G-1 cell cycle arrest, and can inhibit replicative senescence (Aoubala M, 2010).

In normal breast tissue is detectable the expression of p53/p53β/p53γ, but not ΔN133p53; in breast cancer ΔN133p53 is over-expressed, and p53β is less detectable (Bourdon JC, 2005).

p53 knock-out mice. In order to assess the role of p53, mice lacking p53 have been generated (Blackburn AC, Jerry DJ, 2002). Mice homozygous for the Trp53 null allele (Trp53-/-) die at 4-6 month of age, primarily from lymphomas. Mammary tumours are not detected, because the early death, due to lymphoma, obscures the mammary tumor development. Trp53-/- mammary epithelium was transplanted into cleared fat pads of wild-type mice, demonstrating the formation of spontaneous mammary tumors (60%). p53 deficiency can promote mammary tumor formation in mice as well as in humans. The tumors formed were moderately to poorly differentiated adenocarcinomas, with high levels of aneuploidy. Trp53+/- mice survived much longer than Trp53-/-, but they also succumbed to lymphomas, and sarcomas (Blackburn AC, Jerry DJ, 2002).

A significant proportion (23%) of Trp53-/- female mice die during embryogenesis, or in the period between birth and weaning, being subjected to a spectrum of abnormalities. In these mice the normal process of neural tube closure fails, leading to exencephaly and subsequent anencephaly. In addition, many of these embryos exhibit craniofacial malformations, including ocular abnormalities and defects in upper incisor tooth formation (Murray-Zmijewski F, 2006).

Trp53-/- female mice, but not Trp53 null male mice, have a significant decrease of fertility (Hu W, 2009).

The p53 protein regulates maternal reproduction in mice, through regulation of leukemia inhibitory factor (Lif) gene. LIF protein plays a crucial role in blastocyst implantation. Implantation is a critical stage in embryonic development, during which the blastocyst establishes a close interaction with the uterine tissues, which lead to the formation of the placenta. Increase of expression of Lif gene, regulated by estrogen and p53, is coincident with the onset of implantation. Trp53 female null mice have an impaired implantation function, due to the decrease levels of LIF protein (Hu W, 2009).

p63 isoforms. The human Tp63 gene consists of 15 exons, spanning over 270 Kb in the chromosome 3q27 (GenBank Accession Number: AC078809, AC117486 and AC063939) (Fig. 1.18) (Murray-Zmijewski F, 2006).

The Tp63 gene is characterized by one promoters upstream to the exon1 (P1), and by an internal promoter located in intron 3 (P2). The transactivating isoforms (Tap63) are generated starting from the promoter P1, while the N-terminally truncated isoforms (ΔNp63), lacking the TA domain, are generated by the alternative P2 promoter (Murray-Zmijewski F, 2006).

Altogether, Tp63 expresses at least six mRNA variants which encode for six different p63 protein isoforms, TAp63α, TAp63β, TAp63γ, DNp63α, DNp63β, DNp63γ, in which α, β, γ result from an alternatively spliced C-terminal protein (Murray-Zmijewski F, 2006).

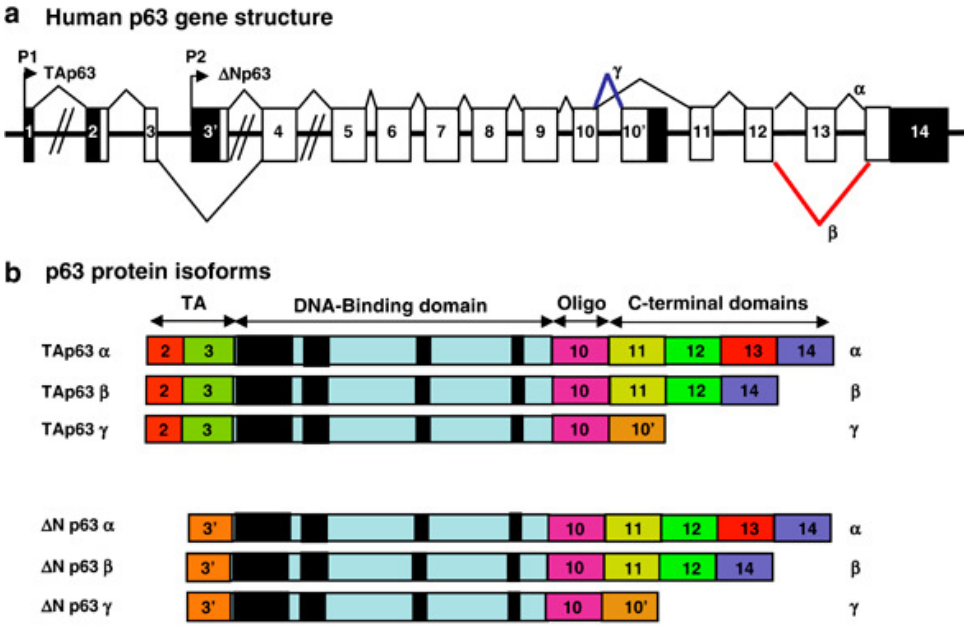


Figure 1.18. Schematic representation of the human Tp63 gene (a) and the human p63 protein isoforms (b).

The TAp63 isoforms are able to bind to DNA through p53REs, and activate transcription factors of target genes. This activation induces cell cycle arrest or apoptosis (Murray-Zmijewski F, 2006). However, recent studies indicate that p63 proteins can bind to the DNA through specific

p63RE conferring responsiveness only to the p63 protein but not to the p53 protein (Murray-Zmijewski F, 2006).

TAp63 has a role in female reproduction. In response to genotoxic stress, Tap63 induces apoptosis of oocytes, having a crucial role in genome protection of female germ line. A more recent study established a function of Tap63 in mediating senescence and preventing tumorigenesis. In addition, Tap63 has a crucial role in preventing invasiveness and metastasis of epithelial tumors by controlling expression of a crucial set of metastasis-inhibitor genes (Collavin L, 2010). The ΔNp63 isoforms can bind to DNA through the p53RE and can exert dominant-negative effects over p53, Tap63 and Tap73 activities, by either competition for DNA binding sites or by direct protein-protein interaction (Murray-Zmijewski F, 2006).

ΔNp63 isoforms directly activate specific gene targets not induced by TA isoforms, since they contain a unique 14 amino acid sequence that contributes to the formation of an alternative TA2 transactivation domain. ΔNp63α is an epithelial progenitor cell marker able to maintain epidermal SC self-renewal capacity, promoting cell proliferation and during embryogenesis are required for the initiation of the epithelial stratification (Craig AL, 2010).

p63 knock-out mice. Trp63-null mice are alive, but die immediately after birth. The mice show a severe phenotype, with defects in the apical ectodermal ridge essential to limb development resulting in truncated limbs. In addition, Trp63-/- mice do not have hair follicles, teeth, mammary, lachrymal and salivar glands. The skin does not progress past an early developmental stage: it lacks stratification and does not express differentiation markers (Fig. 1.19.A-D) (Levrero M, 2000).

The phenotype of Trp63-deficient mice suggests that the primary biological function of p63 proteins is to regulate development of epithelia and limbs (Levrero M, 2000).

Recently, germ line mutations of Trp63 are found in humans and cause six rare autosomal dominant developmental disease which include: Ectrodactyly Ectodermal dysplasia-Clefting syndrome (EEC); Acro-dermato-ungual-lacrimal-tooth malformations (ADULT); Lymb-Mammary Syndrome (LMS); Hay-Wells syndrome, also named AEC syndrome for Ankyloblepharon and Ectodermal dysplasia-Clefting; Split-hand/foot malformations (SHFM); Rapp-Hodgkin Syndrome (Fig. 1.19.E) (Murray-Zmijewski F, 2006).

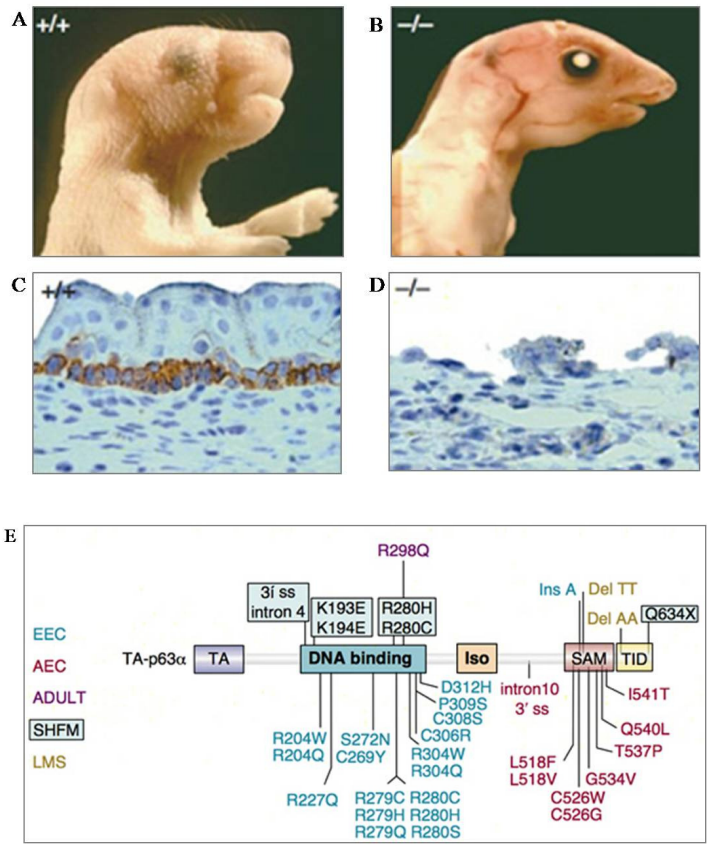


Figure 1.19. A-D. p63 knock-out mice. p63 knock-out mice exhibit developmental defects **(B)** and does not express differentiation markers **(D)**, as the control **(A, C)**.

Figure 1.19.E. Mutations of p63 in human disease. Location of p63 mutations described in different diseases. These include ectrodactyly ectodermal dysplasia and cleft lip/palate syndrome (EEC), ankyloblepharon ectodermal dysplasia-clefting (AEC or Hay-Wells syndrome), split-hand/split-foot malformations (SHFM), limb-mammary syndrome (LMS) and acro-dermato-ungual-larimal-tooth (ADULT) syndrome. Mutations are mainly found in the DBD for EEC, ADULT and SHFM patients, and in the SAM domain for AEC and LMS patients. Abbreviations: DBD, DNA-binding domain; OD, oligomerization domain; PR, proline-rich regulatory region; SAM, sterile a motif; TA, transactivation domain; TA2, second transactivation domain; TI, transactivation inhibitory domain.

p73 isoforms. The human Tp73 genes consists of 15 exons spanning over 80 kb (Gen Bank Accession Number: AL136528) located in chromosome 1p36.3 (Fig. 1.20) (Murray-Zmijewski F, 2006). Tp73 express at least seven alternatively spliced C-terminal isoforms (α, β, γ, δ, ε, ζ and η), and at least four alternatively spliced N-terminal isoforms initiated at different start codon. Like Tp53 and Tp63, Tp73 can be transcribed from an internal P2 promoter located in the intron 3. The TA isoforms are generated by the activity of the promoter upstream of exon 1,

while the alternative promoter in intron 3 leads to the expression of N-terminally truncated isoforms (ΔNp73) lacking the TA domain. Altogether, Tp73 expresses at least 35 mRNA variants, which may encode for 29 different p73 protein isoforms. So far, 14 different isoforms have been described. In contrast to p63, p73 isoforms can start from different ATG codon (ex2 p73, ex2/3 p73 and DN'p73) and contain different part of the N-terminal domain, suggesting that they can have distinct protein interactions and specific activities (Murray-Zmijewski F, 2006).

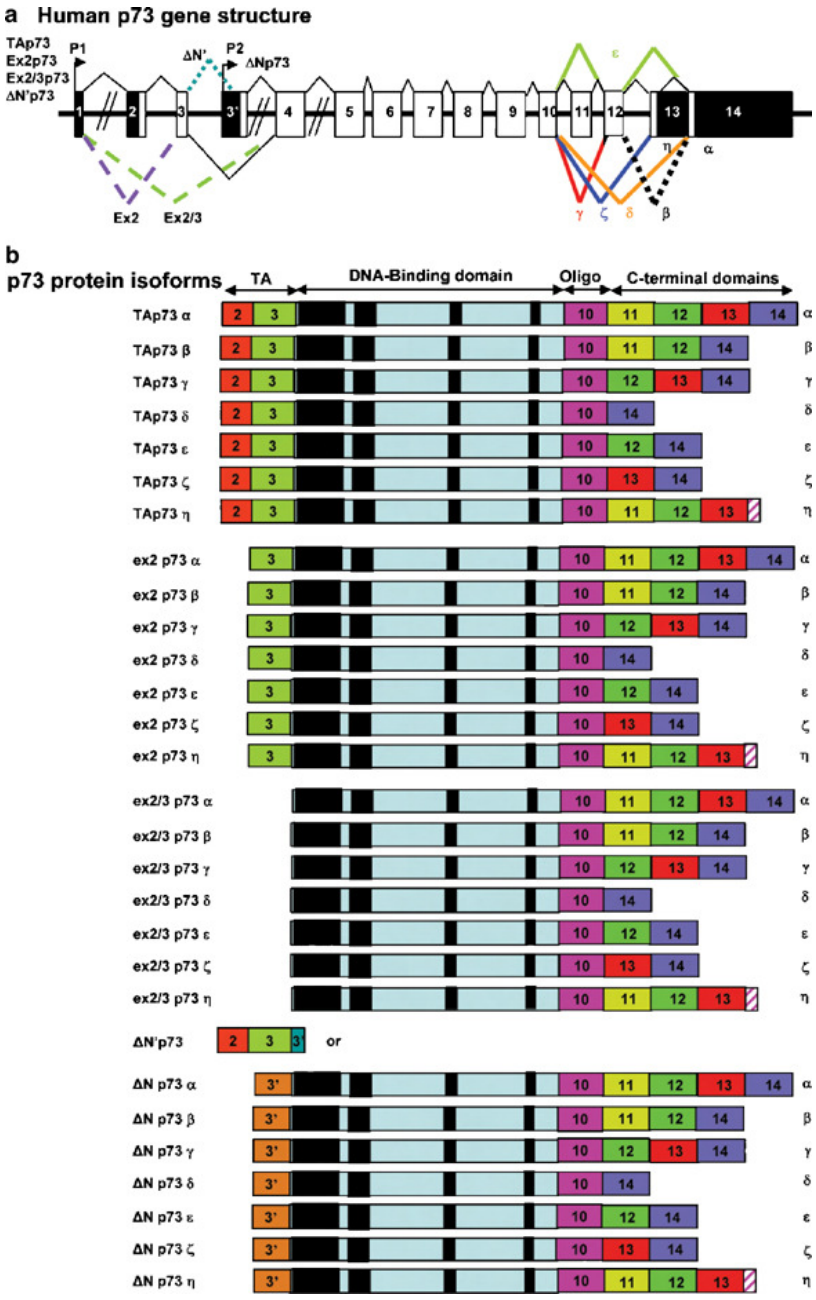


Figure 1.20. Schematic representation of the human Tp73 gene (a) and the human p73 protein isoforms (b).

Tap73 isoforms, like Tap63 isoforms, are able to bind specifically to the DNA through the p53 RE and activate transcription of the target genes, involved in the cell cycle arrest and apoptosis. These isoforms are able to bind the P2 promoter on the Tp73 gene and activate transcription of the ΔNp73 isoforms, in response to genotoxic stresses. The ΔNp73 isoforms bind to the DNA through the p53REs and exert dominant-negative effects over the p53, Tap63 and Tap73 activities, and may bind to specific p73REs directly activating specific gene targets not induced by TA isoforms. ΔNp73 is the predominant form in the developing mouse brain and is the only form of p73 in the neonatal brain and sympathetic ganglia. ΔNp73 plays an essential antiapoptotic role during development, acting as a dominant-negative inhibitor of p53, during the normal "sculpting" of developing neuronal system (Zaika AI, 2002).

TAp73 isoforms represent important mediators of apoptosis in particular following the chemotherapy-induced DNA damage. TAp63 is also known to play a role in the female oogenesis, in which it is essential to eliminate the DNA damage-oocytes. Tap73 prevents genomic stress, and loss of Tap73 during aging may contribute to the decline in oocytes viability (Gonfloni S, 2009).

p73 knock-out mice. Trp73 null mice, like p63 null mice, are born viable, but show developmental defects including congenital hydrocephalus, hippocampal dysgenesis, due to disappearance of Cajal-Retzius neurons, and defects of pheromone detection that lead to lack of interest in sexually mature females. In addition to these severe neurogical defects, Trp73-/- mice show a generalized pan-mucositis, with consequent microbiological infections, which are characterized by massive neutrophil infiltration at the affected sites. The pathogenesis of this massive inflammation, however, is not clear, since no major defects of the lymphoid and granulocyte populations are present in these mice; this raises the possibility that the inflammation and the infections are related to the epithelial barrier dysfunctions, as in cystic fibrosis. Interestingly, Trp73-/- mice did not show spontaneous tumors up to 15 months of age. (Levrero M, 2000).

Studies of different tumors types have demonstrated that the p73 gene is over-expressed, like p63, rather than mutated or deleted in human cancers. ΔNp73 forms a complex with ΔNp63 counteracting with TAp73, inhibiting p73-dependent proapoptotic activity (De Young MP and Ellisen LW, 2007).

Structural motifs in p53/p63/p73 proteins. The three members of the p53 family share high homology both at the nucleotidic and aminoacidic level (Fig. 1.21). Each contain the TA domain, the DBD and the OD. The highest level of homology is reached in the DBD, suggesting that the three proteins can bind to the same DNA sequence and transactivate the same promoters. The high homology is also at the level the OD, suggesting that members of this family form hetero-oligomers as well as homo-oligomers. In addition, p63 and p73, but not p53, contain a long C-termini, that contains a sterile alpha motif (SAM), which is a protein-protein interaction domain. The SAM domain is a globular domain composed by four α-helices and a small 3_{10}-helix. The majority of the SAM-domain-containing proteins are involved in the regulation of the development. Unlike other SAM domains, those present in p63 and p73 do not mediate homo-hetero-dimerization, indicating that they are important for interactions with other, as not yet identified proteins (Levrero M, 2000). An additional post-SAM region known as the TA inhibitory domain (TID) has been identified in the p63α and p73α isoforms; its function could be the inhibition of the transcriptional activity of both Tap63α and Tap73α through inter- or intra-molecular association with the TA domain (Fig. 1.22). The presence of TID domain within the ΔNp63α and ΔNp73α isoforms may allow trans-repression of associated TA isoforms, thereby explaining their potent inhibitory effect when bound to DNA as hetero-tetramers composed of both ΔN-α and TA isoforms (De Young MP and Ellisen LW, 2007).

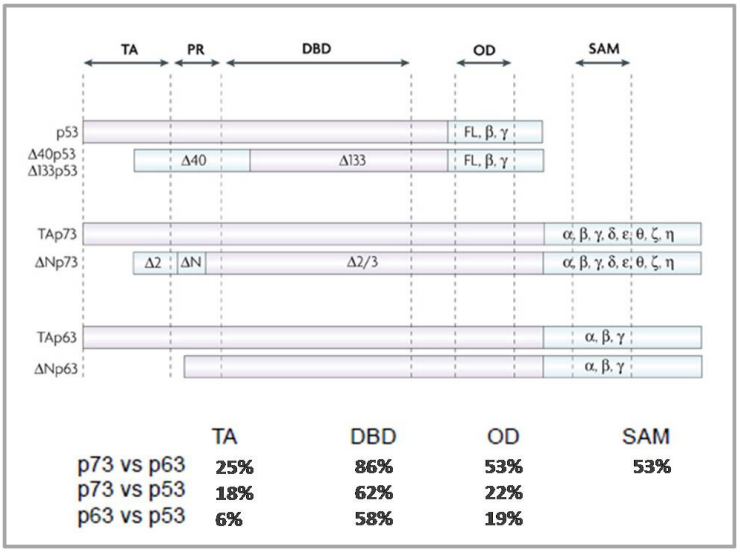


Figure 1.21. Percentage of homology between the p53, p63 and p73 domains. The highest degree of homology is seen within the DBD. The percentage of DBD homology is higher between p63 and p73, than with p53.

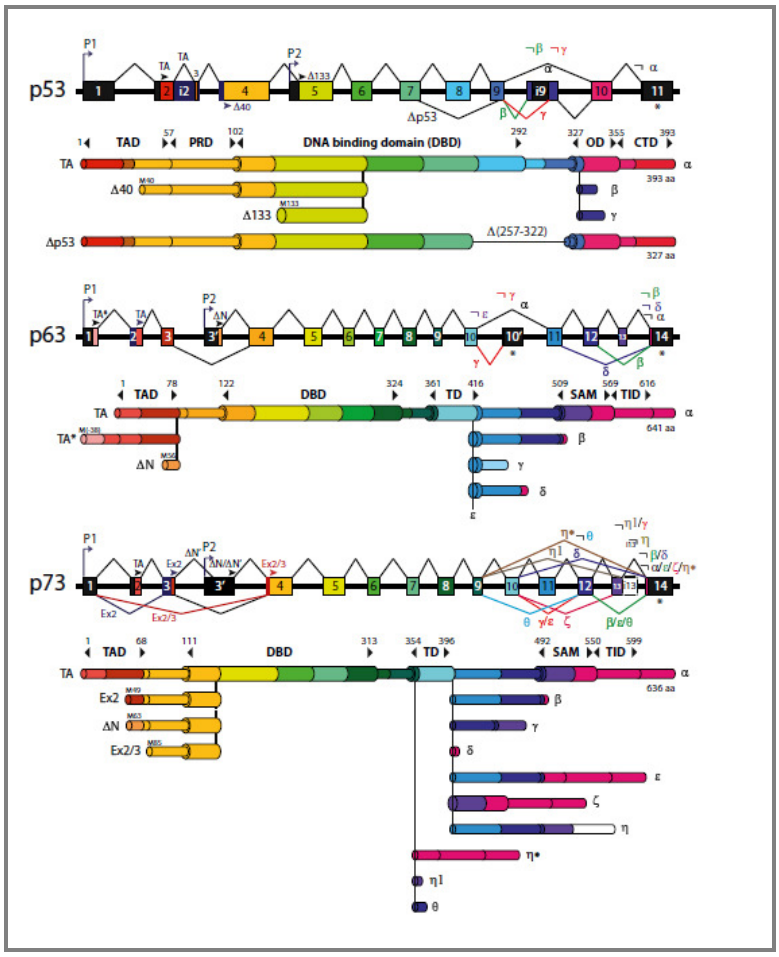


Figure 1.22. p53, p63 and p73 isoforms. TAD, transcription activation domain; PRD, proline-rich domain; DBD, DNA binding domain; OD, oligomerization domain; TD, tetramerization domain (differs from the OD by the existence of an additional C-terminal helix); SAM , sterile α motif; TID, transcription inhibition domain; CTD, C-terminal regulatory domain.

Interplay between p53/p63/p73 isoforms. The p53 family members interact each other to induce the transcription of common target genes, and can regulate the expression each other's. p53 and TA isoforms of p63 and p73, can bind to the P2 promoter of p53, p63 and p73, and induce the transcription of ΔN isoforms, that can inhibit the TA isoforms activity with negative feed-back. In head and neck squamous cell carcinoma and in basal-like breast cancer cell line physical ΔNp63/Tap73 complex that inactivate Tap73 has been demonstrated. In conclusion it is evident that during normal development and tissue renewal the ratio of these isoforms leads to cell proliferation, survival and differentiation, and down-regulation of this balance is involved in tumorigenesis (De Young MP and Ellisen LW, 2007)

2 AIM OF THE STUDY

The aim of the study was to evaluate the correlation between the homeobox gene Otx1 and the tumor suppressor proteins p53, p63, p73 and their isoforms, to find new pathways in human breast cancer and during mammary gland development.

In order to verify if the molecular interaction between these genes could be involved in the differentiation of breast cancer stem cells, we analyzed their expression in human ductal and lobular invasive breast cancer and in LA7 cell lines. The LA7 cells, isolated by Professor Dulbecco from a rat mammary adenocarcinoma, show the self-renewal stem cells properties, and the capacity to differentiate in all the cell lineages of the mammary gland. They can be used as a model to study cancer stem cells and mammary gland differentiation.

Furthermore, the Otx1, Tp53, Tp63 and Tp73 gene expression was evaluated in adult mice during linear and cyclical mammary gland development (pregnancy, lactation, involution), with the aim to study their involvement in the physiological development, and to verify their role in cell differentiation.

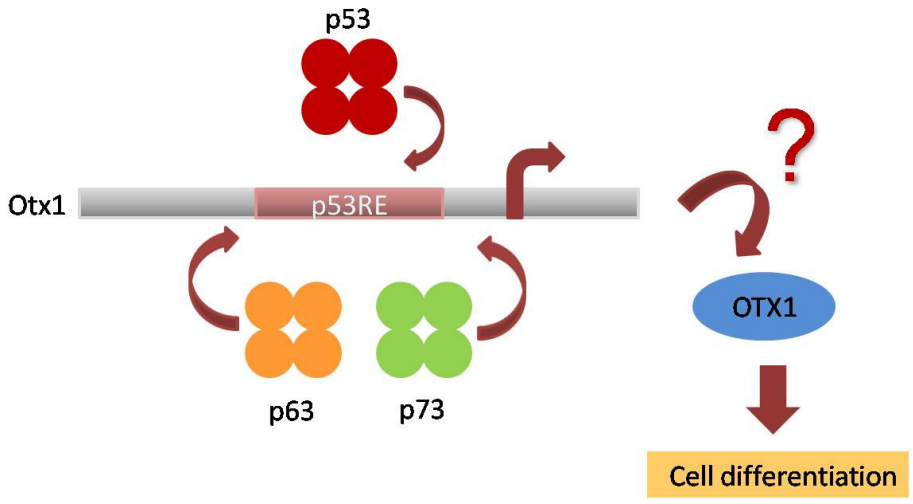


Figure 2.1. Aim of the study. The purpose of the study was to evaluate if p53, p63 and p73 proteins could transactivate the Otx1 gene expression, directly binding to the Otx1 promoter, through the p53REs, and was demonstrate their involvement in cell differentiation.

3 MATERIALS AND METHODS

3.1 Human tissue samples

Tissues from breast carcinoma, over two centimeters in diameter, and non-neoplastic tissues were obtained from 78 women, after informed consent ("Ospedale di Circolo, Varese, Italy"), between January 2005 and December 2009. The experimental protocol was approved by the Ethic Committee of the Insubria University. We selected 43 ductal and lobular invasive breast carcinoma for our studies. All tumors were histologically examined by the anatomo-pathologist to confirm the diagnosis, and identify the type and grade, as reported in the results (table 4.1 and table 4.4).

Tumor and non-neoplastic tissues were divided in two parts, one placed in RNA later (Ambion, Austin, TX, USA) until RNA extraction, and the other fixed in formalin and embedded in paraffin for immunohistochemical analysis.

3.2 Mice and mammary gland tissue

CD1 mice were from the Charles River Laboratories. Mice were maintained in accordance with Italian Ministry of Health according to Art. 12, D-L. 116/92, and European Community guidelines. All female mice were deeply anesthetized and sacrificed at 1 month, 8 month of age, after 2 weeks of gestation and 2 weeks of lactation, and during mammary gland involution, i.e. 3 weeks post-lactation. For each analysis were used three mice.

The samples were examined by anatomo-pathological analysis.

Mammary gland tissue was preserved in RNA later (Ambion, Austin, TX, USA) until RNA extraction.

3.3 p53-deficient mice

RNA from p53-deficient mice mammary gland tissues (Donehower LA, 1992) were kindly supplied by Professor A. Simeone (International Institute of Genetics and Biophysics, CNR, Naples, Italy).

3.4 Cell lines and culture

3.4.1 MCF7 cells treated with all-trans retinoic acid (ATRA)

Breast cancer MCF7 cell lines (ATCC, Middlesex, UK) were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with sodium pyruvate (110 mg/l), L-glutamine (300 mg/l), sodium bicarbonate (2,2 g/l), gentamicin (50 mg/l) and 5% fetal calf serum (Hyclone SH30072).

ATRA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 95% ethanol and was used at final concentration of 1μM for 24 hours.

Cells were then harvested in Trizol (Invitrogen, Paisley, UK) for RNA isolation.

3.4.2 Doxycycline (Dox) inducible tet-on/p53, -/p63, -/p73 SaOs-2 cells

Human osteosarcoma SaOs-2 cell lines (ATCC, Middlesex, UK), with Dox-inducible expression of Tp53, TAp63α, ΔNp63α, TAp73α, or ΔNp73α, were generate using the Tet-On Advances Inducible Gene Expression System (Clontech, Mountain View, CA, USA) (Fig. 3.1). Cells were cultured in a 1:1 mixture of Ham's F-12/DMEM, supplemented with 10% heat-inactivated, tetracycline (Tet) system-approved fetal bovine serum (Clontech, Mountain View, CA, USA).

The induction of the genes expression, was obtained treating the SaOs2 cells with Dox at 2,5 μg/ml, for 12, 24 and 48 hours. Cells without induction was used as control.

Cells were harvested in Trizol (Invitrogen, Paisley, UK) for RNA and protein extraction.

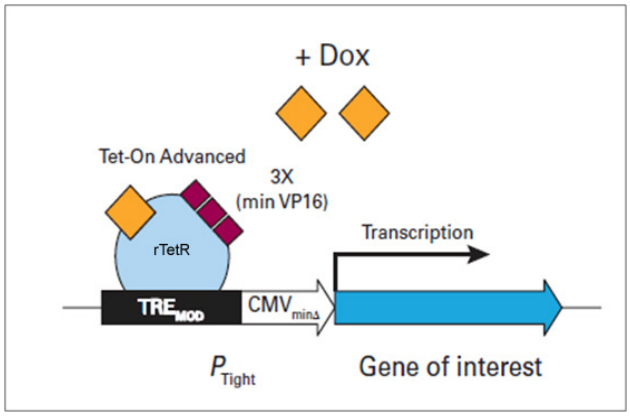


Figure 3.1. Tet-On Advanced system. The Tet-controlled transactivator (Tet-On Advanced) is a fusion protein derived from a mutant version of the *E. Coli* Tet repressor protein, rTetR, which is joined to three minimal transcription activation domains from the herpes simplex virus VP16 protein. The P_{Tight} promoter is an inducible promoter that control the transcription of your gene of interest. It consists of a modified Tet-Responsive element (TRE_{mod}) containing seven direct repeats of the *tet* operator sequence, *tetO*, which is joined to a minimal CMV promoter. In the presence of doxycycline (Dox), Tet-On Advanced protein binds to the TRE_{MOD}, and produces high-level transcription of the downstream gene of interest.

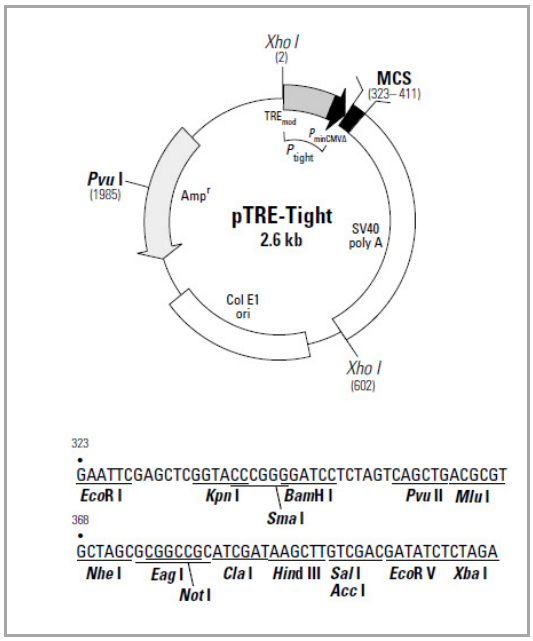


Figure 3.2. Map and Multiple Cloning Site (MCS) of pTRE-Tight Vector. pTRE-Tight is a response plasmid that can be used to express a gene of interest (in our experiments Tp53, TAp63α, ΔNp63α, TAp73α, or ΔNp73α) in the Tet-On Gene Expression Systems. pTRE-Tight contains an MCS immediately downstream of the Tet-responsive *P*tight promoter. cDNAs or genes inserted into the MCS will be responsive to the Tet-On Advanced protein, after Dox administration.

3.4.3 LA7 cell lines

The LA7 cell line, the clonal derivative from the Rama-25 line, was cultured in undifferentiating conditions using DMEM (Zucchi I, 2007).

For differentiation experiments LA7 cells, in confluence, were exposed to 1.8% DMSO for 48 hours.

Cells were harvested in Trizol (Invitrogen, Paisley, UK) for RNA isolation.

3.4 LA7 transplantation in NOD-SCID mice

In according to Zucchi et al. (Zucchi I, 2007), sub-confluent undifferentiated (LA7) and differentiated (LA7D: LA7 plus DMSO) cells, were trypsinized, counted and diluted in 1X PBS.

LA7 and LA7D cells, ranging from 1 cell to 100.000cells, were injected into the fat pads of female NOD-SCID mice six weeks old, anesthetized with ketamine/xilazine. Tumor growth was monitored three times for week, and visible nodules were measured with a caliper.

The animals was killed after 63 days of observation. Autopsy was performed to score for possible metastasis.

The tumor removed were used to establish cell lines or fixed in formalin and embedded in paraffin for immunohistochemical analysis.

3.5 Mammary tumors and metastasis dissociation

Tumors and metastasis, generated through LA7 and LA7D injection, were dissociated mechanically and enzymatically, as described by Stingl *et al*. (Stingl J, 1998).

3.6 RNA extraction

The tissues, from human breast tumors and mice mammary gland, were dissociated mechanically and homoginezed with Mixermill Retsch MM301 for 10 minutes at 4°C.

The RNA was extracted with RNeasy Fibrous Tissue mini Kit from Qiagen (Valencia, CA, USA).

The RNA from cell lines (SaOs2, MCF7, LA7) was extracted using EuroGold Total RNA Kit (Euroclone, Milan, Italy).

The concentration of total RNA was determined using nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).

Equal amount (1µg) of total RNA from each sample was reverse transcribed using random primers (hexamers), according to the protocol of High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA , USA).

3.7 Tp53 mutation and polymorphism analysis

The coding regions of Tp53 was amplified by PCR using the primers reported in table 3.1. All the reactions were performed in 25 µl of final volume, with 2.5 µl of 10X buffer, 0.3 µl of 10 mM dNTPs, 1 µl of 10 µM primer F, 1 µl of 10 µM primer R, 1U/µl of RedTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA).

Using the primers p53-DBD-378F and p53-DBD-1161R, the termocycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, with final extension at 72°C for 3 min. The length of the amplicon was 800 bp.

For the p53-148F/p53-539R and p53-1004F/p53-1461R primers, the termocycling conditions was of an initial denaturation step at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec, with final extension at 72°C for 3 min. The lengths of amplicons were of 391 bp and 457 bp, respectively

Table 3.1. Primer sequences for p53 mutational analysis

Primer	Sequence
p53-148F	5'-GTGACACGCTTCCCTGGATT-3'
p53-539R	5'-CAAGAAGCCCAGACGGAAAC-3'
p53-DBD-378F	5'-GATGAAGCTCCCAGAATGCC-3'
p53-DBD-1161R	5'- GTTTCTTCTTTGGCTGGGGA-3'
p53-1004F	5'-CTTTGAGGTGCGTGTTTG-3'
p53-1461R	5'-TCAAAGACCCAAAACCCAAA-3

The numeration is referred to the p53 sequence accession number NM_000546.

The PCR products were purified from gel using the Gen Elute™ Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA), and were automatically sequenced (Bio-Fab Research, Rome, Italy).

The sequences was aligned with the p53 sequence accession number NM_000546, using ClustalW program (EMBL-EBI, www.ebi.ac.uk/tools/msa/clustalw2).

The mutations and polymorphisms were compared with the p53 databases: we used "swiss prot" (www.uniprot.org/uniprot/P04637) for mutations analysis, and "Ensembl" (www.ensembl.org/index.html) or "SNP-NCBI" (www.ncbi.nlm.nih.gov/projects/SNP) for polymorphisms analysis.

3.8 Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed with TaqMan and Sybr Green technology using the ABI Prism 7000 apparatus (Applied Biosystems, Carsbad, California, USA).

Gene expression analysis was performed using TaqMan® Assays-on-Demand probes (Table 3.2 and table 3.4). All the reactions were performed in 25 µl of final volume. The PCR reaction mix

contained 12.5 µl of TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, Carsbad, California, USA), 1.25 µl of Assay-on-Demand (primers and probe), 8.75 µl of nuclease-free water, and 2.5 µl cDNA. The PCR program consisted in an initial hot start at 50 °C for 2 min and 95°C for 10 min, followed by 95°C for 15 sec, 60°C for 60 sec, for 40 cycles.

Sybr Green assays was performed using the specific primers, designed with "Primer 3" program, and then validated.

All the reactions were performed in 25 µl of final volume. The PCR reaction mix contained 12.5 µl of Power Sybr Green PCR Master Mix (Applied Biosystems, Carsbad, California, USA), X µl primers at specific concentrations (Table 3.3 and 3.5), X µl of nuclease-free water, and 2.5 µl cDNA. The PCR program consisted of an initial hot start at 50°C for 2 min and 95°C for 10 min, followed by 95°C for 15 sec, 60°C for 60 sec for 40 cycles, and a final stage of 95°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec.

All the reactions, using Taq Man and Sybr Green assays, were in triplicate. Human, mouse and rat beta-actin (Actb) was used as endogenous control to normalize gene expression levels in the relative quantitative analysis, using the $2^{-\Delta\Delta Ct}$ method:

Ct = number of cycle of exponential fluorescence beginning

ΔCt = Ct (target) – Ct (endogenous control)

$\Delta\Delta Ct = \Delta Ct$ (sample) $-\Delta Ct$ (calibrator).

In human breast cancer analysis the calibrator was the non-neoplastic tissue, in mouse mammary gland development, the calibrator was the mammary tissue from non-pregnant 1 month old female, and in cell lines experiments the calibrators were the LA7 cells, and the cells MCF7 non treated with ATRA.

Table 3.2- Human ductal and lobular invasive breast cancer primers - Sybr Green technology

Primers	Sequence	Concentration	Sequence accession number
Otx1 F	5'-ACCCATCCGTGGGCTATC-3'	100 nM	NM_014562.2
Otx1 R	5'-TGTGAACGCGTGAAGGTG-3'	100 nM	
Tp53 F	5'- CCCCAGCCAAAGAAGAAAC-3'	50 nM	NM_000546
Tp53 R	5'- AACATCTCGAAGCGCTCAC-3'	50 nM	
TAp63α F	5'- TTTGAAACTTCACGGTGTGC-3'	200 nM	NM_003722.4
TAp63α R	5'- TGAGCTGGGGTTTCTACGA-3'	200 nM	
ΔNp63α F	5'- GGTTGGCAAAATCCTGGAG-3'	40 nM	NM_001114980.1
ΔNp63α R	5'- GGTTCGTGTACTGTGGCTCA-3'	40 nM	
Tap73α F	5'- AACCAGACAGCACCTACTTCG-3'	40 nM	NM_005427.2
Tap73α R	5'- CGCCCACCTCATTATT-3'	40 nM	
ΔNp73α F	5'- AAGCGAAAATGCCAACAAAC-3'	200 nM	NM_001126240.1
ΔNp73α R	5'- AGGCTCCGCAGCTAGTGA-3'	200 nM	
Actb F	5'-CGCGAGAAGATGACCCAGAT-3'	40 nM	NM_001101.3
Actb R	5'-ACAGCCTGGATAGCAACGTACA-3'	40 nM	

Table 3.3- Human ductal and lobular invasive breast cancer assays - Taq Man technology

Gene	Taq Man assay	Exon boundary	Amplicon length (bp)
Tp53	Hs 00153340_m1	1-2	81
Otx1	Hs 00293035_m1	2-3	90
Tp63	Hs 00978343_m1	7-8	85
Tp73	Hs 01056231_m1	5-6	65
Actb	Hs 99999903_m1	1-1	171

Table 3.4- Rat LA7 primers - Sybr Green technology

Primers	Sequence	Concentration	Exon boundary	Sequence accession number
Otx1 F	5'-GCTACCCAGACATCTTCATGC-3'	300 nM	1-1	NM_013109.1
Otx1 R	5'-GGCGGTTCTTGAACCAAAC-3'	300 nM		
TRp53 F	5'-GTTAGGGGGTACCTGGATC-3'	300 nM	1-1	NM_030989.3
TRp53 R	5'-CGACTGTGAATCCTCCATGA-3'	300 nM		
TAp63α F	5'-CAAAGCCTTCTCGCGCTAGCTC-3'	600 nM	14-14	NM_019221.3
TAp63α R	5'-TCCGTCCCTTTAGAAACCAG-3'	600 nM		
ΔNp63α F	5'-TACCAGCCCCTATAACA-3'	600 nM	2-2	NM_001127342.1
ΔNp63α R	5'-GACTGCTGGAAGGACACATC-3'	600 nM		
Trp73F	5'-CAACAACTGCCCTCTGTCA-3'	300 nM	11-12	NM_001108696.1
Trp73 R	5'-CTGTTGAGTATCCCGGAACC-3'	300 nM		
Actb F	5'-AGGCCCCTCTGAACCCTA-3'	300 nM	3-4	NM_031144.2
Actb R	5'-GGGGTGTTGAAGGTCTCAAA-3'	300 nM		

Table 3.5- Mouse mammary gland- Taq Man technology

Gene	Taq Man assay	Exon boundary	Amplicon length (bp)
Otx1	Mm 00550304_m1	2-3	77
Trp53	Mm 01731281_m1	1-2	133
Trp63	Mm 00495788_m1	4-5	64
Trp73	Mm 00660220_m1	13-14	83
Actb	Mm 01205647_g1	2-3	72

3.9 Statistical analysis

The statistical analysis (Student's t test and correlation) was performed using the GraphPad software (www.graphpad.com/quick.calcs/ttest1.cfm).

3.10 Immunohistochemical assay

The immunohistochemical analysis was performed on the formalin-fixed, paraffin-embedded tumor samples. Three µm sections were mounted on poly-L-lysine coated slides, deparaffinized and hydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 10 minutes. Antigen retrieval was performed with citrate buffer (10 mM, pH 6,0) in a domestic 720 W microwave oven. The sections were incubated overnight at 4°C with mouse anti-p53 monoclonal antibody (clone DO-7, Ventana, Tucson, Arizona, USA) not diluted, and other sections were incubated with rabbit anti-OTX2 polyclonal antibody (Chemicon International, Temecula, CA, cat. #AB9566) at a dilution of 1:2000. This antibody was produced using full length recombinant human OTX2 as immunogen. Due to amino acid sequence homology between the OTX1 and the OTX2 proteins, the antibody react with OTX1. Since Otx2 mRNA has not been detected in any tumors, we considered that the immunoreactivity was due to the presence of OTX1 protein. The sections were incubated with Ultravision Detection System kit (Thermo Scientific, Fremont, CA) according to manufacture suggestion. The immunoreactions were developed using 0,03% 3,3-diaminobenzedine

tetrahydrochloridre (Sigma-Aldrich, St. Louis, MO, USA), and nuclei were counterstained with Harris' hematoxylin.

As controls we use tissues with or without the pertinent antigen, omission of the primary antibody and substitution with a non-immune serum at the same dilution.

3.11 Protein extraction from SaOs2 cell lines and western-blotting

SaOs2-inducible cells were harvested and lysed with lysis buffer, containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 50 mM NaF, 1 mM EDTA pH 6 and 20% Triton-X 100, at time zero or after 24, and 48 hours from the addiction of Dox. Protease inhibitors (Sigma-Aldrich, St. Louis, Mo, USA) and 1 mM DTT (Sigma) were addicted. The total lysated proteins were separated by SDS-PAGE and transferred into PVDF membranes. Blot were performed using standard procedures. The membranes were then incubated with monoclonal anti-OTX1 (Abcam, Cambridge, MA, USA) at dilution of 1:200, and with polyclonal anti-p53 (clone DO-1, Santa Cruz, CA, USA) at dilution of 1:500. Goat anti-actin (C-11, Santa Cruz, CA, USA), at dilution of 1:1 .000, was used as loading control.

Proteins were detected with electochemiluminescence (ECL).

3.12 Chromatin immunoprecipitation (Chip) assay

$12x10^6$ SaOs2 cells were cross-linked for 10 min, in a solution containing 1% formaldehyde. Cross-linking was stopped by replacing the solution with 0.125 M glycine for 5 min at room-temperature. After washing with ice-cold PBS, cells were harvested in lysis buffer (50 mM Hepes-KOH pH 8.0, 1 mM EDTA, 140 mM NaCl, 25% glycerol, 0.5% NP-40, 0.25% Triton X-100), plus protease inhibitors. Nuclei were collected by centrifugation and re-suspended in washing buffer (10mM Tris-HCl pH 8.0, 1 mM EDTA, 200 mM NaCl), plus protease inhibitors. After centrifugation, the nuclei were re-suspended in 1,8 ml ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 0.0167M Tris-HCl, 0.167 M NaCl), plus 200 ml SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1).

Cell lysated were sonicated in order to obtain chromatin fragments of ~700 bp. After centrifugation at 13 000 rpm for 10 min to remove any cell debris, total proteins (cross-linked to

DNA and not) were pre-cleared with protein G agarose/salmon sperm DNA (Upstate, Charlottesville, VA, USA) for 1h at 4°C, in a ratio 50 µg/µl. Then, the pre-cleared extracts were incubated with 3µg polyclonal antibody anti-p53 (DO-1, Santa Cruz, CA, USA).

The negative control was incubate with 3 mg of mouse anti-IgG1k (BD, Franklin Lakes, NJ, USA) overnight at 4°C, followed by incubation with protein G-agarose/salmon sperm DNA (40 µl) for 3 h at 4°C.

The immuno-complexes were washed twice with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 0.15 M NaCl), twice with high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 0.15 M NaCl), then once with LiCl salt wash buffer (1 mM EDTA, 10 mM Tris-HCl, 0.25 M LiCl, 1% NP-40, 1% deoxycholate), and twice with TE buffer.

The immuno-precipitates were extracted, twice using 250 µl of IP elution buffer (1% SDS, 0.1 M $NaHCO_3$). The total eluates (500 µl) were pooled by adding 20 µl of 5 M NaCl, and incubated at 65°C over-night to reverse the formaldehyde cross-linking. DNA fragments were purified from proteins, using phenol-chloroform extraction and ethanol precipitation. Then they were dissolved in 30 µl of sterile water.

DNA samples were analyzed with 25 cycles of PCR to amplify Otx1 promoter sequence. The termocycling conditions used for amplification consisted of an initial denaturation step at 94°C for 4 min, followed by 25 cycles of denaturation at 94°C for 35sec, annealing at 61°C for 35 sec and extension at 72°C for 2 sec, with final extension at 72°C for 3 min. In table 3.6 are reported the primers used for Otx1 amplifications.

Table 3.6. Primer sequences for PCR analysis of the samples after CHIP

Primers	Sequence
5'p53RE-F	5'- TTCTCCTC TCCTCACCCAGC-3'
5'p53RE-R	5'-ATCTCCCCCACTCCC CCACC-3'
3'p53RE-F	5'-AGAAGACGGGGGCGGGAACG-3'
3'p53RE-R	5'-CAGGGAGGGGCGCTGGAAAC-3'

3.13 Luciferase assay

Otx1 promoter with 3'p53RE was cloned into pGL3 Luciferase (Luc) Basic Vector (Promega, Madison, WI, USA).

Luc assay was performed by co-transfecting pGL3-OTX1 with increasing amounts of Dox/inducible p53 expression vector, from a ratio of 1:1 to 1:10, in the SaOs2 cells. We used Lipofectamine 2000 transfection reagent (Invitrogen, Paisley, UK) following the manufacture instructions.

Luciferase activity were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Light emission was measured over 10 sec using OPTOCOMP I luminometer (MGM Instruments Inc, Hamden, CT, USA).

4 RESULTS

4.1 Tp53 mutation and polymorphism analysis

A pool of 33 invasive human breast cancer (30 ductal and 3 lobular) was sequenced to identify the presence of mutations in the Tp53 gene (Table 4.1). We found mutations in the p53 DNA binding domain in 3 breast cancer samples with a frequency of 9,1% versus 20% reported in literature (Borresen-Dale AL, 2003). The mutations in heterozygous state were p.193 H>R, p.238 C>F and p.195 I>T.

In 16 out of 33 (51.5%) tumors we found the polymorphism R72 (c.412 G) in homozygous state (Fig. 4.1.A). 14 out of 33 (39.4%) carried the R72P (c.412 C>G) polymorphism in heterozygous state (Fig. 4.1.B), and 3 out of 33 (9.1%) were homozygous for the codon P72 (c.412 C, the wild-type form) (Fig. 4.1.C). Sample 8T homozygous for the P72, and sample 11T homozygous for the R72 were heterozygous for polymorphisms c.305 G>A and c.836 A>G, respectively identified in the same amplicon. 4T and 7T samples heterozygous for the R72P, were heterozygous for the c.305 G>A and the c.836 A>G polymorphism, respectively (Table 4.1).

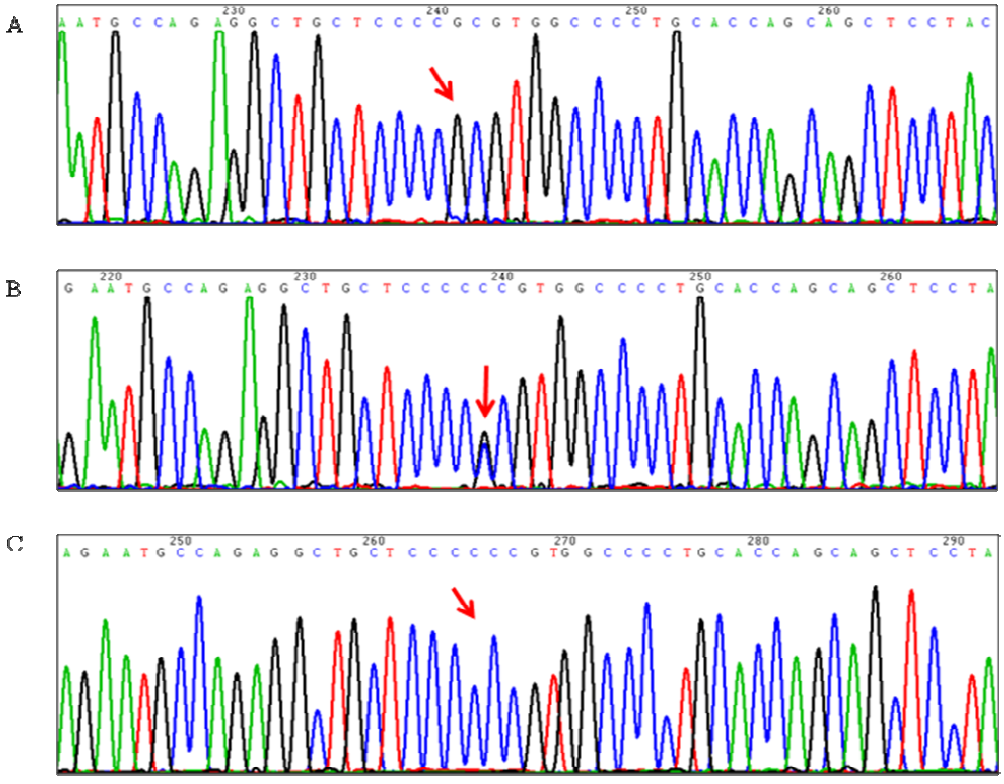


Figure 4.1. Polymorphism at codon 72 of the p53 protein. The chromatograms show the polymorphisms at codon 72 of p53 (red arrows), R72 **(A)** and P72 **(C)** in homozygous state, and R72 P **(B)** in heterozygous state.

Table 4.1. Histological type, tumor grade, pT, pN, p53 mutations and polymorphisms of patient samples

Samples	Histological type	Grade	pT	pN	p53 mutations	p53 polymorphisms
3T	IDC	2	1c	X		R72P
4T	IDC	2	1c	0 sn		R72P c. 305 G>A p.P36 in heterozygous state
5T	IDC	2	1c	0		R72P c. 836 A>G p. R213 in heterozygous state
7T	IDC	3	2	0	c.775A>G p.193H>R	R72P
8T	IDC	2	2	X		P72 c. 305 G>A p.P36 in heterozygous state
11T	IDC	2	1b	X		R72 c. 836 A>G p. R213 in heterozygous state
12T	IDC	3	X	3a	c.910A>T p.238C>F	P72
16T	IDC	3	X	X		R72P
18T	**ILC**	2	X	X		R72
20T	IDC	X	X	X	c.781T>C p.195I>T	R72
23T	IDC	2	2	X		P72
26T	IDC	2	2	1 sn		R72
27T	IDC	3	1c	X		R72P
29T	IDC	3	2	0 sn		R72
31T	**ILC**	3	2	0		R72
33T	IDC	2	2	0 sn		R72
35T	IDC	2	2	0		R72P
37T	IDC	3	2	1a		R72
41T	IDC	2	1	0 sn		R72P
43T	IDC	3	2	2a		R72
44T	IDC	2	2	1		R72
47T	IDC	2	2	1 mic		R72P
49T	IDC	2	2	1a		R72
50T	IDC	2	2	1a		R72P
51T	IDC	3	2	1a		R72
53T	IDC	2	2	0 ls		R72P
54T	IDC	2	2	1		R72
55T	IDC	2	2	0		R72
56T	IDC	2	2	1a		R72P
57T	IDC	2	2	3		R72
58T	**ILC**	2	2a	2		R72P
62T	IDC	2	2	0 sn		R72
63T	IDC	2	2	1a		R72P

Table 4.1. Histological type, tumor grade, pT, pN, p53 mutations and polymorphisms of patient samples. Legend: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; G1, low-grade; G2, moderate or intermediate grade; G3=high grade; X, not evaluated.

4.2 Expression analysis of Tp53 and Otx1 in breast cancer

Tp53 and Otx1 gene expression levels were analyzed in the wild-type Tp53 (27 ductal and 3 lobular) human invasive breast cancer samples (table 4.1) by quantitative real-time reverse transcriptase PCR (qRT-PCR), using normal breast tissue as control. Tumors showed higher levels of Otx1 and Tp53 gene expression compared with the controls, in which we found basal levels of expression. Statistical analysis was performed to verify the correlation between these values of expression. Tumor tissues showed a correlation coefficient (r= 0,864) significantly different from zero (t= 8,412) with probability p= $1{,}284E^{-0,8}$ (Fig. 4.2). The correlation between Otx1 and Tp53 gene expression was not observed in the normal breast tissue (data not shown).

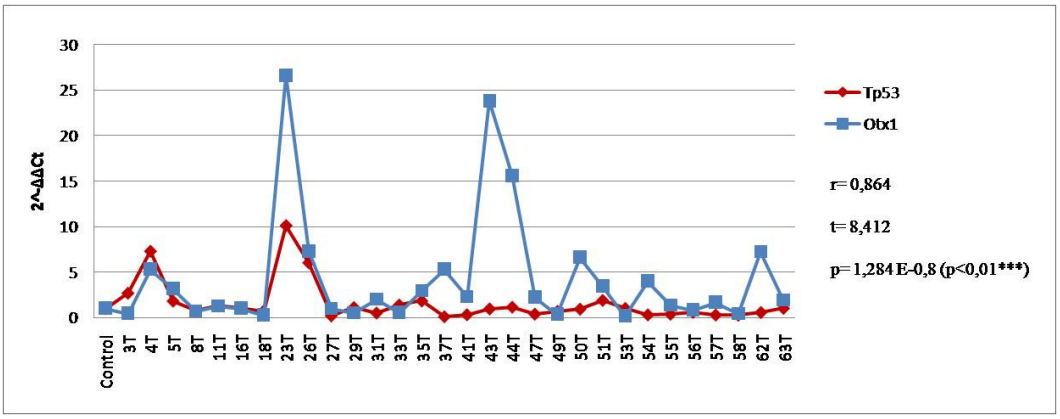


Figure 4.2. Gene expression levels of Tp53 and Otx1 genes in human breast cancer and correlation analysis. Gene expression levels of Tp53 and Otx1 genes was detected by qRT-PCR using Taq Man assays (see Table 3.3) in human ductal and lobular invasive breast cancers (table 4.1). The samples are in abscissa, and in ordinate there are the expression levels of these genes evaluated with the relative quantitative analysis, using the $2^{-\Delta\Delta Ct}$ method.

In the tumors, the presence of the Otx1 protein was confirmed by immunohistochemical (IHC) analysis (Table 4.2). We used rabbit anti-OTX2 polyclonal antibody, which also identified OTX1, due to amino acid sequence homology between these proteins. Since Otx2 mRNA was

not detected in any tumors by qRT-PCR (data not shown), we considered that the observed immunoreactivity was specific for the OTX1. We detected nuclear positivity for OTX1 in few cases (Fig. 4.3.A), while the majority of the tumors showed OTX1 cytoplasmic localization (Fig. 4.3.B). In the non neoplastic tissue adjacent to carcinoma the OTX1 protein was localized in the nucleus of some epithelial cells (Fig. 4.3.C).

Table 4.2. OTX1 subcellular localization

Samples	OTX1* Nuclear Localization	OTX1* Cytoplasmic Localization
3T	1	20 (++)
4T	<1	neg
5T	neg	5 (+++)
7T	neg	40 (++/+++)
8T	20	<5 (+)
11T	20	20 (+)
12T	2	>50 (+/+++)
16T	n.e.	n.e.
18T	n.e.	n.e.
20T	n.e.	n.e.
23T	>2	rare (+++)
26T	<1	<5 (+/++)
27T	<1	100 (+)
29T	neg	10 (+)
31T	neg	100 (++)
33T	2	80 (+/+++)
35T	10	20 (+/+++)
37T	20	40 (+/++)
41T	<1	40/50 (+/++)
43T	<1	40 (++/+++)
44T	20	90 (+/+++)
47T	30	neg
49T	1	80
50T	2	diffuse (++)
51T	5	diffuse (++)
53T	50 (+/++)	5
54T	10	20/30 (++)
55T	10	90 (++)
56T	2/3	neg
57T	3	60 (++)
58T	3	90 (+++)
62T	10	70 (+/++)
63T	neg	90 (+++)

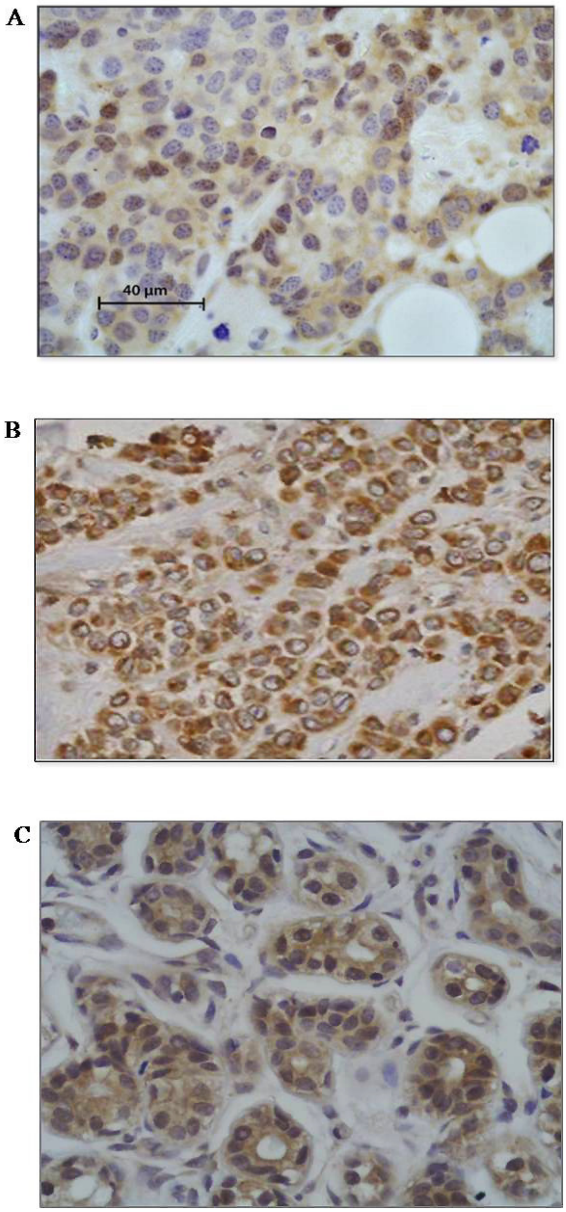


Table 4.2. OTX1 subcellular localization. Legend: *, % of positive cells; +/++/+++, weak/moderate/intense immunoreactivity; n.e., not evaluated.

Figure 4.3. A-C. OTX1 subcellular localization. The immunohistochemistry shows the nuclear localization for the OTX1 protein in the sample 53T (invasive ductal breast carcinoma) **(A)** and cytoplasmic localization in the sample 27T (invasive ductal breast carcinoma) **(B)**. In the non neoplastic tissue adjacent to carcinoma (29S) the OTX1 protein was localized in the nucleus of some epithelial cells **(C)**.

4.3 Molecular interaction between p53 and Otx1

In order to demonstrate the direct molecular interaction between Tp53 and Otx1 genes we used the doxycycline (Dox) inducible tet-on/p53 SaOs-2 cells (Riley T, 2008). Tp53 expression in this cell line was induced by adding Dox to the culture medium. The expression of Tp53 at 12, 24, and 48 hours (and its absence in control cells), was confirmed by western blot using an anti-p53 antibody (Fig. 4.4.A). To evaluate Otx1 expression levels, we used qRT-PCR and western blot analysis. Both techniques confirmed the expression of the Otx1 gene at the transcriptional (Fig. 4.4.B) and at the protein level (Fig. 4.4.A), after induction of p53 (+Dox). The expression of Otx1 clearly increased at 12 and 24 hours after the addition of the antibiotic (Fig. 4.4.B), according to the expression of Tp53. The western blot analysis demonstrated the presence of the OTX1 protein at 48 hours after p53 induction (Fig. 4.4.A).

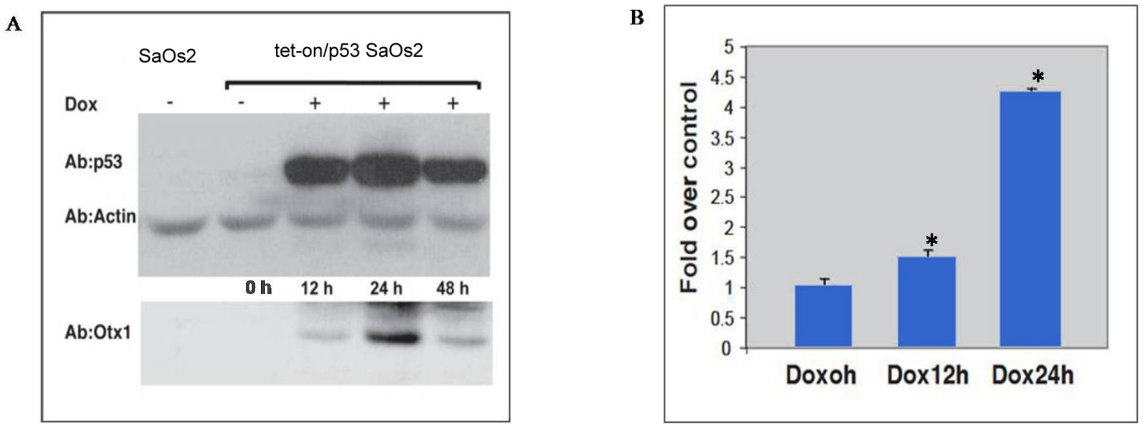


Figure 4.4.A. Western blot. The p53 and OTX1 proteins were detected in the Dox inducible tet-on/p53 SaOs-2 cells treated with Dox at 12 h, 24 h and 48 h, using the untreated cells (0 h) and the SaOs2 not transfected as control.

Figure 4.4. B. qRT-PCR. Increase of Otx1 gene expression levels in Dox inducible tet-on/p53 SaOs-2 cells treated with Dox at 12 h and 24 h. qRT-PCR was performed using Taq Man assays (see Table 3.3). In abscissa there are the samples untreated and treated with Dox, and in ordinate there are the gene expression levels.

To clarify the nature of interaction between p53 and Otx1 we performed an "*in silico"* analysis of an approximate 4.8 Kb CpG island in the 5' region of the Otx1 gene, looking for p53 regulatory sequences in the OTX1 promoter. The CpG island extends from approximate 3.6 Kb upstream of the OTX1 transcription starting site (TSS), up to 1.2 Kb downstream to the TSS. The OTX1 transcript contains five exons: the first two represent the 5'-UTR, while the ATG is located at the beginning of the third exon. It is known that regulatory elements could lie inside introns and exons of UTR mRNA (Gressner O, 2005); indeed the analysis was extended to include the first two exons/introns. Bioinformatic analysis of the region was performed using the MatInspector program by Genomatix (Genomatix Software GmbH), looking for the transcription factors with core and matrix similarity above 0.8. The "*in silico*" analysis showed the presence of two putative p53 responsive elements (REs). The first is located at -3435 bp upstream the ATG (5' p53RE), with matrix similarity of 0.942 and core similarity of 0.921. The second (3' p53RE) is located in the second intron at +1268 bp downstream the ATG, with matrix similarity of 0.898 and a core similarity of 1.0 (Fig. 4.5.A).

In order to understand if one of these two putative REs represented a functional p53 binding site, chromatin immunoprecipitation analysis (ChIP) was carried out. Tet-on/p53 SaOs-2 cells were treated with Dox at 12 and 24 hours, using untreated cells as control. Cells were cross-linked, lysated and sonicated in order to obtain chromatin fragments of ~700 bp. Total proteins (cross-linked and not) were extracted and immunoprecipitated using anti-p53 antibody and anti-IgG as non-specific control. The immuno-precipitates (IPs) were extracted, and the cross-linking was reverted. DNA fragments was purified from proteins, and the sequence containing the two p53RE sites (approximately 160-170 bp) in the promoter of Otx1 was amplified by PCR. No PCR product was obtained for the 5'p53RE, indicating that this sequence was not the bindig site for p53. A positive signal was obtained for the 3'p53RE, at 24 hours from p53 induction. No signal was obtained with the immunoprecipitation using IgG, indicating the specificity of the p53 antibody (Fig. 4.5.B).

These results clearly demonstrated the direct interaction between p53 and the OTX1 promoter through the 3'p53RE. Interestingly, the putative functional p53RE is located after the TSS, into the transcribed region.

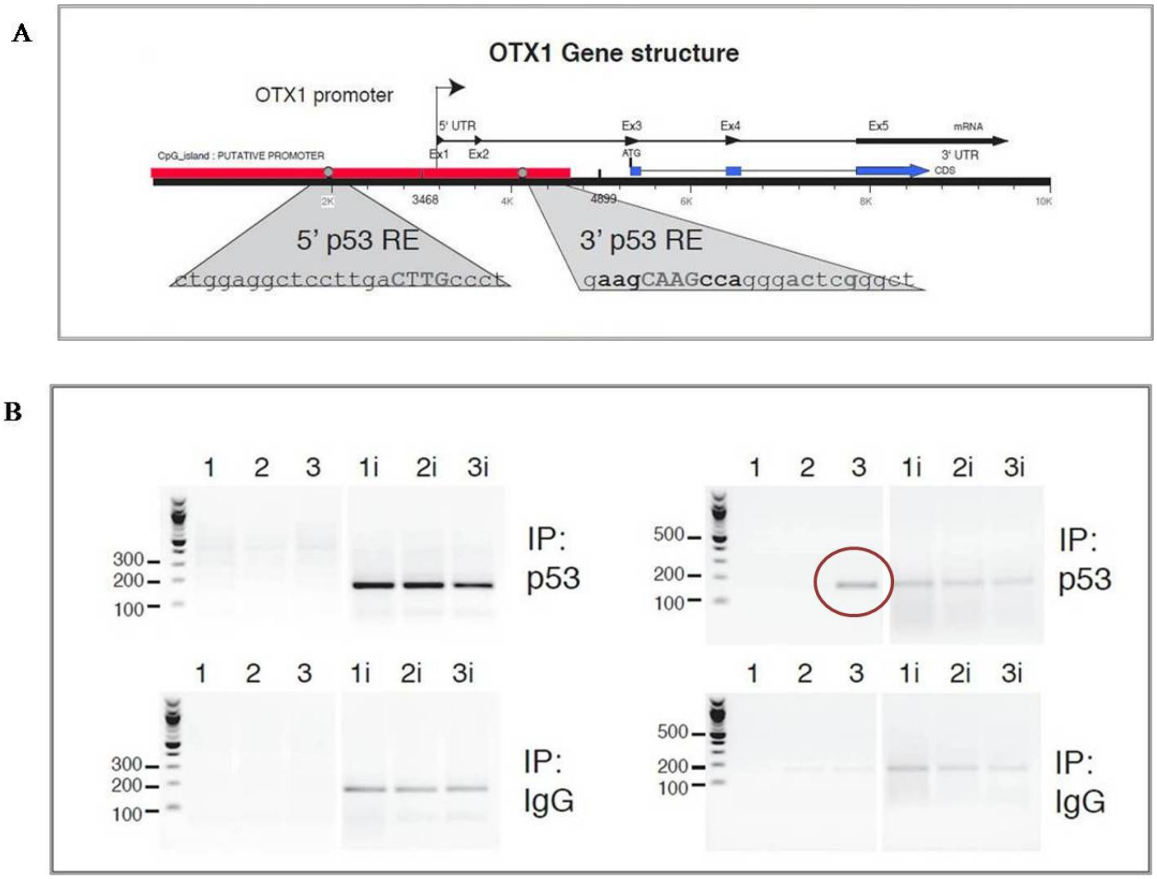


Figure 4.5.A. *In silico* analysis of the Otx1 promoter. The *in silico* analysis showed two putative p53 responsive elements (REs) in the CpG island: 5'p53RE and 3'p53RE.

Figure 4.5.B. PCR analysis of the 5'p53RE and 3'p53RE after immunoprecipitation with p53 protein. Left panels show the PCR analysis performed on the 5'p53RE, and right panels show the PCR analysis performed on the 3'p53RE (primers in the table 3.6). The ChIP assay was performed on the tet-on/p53 SaOs-2 cells untreated (1) and treated with Dox at 12h (2) and 24h (3.). 1i ,2i and 3i represent the chromatin sonicated after cross-linking (positive control), were the positive signal for p53 is detected. 1,2 and 3 represent the chromatin after cross-linking and immuno-precipitation with specific antibody anti-p53. The positive signal is detected only in 3'p53RE after 24h of Dox administration. The IgG was used as non-specific control.

To clarify if the 3'p53RE was able to drive the expression of the Otx1 gene, we cloned 1431 bp surrounding this RE in the pGL3 luciferase basic vector (pGL3-Otx1), to be used as minimal promoter. Luciferase (Luc) assay was performed by co-transfecting pGL3-Otx1 with increasing amounts of Dox/inducible p53 expression vector, from a ratio of 1:1 to 1:10, in the SaOs2 cells.

The results indicated that this region was able to drive the Luc transcription, behaving as a minimal promoter. We showed that the Luc activity was directly proportional to the p53 expression (Fig. 4.6).

All these results lead to the conclusion that Otx1 is one target gene for p53, which can regulate its expression through the 3'p53RE.

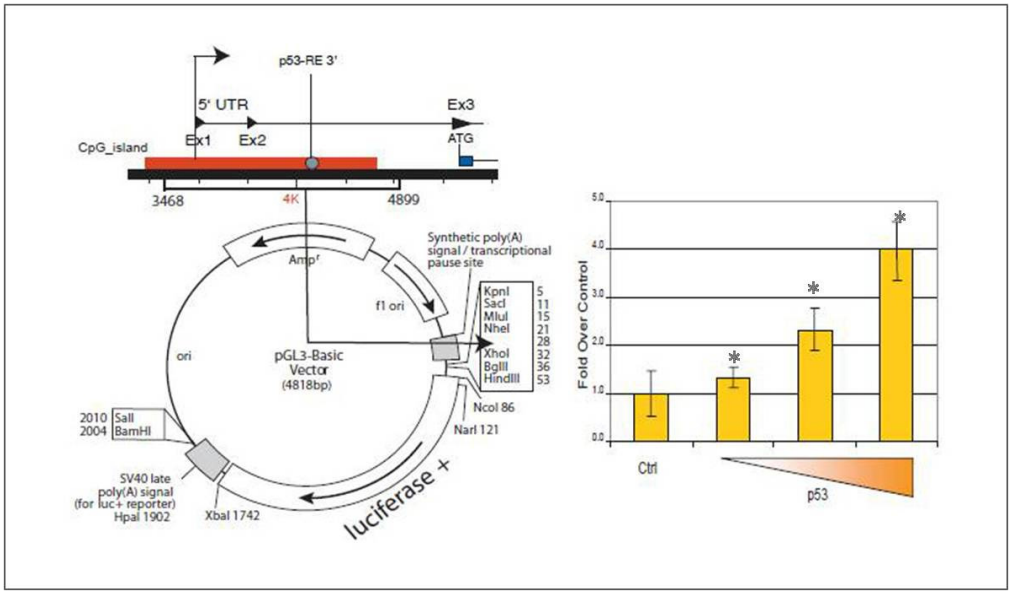


Figure 4.6. Luc assay. Schematic representation of the luciferase (Luc) reporter plasmid (pGL3-Otx1), with the 3'p53RE cloned up-stream to the Luc gene (left). The graph (right) shows the increase of luciferase activity when increasing amount of Dox inducible p53 (from a ratio 1:1 to 1:10) expression vectors are transfected. In abscissa are reported the samples co-transfected with pGL3-Otx1 and Dox inducible p53 expression vector. The empty pGL3-Otx1 vector was used as control. In ordinate there is the luciferase activity.

4.4 Correlation between Tp53 and Otx1 in human MCF7 cell lines differentiated with retinoic acid

Retinoic acid (RA), an active metabolite of vitamin A, triggers antiproliferative effects in tumor cells, inducing differentiation and apoptosis, and therefore has great potential as an anticarcinogenic agent (Hua). Human breast cancer cell lines MCF7, which express p53 wild-type, are responsive to treatment with all-*trans* retinoic acid (ATRA). ATRA inhibits cell cycle progression in MCF7 cells and increases the expression of Tp53 (Mrass). In order to demonstrate

the possible role of Otx1 in tumor cells differentiation, we performed a qRT-PCR assay to evaluate the Otx1 and Tp53 gene expression levels in MCF7 treated with ATRA. Drug treatment increased levels of both Otx1 and Tp53, confirming the Otx1 activation by p53 during differentiation of cancer cells (Fig. 4.7). The increase of both Otx1 and Tp53 in the MCF7 in comparison with the untreated control, was statistically significant with t=7,0545 and p=0,0021 (p<0,01**), and t= 7,2028 and p= 0,0020 (p<0,01**), respectively.

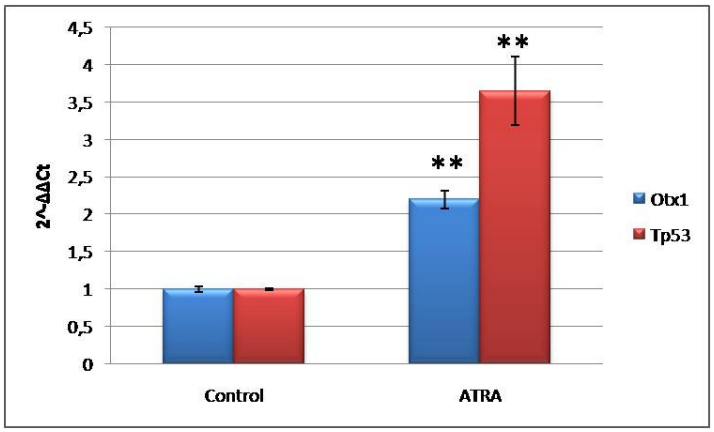


Figure 4.7. Increase of Otx1 and Tp53 gene expression levels detected by qRT-PCR in MCF7 cells treated with ATRA. In abscissa are reported the MCF7 cells treated and untreated with ATRA, and in ordinate there are the expression levels of the Otx1 and Tp53 genes.

4.5 The p53 family in Otx1 activation

p53, p63 and p73 are a family of transcription factors, that play a pivotal role in the control of the cellular response to DNA damaging agents. p53, Tap73 and Tap63 have proapoptotic function while ΔNp73 and ΔNp63 play anti-apoptotic roles (Lonza M, 2006). Tp73 and Tp63 isoforms are able to bind to p53RE on the target genes. To verify if p63, p73 and their isoforms were able to transactivate the Otx1 gene, through the p53RE, a Luc assay was performed. SaOs2 cell lines were co-transfected with Dox inducible tet-on/TAp63α, /ΔNp63α, /TAp73α, /ΔNp73α expression vectors and pGL3-OTX1 vector. We showed that only TAp73α was able to transactivate Otx1. (Fig. 4.8).

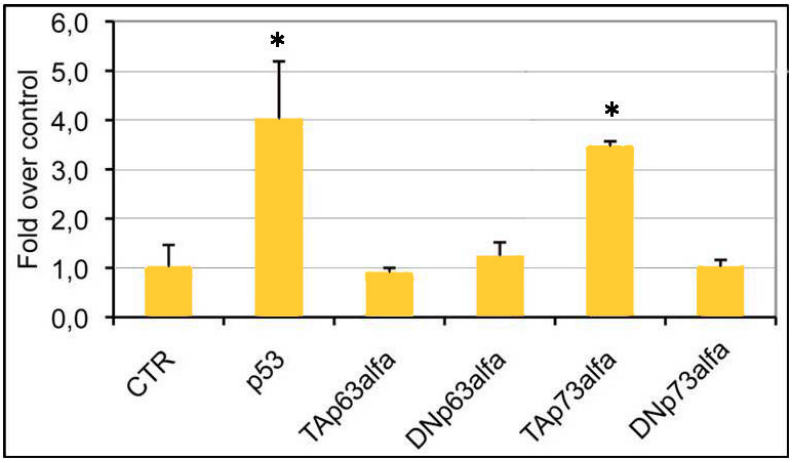


Figure 4.8. Luc assay performed with pGL3-Otx1 and Dox inducible p53, TAp63α, ΔNp63α, TAp73α and ΔNp73α expression vectors. In abscissa are reported the samples co-transfected with pGL3-Otx1 and the Dox inducible expression vectors. The empty pGL3-Otx1 vector was used as control. In ordinate there is the luciferase activity.

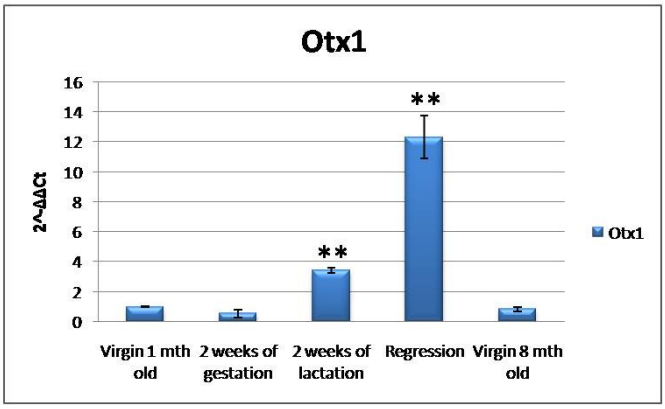
4.6 Role of Otx1 and p53 family in mammary gland development

Unlike most mammalian organs, which develop primarily embryonically with a more or less linear progression toward functional maturity, development of the mammary gland is primarily postpuberal and may divided into both linear and cyclical phases (pregnancy, lactation and involution) (Lewis MT, 2000). In every stage of mammary development are activated different pathways, that regulate cell proliferation and organ remodeling. A qRT-PCR assay was performed to evaluate changes in Otx1 (Fig. 4.9.A), Trp53 (Fig. 4.9.B), Trp73 (Fig. 4.9.C) and Trp63 (Fig. 4.9.D) gene expression in linear and cyclical mammary gland development in mice. Mammary tissues from virgin female CD1 mice 1 month old and 8 months old, and from female CD1 mice at 2 weeks of pregnancy, at 2 weeks of lactation and in regression, were obtained. RNA was extracted and retro-transcribed, and cDNA was analyzed by qRT-PCR, using the gene expression level of virgin 1 month old as control

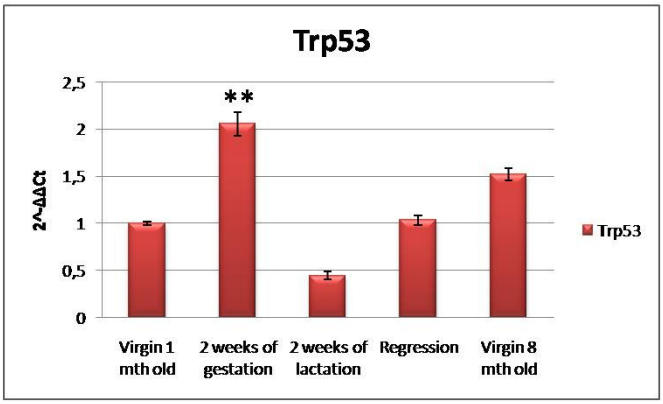
The Otx1 expression levels increased in lactation ($t=5,4393$, $p=0,0055$-$p<0,01$**) and in regression ($t=8,1336$, $p=0,0012$-$p<0,01$**). Trp73 and Trp63 also increased in lactation ($t=2,4238$, $p=0,048$-$p<0,05$*; $t=4,7616$, $p= 0,0089$-$p<0,01$**, respectively) and in regression ($t=8,9239$, $p=0,0009$-$p<0,01$***; $t=27,0131$, $p=0,0001$-$p<0,01$***). Instead, Trp53 expression levels increased only in pregnancy ($t=8,5622$, $p=0,0010$-$p<0,01$**).

These data showed that the Otx1 expression in mouse mammary gland development is statistically correlated with the Trp63 and the Trp73 expression, but not with Trp53.

A



B



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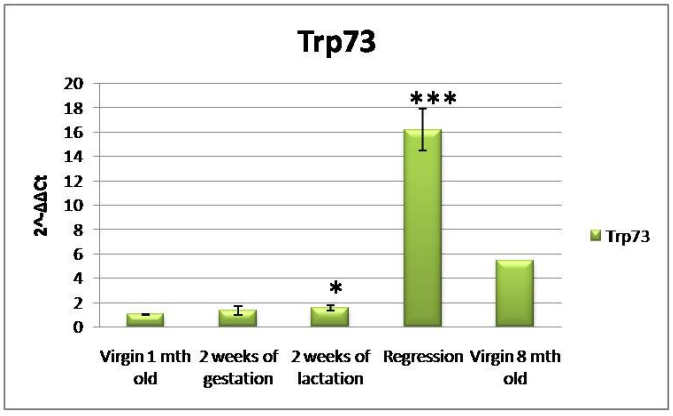


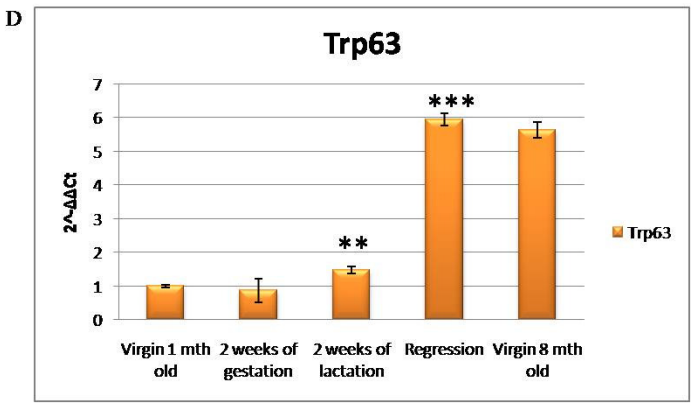


Figure 4.9.A-D. Gene expression levels of Otx1 (A), Trp53 (B), Trp73 (C) and Tr63 (D) genes during the linear and cyclical (gestation, lactation, involution) development of adult mice mammary gland. The gene expression levels were detected by qRT-PCR. The mice virgin 1 month (mth) old were used as control. In abscissa are reported the development stage of the mammary gland, and in ordinate there are the gene expression levels in these samples.

4.7 Otx1, Trp63 and Trp73 expression in p53 knock-out mouse in lactation

In order to confirm the regulation of Otx1 by p53 family members, we evaluated RNA levels of Otx1, Trp73 and Trp63 in breast tissues derived from p53-deficient mice at 7 days of lactation by qRT-PCR. Donehower and coworkers in 1992 generated the mouse strain carrying a germline mutation in the Trp53 gene. The *neo* cassette replaced a part of the Trp53 intron 4 and the 5' portion of exon 5, deleting approximately 40% of the Trp53 coding region, resulting in the lack of the p53 protein (Donehower LA, 1992). In these mice we showed the statistically significant increase of Otx1 (t=11,1879, p=0,0004-p<0,01***), Trp73 (t=6,6875, p=0,0026-p<0,01**) and Trp63 (t=14,4135, p=0,0001-p<0,01***) gene expression in lactation. We confirmed also the absence of the Trp53 expression. All the data were referred to virgin female 1 month old (Fig. 4.10).

We demonstrated that in physiological conditions Otx1 regulation was independent from Trp53, but it was related to Trp73 and Trp63.

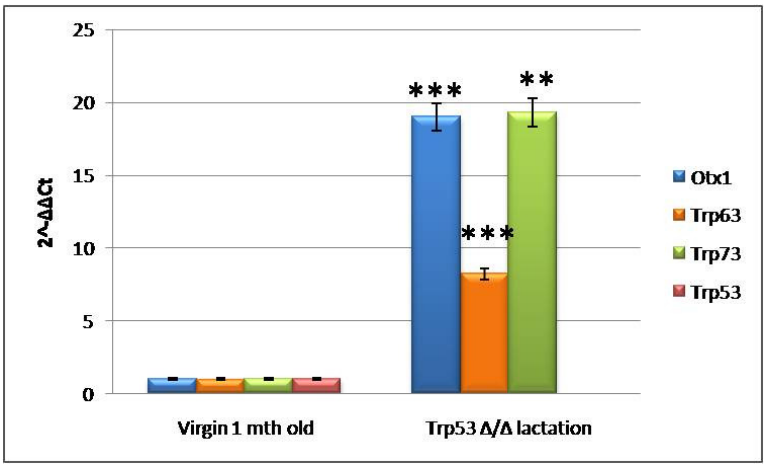


Figure 4.10. Gene expression levels of Otx1, Trp63, Trp73 and Trp53 in p53 knock-out mice in lactation. The p53 knock-out mice was generate carrying a germline mutation in the Trp53 gene. The *neo* cassette replaced a part of the Trp53 intron 4 and the 5' portion of exon 5, deleting approximately 40% of the Trp53 coding region, resulting in the lack of the p53 protein. The gene expression levels were detected by qRT-PCR. The p53 wild-type mice virgin 1 mth old were used as control. In abscissa are reported the samples obtained from the p53 knock-out and the virgin mice, and in ordinate there are the gene expression levels.

4.8 Otx1 and Tp53 family in differentiation of breast cancer stem cells

Mammary gland LA7 cells, isolated by rat mammary adenocarcinoma Rama-25 cell line, are cancer stem cells (CSCs), with self-renewal properties. They have the capacity to differentiate into all of the cell lineages of the mammary gland, and to form heterogeneous tumors and metastasis in non obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. LA7 cells treated with differentiating agents, such as DMSO, are able to form hemispherical polarized dome-shaped structures. These structures recapitulate the cellular changes occurring during the mammary gland development in pregnancy (Zucchi I, 2007). We analyzed by qRT-PCR the relation between Otx1, Trp53, TAp63, ΔNp63 and Trp73 expression levels in LA7 cells and in LA7 differentiated (LA7D) with DMSO. We identified a statistically significant increase of Otx1 ($t=8,4280$, $p=0,0138$-$p<0,05$*), Trp73 ($t=2,1465$, $p=0,0391$-$p<0,05$*) and Trp53 ($t=2,7924$, $p=0,0315$-$p<0,05$*) in LA7D, compare to LA7, suggesting the role of these genes in the differentiation of cancer stem cells. While, ΔNp63 ($t=106,4729$, $p=0,0001$-$p<0,01$***) showed a statistically significant decrease of expression (Fig. 4.11).

IHC performed with anti-OTX1 antibody, showed cytoplasmic and nuclear positivity for OTX1 in the LA7 cells, with an increase of cytoplasmic positivity in LA7D (data not shown).

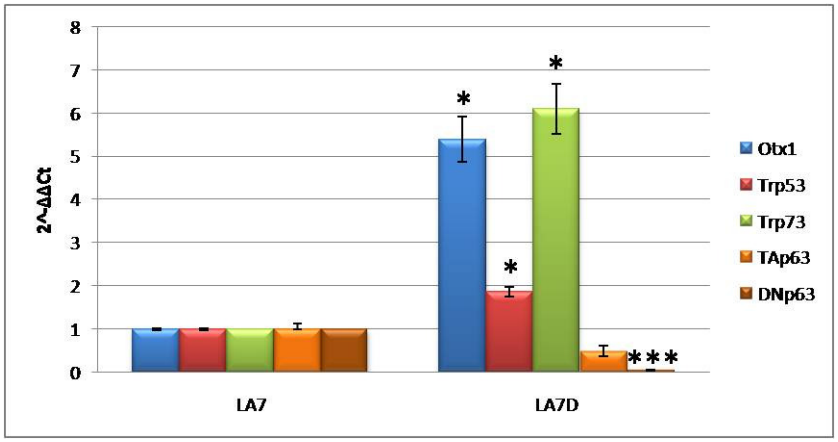


Figure 4.11. Gene expression levels of Otx1, Trp53, Trp73, TAp63 and ΔNp63 in LA7 cells undifferentiated and differentiated (LA7D) with DMSO. The gene expression levels were detected by qRT-PCR. The LA7 cells was used as control. In abscissa are reported the LA7 and LA7D cells, and in ordinate there are the gene expression levels.

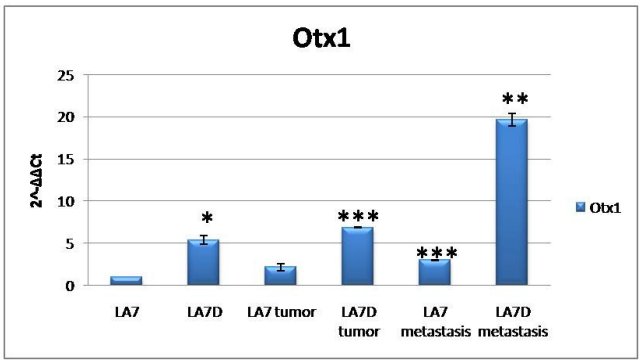
Cells derived from tumors and metastasis generated through the subcutaneous injection of a single or few LA7 and LA7D cells, in NOD-SCID mice, were cultured and maintained under selective conditions.

Cell derived from LA7 tumors and metastasis are able to form, in vitro, mammospheres (non-adherent spherical clusters of stem cancer cells), while those obtained from LA7D tumors and metastasis are not (Zucchi I, 2007).

RNA obtained from these cell cultures, was extracted and retro-transcribed to perform qRT-PCR.

We analyzed the expression levels of Otx1 (Fig. 4.12.A), Trp53 (Fig. 4.12.B), TAp63 (Fig. 4.12.C), ΔNp63 (Fig. 4.12.D) and Trp73 (Fig. 4.12.E). The increase of Otx1, Trp53 and Trp73 expression was detected in metastasis and the results were statistically significant. Interestingly, in the tumors induced by LA7D there was the increase of Otx1, Trp53 and Trp73, and the decrease of TAp63 and ΔNp63 gene expression levels (Table 4.3).

A


Otx1
2^-ΔΔCt
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LA7
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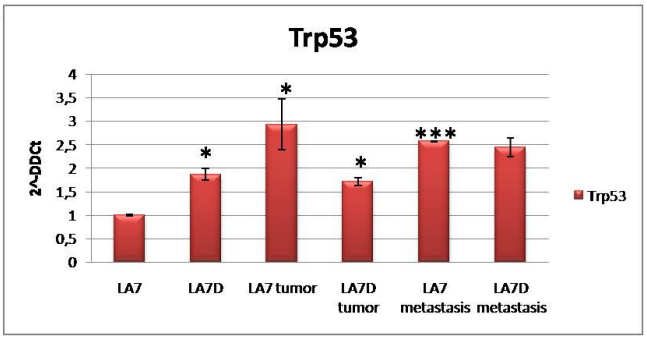
LA7 tumor
LA7D tumor
LA7 metastasis
LA7D metastasis
Otx1

B


Trp53
2^-DDCt
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LA7
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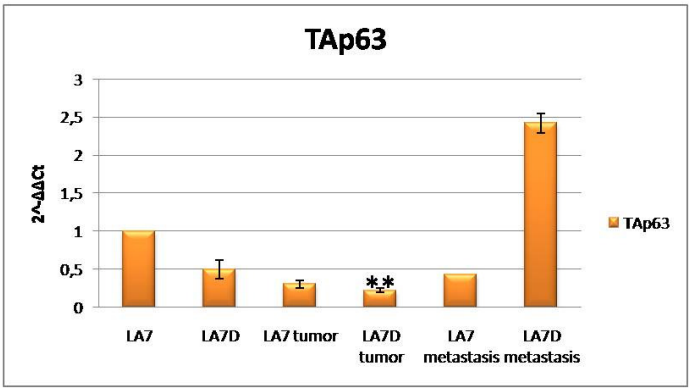
LA7D tumor
LA7 metastasis
LA7D metastasis
Trp53

C


TAp63
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LA7
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TAp63

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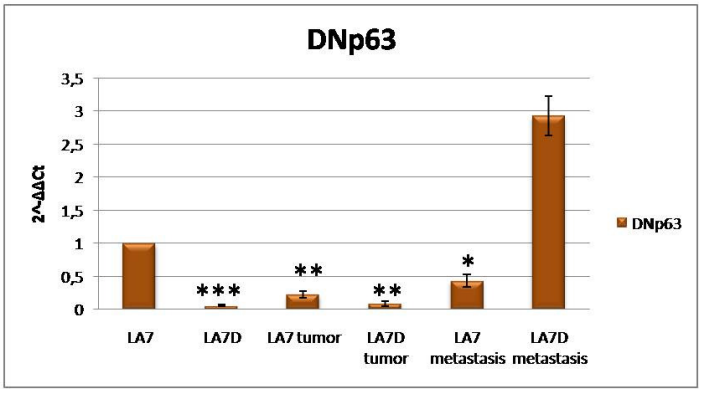
DNp63
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LA7 tumor
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LA7 metastasis
LA7D metastasis
DNp63

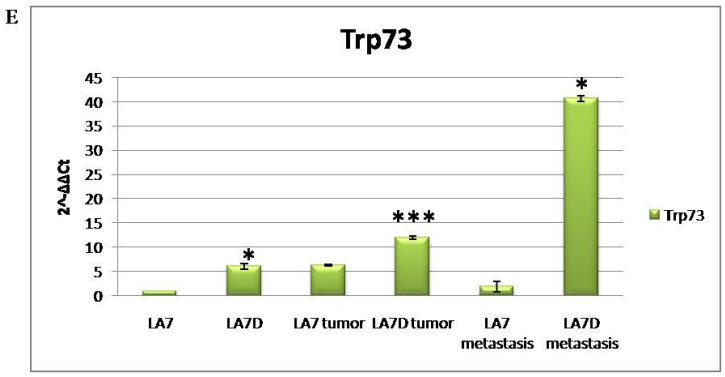


Figure 4.12.A-E. Otx1, Trp53, TAp63, ΔNp63 and Trp73 gene expression levels in LA7 and LA7D, and in cell cultures obtained from the tumors and metastasis generated by injection of LA7 and LA7D in NOD-SCID mice. The gene expression levels were detected by qRT-PCR. The LA7 cells was used as control. In abscissa are reported the LA7 and LA7D cells, and the cells obtained from tumor and metastasis. In ordinate there are the gene expression levels.

Table 4.3. Statistical analysis

Gene	LA7 tumor	LA7D tumor	LA7 metastasis	LA7D metastasis
Otx1	NSS	t=123,2627 ↑ p=0,0001 (p<0,01***)	t=38,4791 ↑ p=0,0007 (p<0,01 ***)	t=24,9076 ↑↑ p=0,0016 (p<0,01**)
Tp53	t=5,1709 ↑↑ p=0,0354 (p<0,05*)	t=8,5866 ↑ p=0,0133 (p<0,05*)	t=176,1294 ↑↑ p=0,0001 (p<0,01***)	NSS
TAp63	NSS	t=26,1999 ↓ p=0,0015 (p<0,01**)	NSS	NSS
ΔNp63	t=15,7718 ↓ p=0,0040 (p<0,01***)	t=21,8450 ↓↓ p=0,0021 (p<0,01**)	t=5,6337 ↓ p=0,0301 (p<0,05*)	NSS
Trp73	NSS	t=48,5834 ↑ p=0,0004 (p<0,01***)	NSS	t=4,2181 ↑↑ p=0,0419 (p<0,05*)

Table 4.3. Statistical analysis of the gene expression variation in cell cultures obtained from the tumors and metastasis generated by injection of LA7 and LA7D, compared to the gene expression in LA7 cells. ↑: increase of gene expression level; ↓: decrease of gene expression level.

4.9 Otx1 and p53 family in human breast tumors

The analysis of the expression of Otx1, Tp63, and Tp73 was performed on 32 human breast tumor samples (Table 4.4), 22 from the previously described (see table 4.1), and 10 new cases.

RNA obtained from these Tp53 wild-type breast cancer was retro-transcribed, and qRT-PCR was performed using Otx1, Tp63 and Tp73 specific probes. Normal tissue was used as control.

The variations of Tp63 (Fig. 4.13.B) and Tp73 (Fig. 4.13.C) expression levels respected to Otx1 (Fig. 4.13.A), was statistically significant, with t=3,1697 and p=0,0024 (p<0,01 **) for Otx1 vs. Tp63, and t=3,1763 and p=0,0023 (p>0,01 **) for Otx1 vs. Tp73.

Table 4.4. Histological type, tumor grade, pT and pN of patient samples

Samples	Histological type	Grade	pT	pN
3T	IDC	2	1c	X
5T	IDC	2	1c	0
8T	IDC	2	2	X
11T	IDC	2	1b	X
29T	IDC	3	2	0 sn
33T	IDC	2	2	0 sn
35T	IDC	2	2	0
37T	IDC	3	2	1a
41T	IDC	2	1	0 sn
44T	IDC	2	2	1
47T	IDC	2	2	1 mic
49T	IDC	2	2	1a
50T	IDC	2	2	1a
51T	IDC	3	2	1a
53T	IDC	2	2	0 ls
54T	IDC	2	2	1
55T	IDC	2	2	0
56T	IDC	2	2	1a
57T	IDC	2	2	3
58T	**ILC**	2	2a	2
62T	IDC	2	2	0 sn
63T	IDC	2	2	1a
64T	IDC	2	2	1a
65T	IDC	2	1c	1a
68T	IDC	2	2	1a
69T	IDC	2	2	1a
70T	IDC	2	2	0 (i+)
71T	IDC	2	2	0
73T	IDC	2	1B	0
74T	IDC	2	2	0
75T	IDC	3	2	0 sn
78T	IDC	2	2	1 mi

Table 4.4. Histological type, tumor grade, pT and pN of patient samples. Legend: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; G1, low-grade; G2, moderate or intermediate grade; G3=high grade; X, not evaluated.

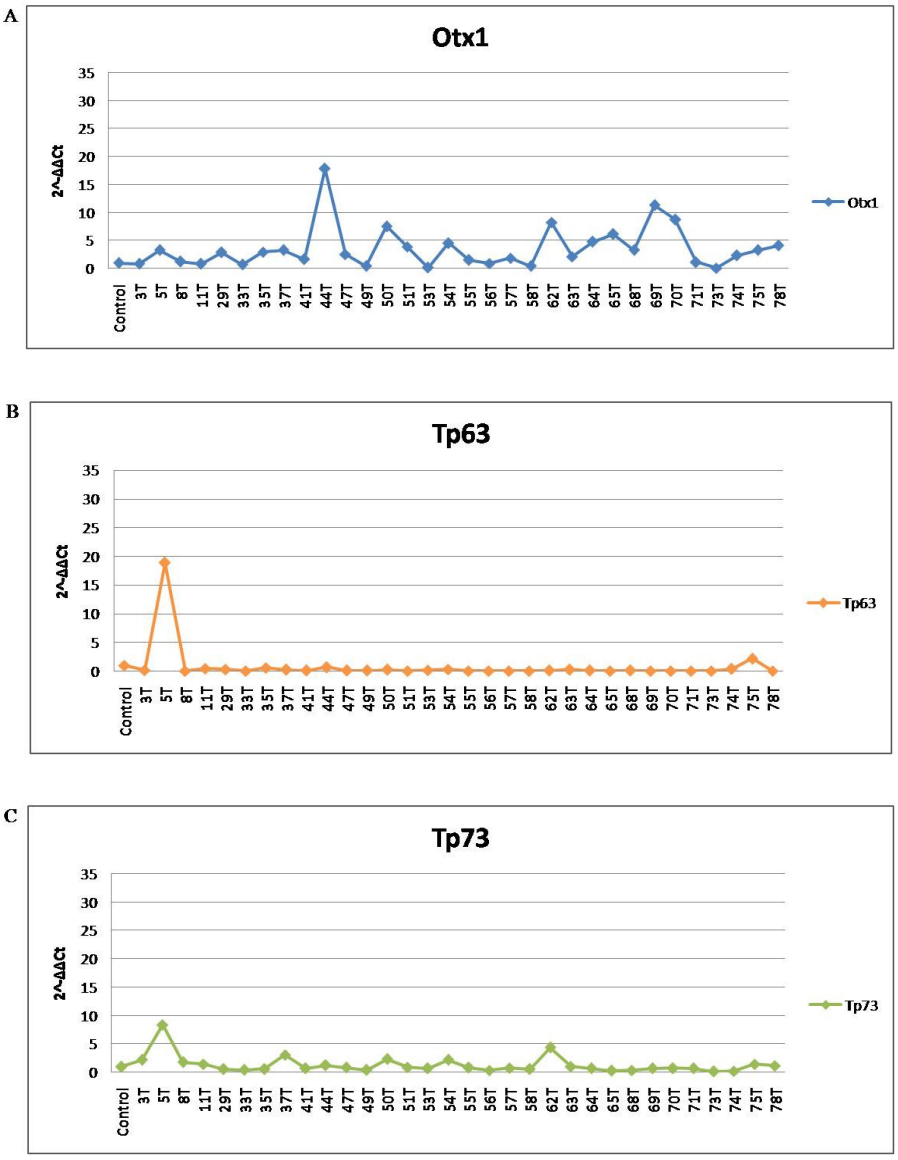
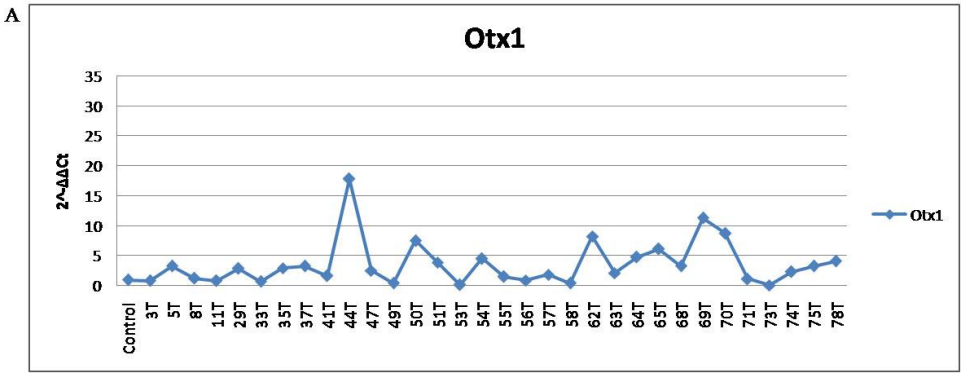


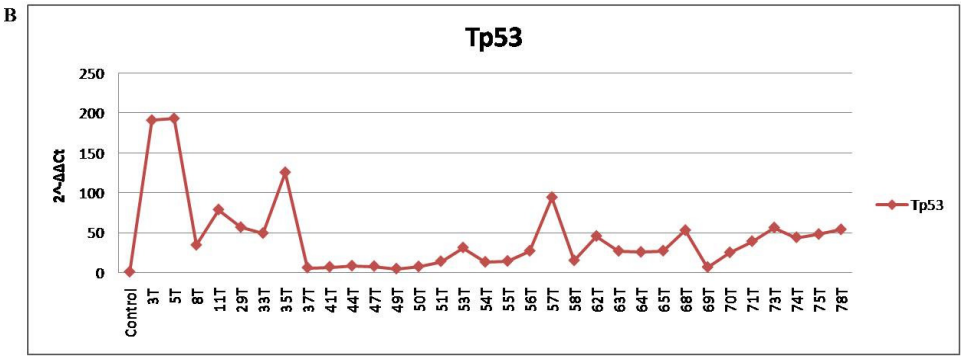
Figure 4.13. A-C. Gene expression levels of Otx1, Tp63 and Tp73 in human ductal and lobular invasive breast cancers (Table 4.4). The gene expression levels were detected by qRT-PCR using Taq Man assays (see table 3.3). The normal mammary tissue was used as control. In abscissa are reported the samples, and in ordinate the gene expression levels.

4.10 Statistical analysis between the expression of Otx1, Tp53, Ta63α, ΔNp63α, Ta73α, ΔNp73α genes in human breast cancer

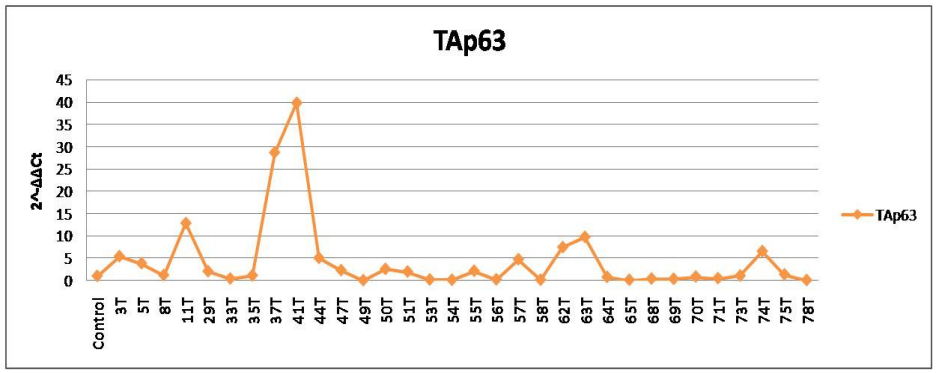
To assess the role of Tp63 and Tp73 in breast cancer, we designed specific primers able to recognize TAp63α, TAp73α, ΔNp63α and ΔNp73α isoforms.

We analyzed the expression levels of Otx1, Tp53 and these isoforms, using normal tissue as control (Fig. 4.14.A-F). qRT-PCR showed statistically significant expression variation between: Otx1 vs. Tp53, TAp73α, ΔNp63α; Tp53 vs. TAp63α, ΔNp63α, TAp73α, ΔNp73α; TAp63α vs. ΔNp63α, TAp73α; TAp73α vs. ΔNp63α, ΔNp73α; ΔNp73α vs. ΔNp63α (Table 4.5). ΔNp73 and ΔNp63 expression are also statistically correlated with linear regression coefficient r=0,87178, t=9,7468 and p=8,2855E-11 (p<0,01 ***) (Fig. 4.14.G).

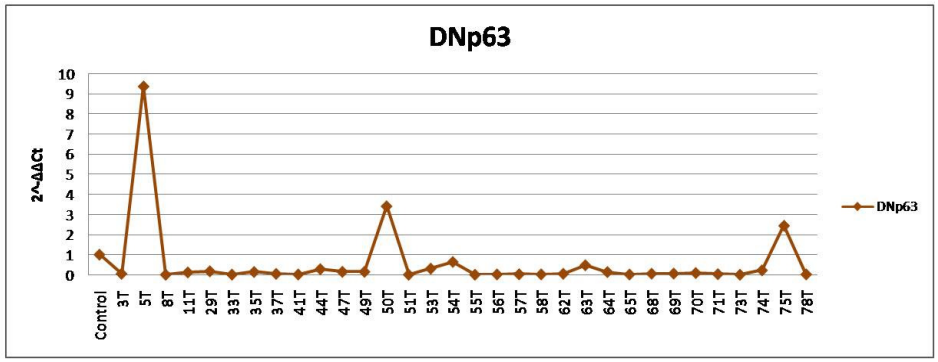




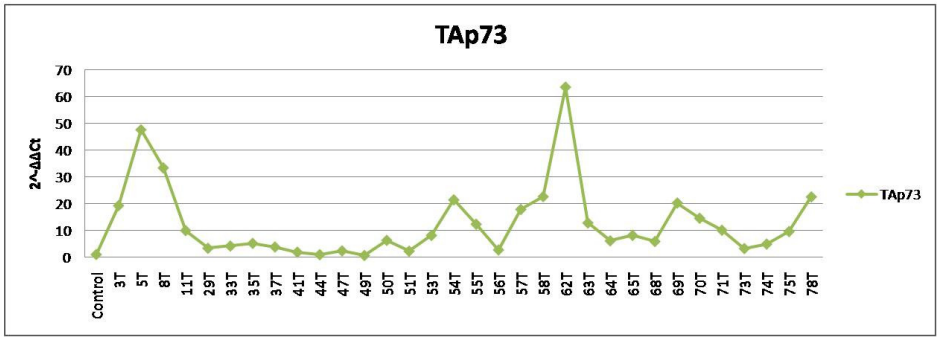
C


TAp63
2^-ΔΔCt
TAp63
Control 3T 5T 8T 11T 29T 33T 35T 37T 41T 44T 47T 49T 50T 51T 53T 54T 55T 56T 57T 58T 62T 63T 64T 65T 68T 69T 70T 71T 73T 74T 75T 78T

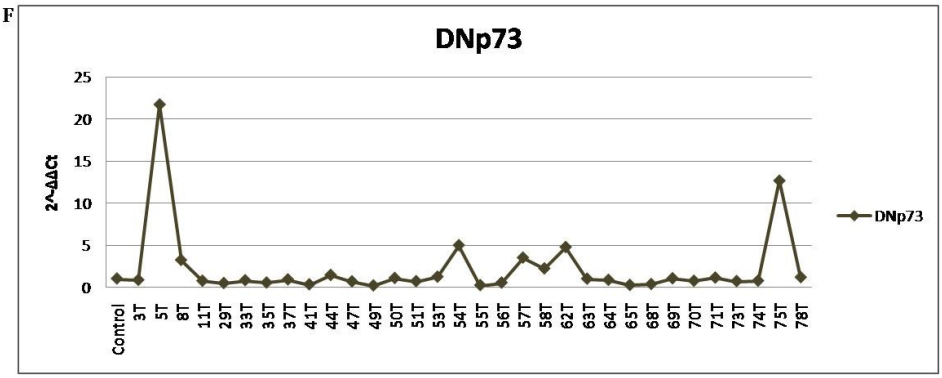
D


DNp63
2^-ΔΔCt
DNp63
Control 3T 5T 8T 11T 29T 33T 35T 37T 41T 44T 47T 49T 50T 51T 53T 54T 55T 56T 57T 58T 62T 63T 64T 65T 68T 69T 70T 71T 73T 74T 75T 78T

E


TAp73
2^-ΔΔCt
TAp73
Control 3T 5T 8T 11T 29T 33T 35T 37T 41T 44T 47T 49T 50T 51T 53T 54T 55T 56T 57T 58T 62T 63T 64T 65T 68T 69T 70T 71T 73T 74T 75T 78T

F


DNp73
2^-ΔΔCt
DNp73
Control 3T 5T 8T 11T 29T 33T 35T 37T 41T 44T 47T 49T 50T 51T 53T 54T 55T 56T 57T 58T 62T 63T 64T 65T 68T 69T 70T 71T 73T 74T 75T 78T

G

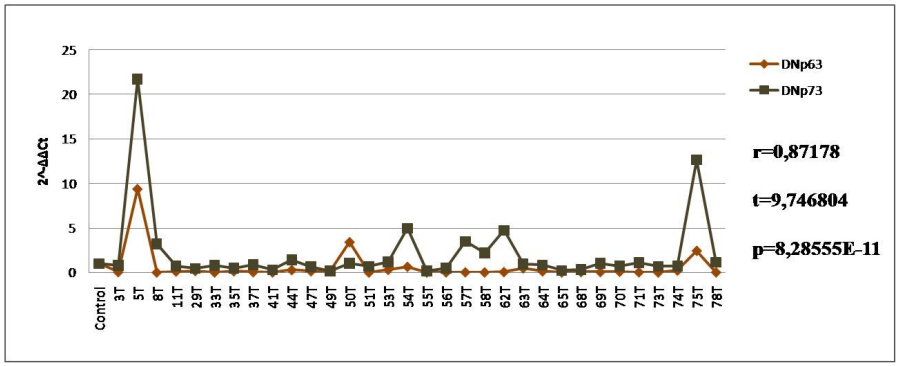


Figure 4.14. A-F. Gene expression levels of Otx1, Tp53, TAp63(α), ΔNp63(α), TAp73(α) and ΔNp73(α) in human ductal and lobular invasive breast cancers (see Table 4.4). The gene expression levels were detected by qRT-PCR using specific primers (Sybr Green technology) (see table 3.2). The normal mammary tissue was used as control. In abscissa are reported the samples, and in ordinate the gene expression levels.

Figure 4.14. G. Correlation analysis between the ΔNp63(α) and ΔNp73(α) gene expression levels.

Table 4.5. Statistical analysis

Genes	t value	p
Otx1/Tp53	4,8693	0,0001 (p<0,01 ***)
Otx1/TAp73α	3,3501	0,0014 (p>0,01 **)
Otx1/ΔNp63α	2,6966	0,0090 (p<0,01 **)
Tp53/TAp63α	7,7407	0,0001(p<0,01 ***)
Tp53/ΔNp63α	5,2795	0,0001 (p<0,01 ***)
Tp53/TAp73α	3,6812	0,0005 (p<0,01 ***)
Tp53/ΔNp73α	5,0661	0,0001 (p<0,01 ***)
TAp63α/ΔNp63α	2,5565	0,0130 (p<0,05 *)
TAp63α/TAp73α	2,8582	0,0058 (p<0,01 **)
TAp73α/ΔNp63α	4,9073	0,0001 (p<0,01 ***)
TAp73α/ΔNp73α	4,0867	0,0001 (p<0,01 ***)
ΔNp73α/ΔNp63α	2,0280	0,0469 (p<0,05 *)

Table 4.3. Statistical analysis of the gene expression variation in breast cancer samples, compared to each other. In the table are reported only the variations statistically significant.

5 DISCUSSION

5.1 Tp53 mutations and polymorphisms: cancer implications

Breast cancer, one of the most common and feared cancers, ranks second after lung cancer among tumor-related death (Ries S, 2000). Indeed, one out of nine women is affected in her life span by breast cancer, and the current therapies for the disease are inadequate once it has metastasized. Normal, as well as malignant, growth is regulated by endocrine hormones and by local tissues factors, such as polypeptide growth factors. Breast cancers progress as hyperplastic ductal and lobular epithelial growth, acquiring progressive genetic changes (including those of oncogenes and tumor suppressor genes) leading to clonal outgrowths of progressive malignant cells (Papadakis EN, 2000).

The Tp53 tumor suppressor gene is commonly altered in human cancer and the spectrum of its mutations in these cancers provide clues to the etiology and molecular pathogenesis of neoplasia (Papadakis EN, 2000). Tp53 is a key player in stress responses that preserve genomic stability, responding to a variety of insults including DNA damage, hypoxia, metabolic stress and oncogene activation. The most well documented mechanism by which p53 exercises its protective roles is as a transcription factor. By binding to specific response elements in DNA, p53 modulates the transcription of genes that govern the major defenses against tumor growth, which include cell cycle arrest, apoptosis, maintenance of genetic integrity, inhibition of angiogenesis and cellular senescence. p53 also interacts with numerous cellular proteins, including several that control programmed cell death, and these molecular interactions might contribute to the inhibitory role of p53 in tumorigenesis (Whibley C, 2009).

Malfunction of the p53 pathway is an almost universal hallmark of human tumors. Somatic mutations of Tp53, that result in the absence or dysfunction of p53, are one of the most common mechanisms by which the p53 pathway is damaged during tumorigenesis. The direct loss of function of p53 is also associated with an unfavorable prognosis in some types of cancer. The most common mutations in Tp53 are single base substitution, which result in amino acid substitution (missense mutations) in the DNA binding domain (DBD) of the p53 protein. Typically, the mutant tumor-associated p53 proteins have lost most or all of the normal p53 wild-type functions. p53 mutant exert a dominant-negative action toward wild-type p53 (Whibley C, 2009). In the Tp53 mutations database it is reported that this gene is mutated in 20%

cases of breast cancer (Fig. 5.1) (IARC TP53 mutations database), but in our study we found 9,1% of Tp53 mutated in ductal and lobular invasive breast carcinomas (see table 4.1).

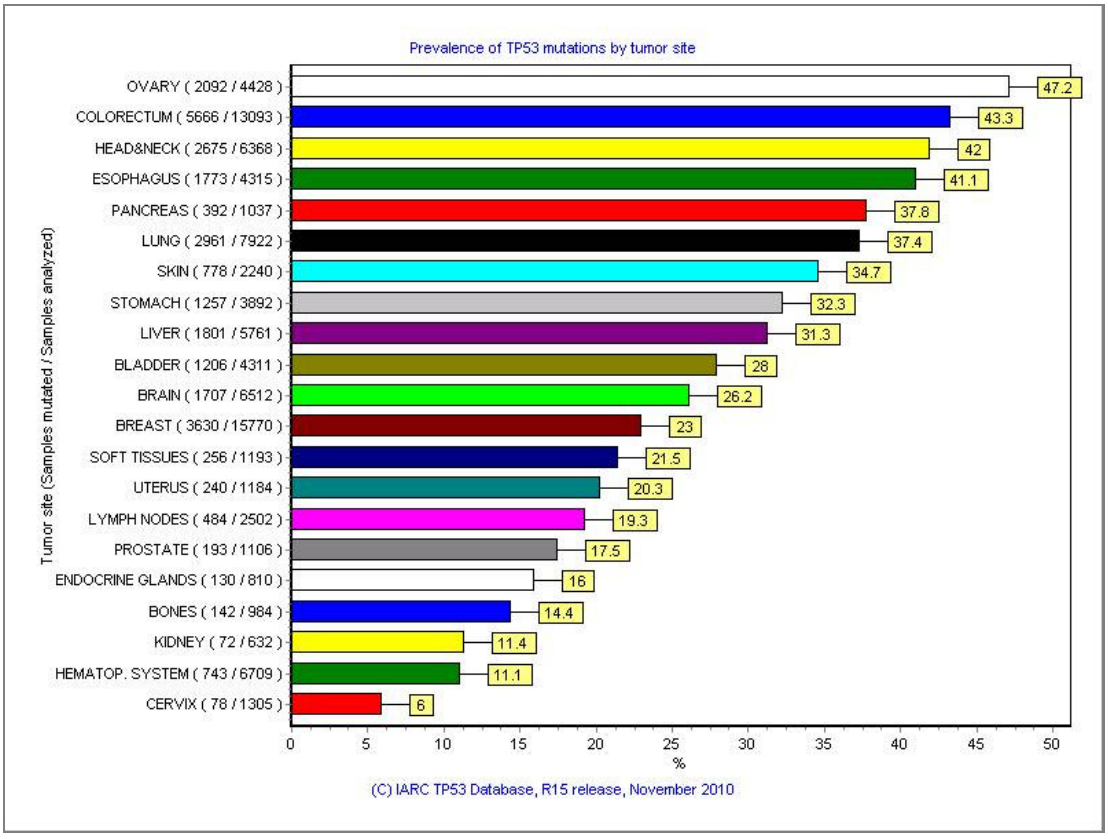


Figure 5.1. Percentage of Tp53 mutations in human cancer (IARC TP53 mutations database).

The loss of function of p53 is obtained by several other mechanism, including polymorphisms at single nucleotide (SNPs), at the Tp53 locus (NCBI SNP database). Nevertheless, most of these variations (80%) are intronic and can be presumed to have no cancer related biological consequences (Whibley C, 2009). The remaining 20% are exonic. Of the 19 exonic polymorphisms that have been reported in Tp53, 8 of them are synonymous. Although these polymorphisms do not change the amino acid sequence or structure of the protein, changes in base sequence could modify protein expression, folding and function, or provoke new splicing events. The remaining 11 polymorphisms in Tp53 are non-synonymous, resulting in an amino

acid change in the protein. Changes in the amino acid sequence can alter the ability of the p53 protein to bind to responsive elements (REs) of target genes, alter the recognition motifs for post-translational modifications, or alter the protein stability and the interactions with other proteins.

In our work, in 2 human ductal breast cancers (4T and 8T) we found a silent exonic polymorphism at codon 36 (CCG to CCA) (see table 4.1), which is located in the region crucial for the MDM2 binding. MDM2 is a key negative regulator of p53, which binds to the TA domain of p53 and ubiquitylates the protein, targeting it for degradation in the proteasome. Because p53 transcriptionally activates MDM2, the expression levels of p53 and MDM2 are balanced through a negative feed-back loop, which is altered by an increase in p53 levels following stresses such as DNA damage. MDM2 also binds to Tp53 mRNA, controlling the rate of translation (Whibley C, 2009). The finding that MDM2 (negative regulator of p53) binds to mRNA and facilitates the translation of Tp53, and that this interaction is abrogated by silent mutations in the N-terminal region, suggests a mechanism by which synonymous polymorphisms can affect p53 functions (Whibley C, 2009). In other 2 human ductal breast cancers (5T and 11T) we found the rare silent exonic polymorphism at codon 213 (CGA to CGG) (see table 4.1), which results in the lost of a TaqI site (Carbone D, 1991).

In each sample we detected the non-synonymous polymorphism at the codon 72, in homozygous state (c.412 C p.P72; c.412 G p.R72) or in heterozygous state (R72P) (see Fig. 4.1 and table 4.1). This polymorphism causes functional change in the p53 pathway and is involved in tumorigenesis (Whibley C, 2009). Residue 72 is located within the proline-rich region, which plays an important role in p53-mediated apoptosis and is required for the growth suppression activity of p53. This polyproline region is an Src homology 3 (SH3) binding domain. This domain is a small non-catalytic domain that has been identified in many signaling proteins, regulating protein-protein interactions. The SH3 domain contain the amino acid motif PXXP (where P indicates proline and X indicates any amino acid). The P72 of p53 constitutes one of the five PXXP SH3 binding motifs defined within this region.

Sharp ethnic differences in codon 72 allele frequencies have been observed, with a latitude gradient in variant frequency from the equator. The P72 allele frequency is approximately 60% in diverse African populations, and 30-35% in Caucasian and in Asian populations (IARC Tp53

database). The P72 allele appears to be the ancestral allele by comparison with DNA of non-human primates (Hu W, 2009).

p53-P72 is structurally different from p53-R72, and this is reflected by its altered electrophoretic mobility. p53-R72 migrates more rapidly on gel than p53-P72 (Papadakis EN, 2000).

Also the functions of these proteins are different. p53-R72 is more efficient in inducing apoptosis and suppressing cellular transformation that the p53-P72. The p53-R72 is also more efficiently target of degradation for the E6 protein of HPV16, suggesting that individuals homozygous for R72 may be at higher risk of HPV-related cervical cancer (IARC Tp53 database). In contrast the p53-P72 appears to induce G1 arrest and senescence at high levels

Patients homozygous for P72 have poorer survival than those with other genotypes. Furthermore, maintenance of the R allele of Tp53 has been associated with reduced disease-free and overall survival in breast cancer. A recent study showed that breast cancer patients with the P72 genotype were less sensitive to chemoterapy than those with R72 or R72P (Toyama T, 2007). This chemoresistance is related to the inability of p53-P72 tumors cells to go into apoptosis.

5.2 Regulation of Otx1 gene expression by p53 tumor suppressor protein: role in breast cancer stem cell differentiation

In our work we demonstrated that the p53 protein regulates the expression of homeogene Otx1, through the 3'p53RE responsive element (p53RE) in tumors (see Fig. 4.5 and Fig 4.6). This direct control raises the question of this interaction's functional role in tumors.

While the function of p53 in the control of cell cycle checkpoints, apoptosis and senescence has been extensively investigated, its role in cellular differentiation is still poorly characterized. Nevertheless, it has been shown in several experimental models that wild-type p53 facilitates cell differentiation (Matas D, 2004).

Tp53 gene is conserved from invertebrates to mammals. Homologs of Tp53 have been described in many different organisms, such as sea anemone, clams, *C. elegans*, *Drosophila*, frogs, zebrafish. The existence of homologs of Tp53 gene in lower organisms such as flies and worms, which have short life spans and do not acquire cancer as adults, suggests that tumor suppression

may not be the original function for p53. There are some evidences for the role of p53 in infection disease, in development and in fecundity. One of the stress responses that activate the p53 pathway program is the production of nitric oxide, which is commonly made during an inflammatory response. This suggests the possibility that p53 is part of the innate immune system's response to inflammation and infections by initiating a program of defenses. Alternatively, the evolutionary origin of the p53 protein in lower organisms utilizes its functions to protect the germ line from DNA damage mutations. Indeed, in *Drosophila* and *C. elegans*, p53 is most commonly expressed in the germ line cells, and its function is the surveillance of damaged germ cells to eliminate defective offspring from the population by apoptosis (Hu W, 2009).

In *Drosophila* and *C.elegans* the only dividing cells in the adult organism are the germ line cells and a few immune cells. The body plan of vertebrates, however, contains many tissues that continue to divide throughout lifetime and renew themselves via tissue specific stem cells (SCs) (Hu W, 2008).

SCs are cells with the capacity to self-renewal and to generate daughter cells, which can differentiate into multiple cell types that are found in the mature tissue (Cicalese A, 2009).

These two functions can be accomplished through single asymmetric self-renewing mitotic division, in which one progeny retains SC identity, and the other undergoes multiple rounds of divisions before entering a postmitotic fully differentiated state (Fig. 5.2.b and d) (Cicalese A, 2009). The cells that form the intermediates between SCs and terminally differentiated cells are usually referred to as progenitor cells, transit cells, and transit amplifying cells. The two cells generated by asymmetric division differ markedly in their proliferative potential: the SC remains quiescent or slowly proliferating, whereas the progenitor cell divides actively. This ensures the production of large numbers of differentiated progeny while maintaining a relatively small pool of long-lived SCs (Smalley M and Ashworth A, 2003). The asymmetric division is a particularly attractive strategy, which the SCs can use to perpetuate themselves and to generate differentiated progeny, maintaining an appropriate number of cells (Morrison SJ and Kimble J, 2006).

SCs can also use symmetric division, when they are expanding in number during development or, in the adult, after tissue injuries during healing and regeneration, to generate two daughter cells that are destined to acquire the same fate (Fig. 5.2.a and c). The capacity to divide

symmetrically has been observed in the nervous and hematopoietic system (Morrison SJ and Kimble J, 2006).

The asymmetric and symmetric divisions can be used together or separately to produce an appropriate number of SCs and/or differentiated daughters (Fig. 5.2.e) (Morrison SJ and Kimble J, 2006).

Recent evidence suggests that symmetric division is necessary for cancer stem cell proliferation, and that asymmetric division functions as a mechanism of tumor suppression in *Drosophila* neuroblasts (Gonzalez C, 2007). Notably, some of the genes that control asymmetric cell division in flies have an evolutionarily conserved role in the regulation of cell polarity and in tumor suppression, suggesting that polarity loss may contribute causally to cancer in mammals (Morrison SJ and Kimble J, 2006). Pelicci and coworkers demonstrated that p53 regulates the polarity of cell division in mammary stem cells, inducing asymmetric division and cell differentiation, while its lost of function leads to the symmetric division, resulting in tumor growth. Pharmacological reactivation of p53 in breast cancer SCs correlates with the restoration of asymmetric division and tumor growth reduction (Cicalese A, 2009).

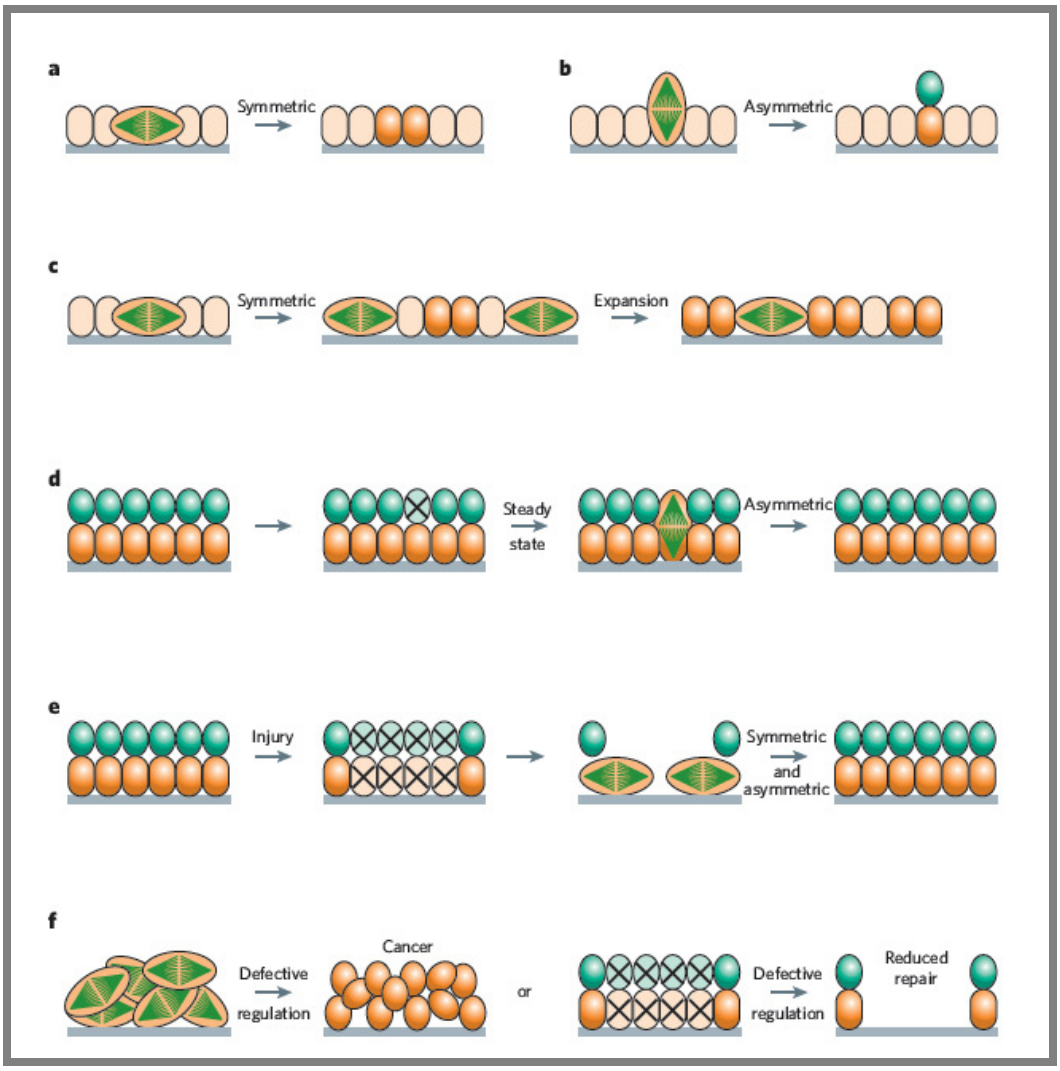


Figure 5.2. Stem cells can facultatively use both symmetric and asymmetric divisions. a. Symmetric division in the plane of the epithelium generates two morphologically similar stem cells daughters (orange). **b.** Asymmetric division perpendicular to the plane of the epithelium generates one stem cell and one differentiated daughter. The asymmetric division predominates during late fetal development, and in the basal layer of the epithelia and in the ventricular zone of the brain, in adult. **c.** During development, symmetric divisions expand the stem-cell pool. **d.** In healthy adults, divisions perpendicular to the epithelial plane typically maintain normal numbers of stem cells and differentiated cells in the basal layer of the epithelia and in the sub-ventricular zone of the brain. **e.** In healthy adults, cells can be lost to injury (X). Symmetric divisions are proposed to regenerate additional stem cells, and asymmetric divisions to regenerate differentiate daughters. F. Defects in the regulation of the switch between symmetric and asymmetric divisions can be involved in cancer formation or in the decrease capacity for tissue repair.

In order to verify if the interaction between p53 and Otx1 could have a role in asymmetric division and differentiation of breast cancer stem cells, we tested their expression levels in LA7 cell lines differentiated (LA7D) with DMSO (see Fig. 4.11). The LA7 cells are clonal lines derived from the cell line RAMA-25 isolated from mammary adenocarcinoma induced in rat by chemicals. These cells are breast cancer stem cells with SCs properties of self-renewal, the capacity to differentiate into all of the lineages of the mammary gland, and to form heterogeneous tumors in mice from a single cells. LA7 cells spontaneously undergo differentiation *in vitro* by forming hemispherical polarized domes, and this differentiation can be strongly promoted by differentiation inducers, such as DMSO. The in *vitro* dome formation is under specific gene regulation, and corresponds to a specific stage in the lobulo-alveolar development of the mammary gland occurring during pregnancy. Distinct steps of cellular differentiation take place during the terminal differentiation of the alveolar epithelial cells; these steps are defined by the sequential activation of genes coding for milk protein. The developmental stage of DMSO-induced domes in LA7 may correlate to an early stage of lobulo-alveolar development during pregnancy that precedes mammary gland terminal differentiation (Zucchi I, 2002).

We found higher levels of Tp53 and Otx1 in LA7D compared to the undifferentiated LA7 cells. These data suggest that the activation of Otx1 by p53 in cancer stem cells could represent an attempt to force neoplastic cells to differentiate, and this raise the hypothesis that Otx1 could be an “tumor suppressor like gene”, involved in the balance between symmetrical and asymmetrical division.

5.3 Regulation of Otx1 gene expression by p53 family in differentiation of breast cancer stem cells

The p53 family includes p63 and p73. The p53, p63 and p73 proteins all contain several conserved domains: an amino-terminal transactivation (TA) domain, a DNA binding domain (DBD), and an oligomerization domain (OD) In addition to these three domains, p63 and p73 contain in their carboxyl terminus a protein-protein interaction domain known as the “sterile alpha motive” (SAM), and a transcription inhibition (TI) domain. The Tp53, Tp63 and Tp73 genes give rise to multiple protein products resulting from both alternative promoter usage and alternative mRNA splicing. They have two promoters: one upstream from exon 1 that generates

isoforms containing TA domain, and one intronic that generates amino-terminal truncated isoforms (ΔN) that function as dominant-negative inhibitors of the TA isoforms. Furthermore, carboxy-terminal splicing generates a variety of TA and ΔN isoforms, that give rise to additional structural and functional diversity (see Fig. 1.22) (Hu W, 2009).

p53, p63 and p73 show high sequence homology both at the nucleotidic and aminoacidic level, even if p63 and p73 are more closely related to each other than to p53. The DBD of p63 and p73 shows high sequence homology with the p53 DBD, so p63 and p73 can bind to p53RE on the target genes (see Fig. 1.21).

In order to verify if the TAp63α, ΔNp63α, TAp73α and ΔNp73α isoforms are able to bind to 3'p53RE in the Otx1 promoter, we performed a Luciferase assay. We showed that only TAp73α, in addition to Tp53, transactivates Otx1 (see Fig. 4.8).

p53 and its homologues TAp73α and Tap63α are tumor suppressor genes, guardians of the genome, but whereas TAp73α is comparable with p53 in potency in the transactivation and apoptosis assays, Tap63α is very weak. One reason for this difference could be that the TI domain of TAp63α isoform drastically reduces its transcriptional activity. The TI domain binds to a region in the amino-terminal TA of p63, which is homologous to the MDM2 binding site in p53, and inhibits the transcriptional activity. Notably, this TI domain is biologically important, because patients with deletions in this region have phenotypes which are very similar to patients with mutations in the DBD (Moll UM and Slade N, 2004).

Although Tp63 and Tp73 are the more recently identified Tp53 family members, it appears that these genes are ancestral to Tp53 and possibly evolved from a Tp63/Tp73-like gene. Tp63 and Tp73, like Tp53, appear to retain their primordial functions in germline surveillance and reproduction (Hu W, 2009). Recent studies inspired by developmental functions of Tp63 and Tp73 indicate that members of the Tp53 family also cooperate in differentiation control (Stiewe T, 2007).

For example, the p63 protein is involved in the specification and differentiation of squamous epithelial cells (Stiewe T, 2007). During development, a single basal layer of primitive epithelium initially covers the inner and outer surface of the organism. As embryogenesis proceeds, epithelia that will be exposed to mechanical stress progressively acquire new layers of suprabasal and differentiated cells which offer better resistance against environmental effects. In

the adult, the skin epidermis maintain an inner layer of proliferating cells that give rise to multiple layers of terminally differentiated cells that continuously reach and are shed from the body surface. A key question is how epithelial stem cells retain this self-renewing capacity, which is so critical for epidermal integrity. Tp63 is involved in this mechanism. Mice lacking Tp63 are severely compromised in their ability to generate epidermis, as well as many other types of stratified epithelia. It has been demonstrated that the two principal isoforms of Tp63, TAp63α and ΔNp63α, have distinct roles in epithelial development. ΔNp63α is expressed in the primitive epithelium, and in the adult its expression persists in the basal layer of the stratified epithelium. This isoform is an epithelial progenitor cell marker that maintains epidermal SCs self-renewal capacity, promoting cell proliferation. Instead, TAp63α is predominant in the suprabasal layer were there are differentiated cells. In conclusion, ΔNp63α governs basal-epidermal gene expression while its interaction with TAp63 promotes terminal differentiation (Blanpain C and Fuchs E, 2007). ΔNp63α maintains the proliferative potential of epithelial progenitors, probably regulating symmetric division of SCs.

Another example, Tp73 is involved in the regulation of SCs differentiation: ΔNp73α isoforms are expressed in proliferating cells, whereas TAp73α isoforms are involved in terminal differentiation. During nephrogenesis, there is a spatiotemporal switch from ΔNp73 in proliferating nephron precursors, to TAp73 in the differentiation domain of the renal cortex (Stiewe T, 2007).

In contrast to apoptosis, differentiation does not eliminate cells from the organism, but rather removes cells from the proliferative compartment at the same time as preserving cellular integrity and function. By preventing the proliferation of damaged cells, differentiation further helps to maintain the genetic stability of the organism. Differentiation therefore adds to the spectrum of p53-based cell fate decisions and could contribute to the tumor suppressor activity of the Tp53 family (Stiewe T, 2007).

In our work we demonstrated that Otx1 activation by p53 is involved in the differentiation of breast cancer SCs, and that TAp73α is also able to transactivate Otx1 expression. To assess if the TAp73α-Otx1 pathway could have a role in cancer SCs differentiation, we evaluated the Otx1 and Trp73 (rat homologous to human Tp73) gene expression levels in rodent LA7D cells using LA7 cells as control. We showed an increase of both Trp73 and Otx1 gene expression, at higher levels than Tp53 (see Fig. 4.11). In its role of genome guardian, Tp73, through Otx1 activation,

induces terminal differentiation of breast cancer SCs, probably leading to asymmetric division as a mechanism of tumor suppression. We hypothesize that both p53 and p73 are involved in cell differentiation, but their expression is probably regulated in a temporal and spatial manner.

Moreover, we evaluated the levels of Trp63α (rat homologous to human Tp63) isoform. During LA7 differentiation, the ΔNp63α RNA levels decrease (see Fig. 4.11), confirming the role of this isoform as a stem cell marker.

5.4 Expression pattern of Otx1 and Tp53 family members in the mammary gland during pregnancy, lactation, and involution: role in mammary SCs differentiation?

The mammary gland is one of the few organs that undergoes numerous rounds of proliferation and regression throughout adult life. Development of the mammary gland occurs in several different stages: it begins in the embryo, progresses after birth, and is completed in the mature individual. The full development of the gland proceeds in distinct phases: embryonic, puberal, pregnancy, lactation, and involution (Zucchi I, 2002).

A rudimentary system of small ducts is present in newborn mice. Later, moderate ductal growth occurs until puberty, when a more pronounced development of the ducts leads to the formation of a ductal tree. In the puberal phase, the growth pattern is characteristically ductal and few alveoli are formed. This process involves an orchestration of hormones, morphogens and growth factors. In virgin mice, the parenchyma consists of a highly organized system of ducts with terminal end-buds which represent the major sites of ductal growth and give rise to alveolar buds. Full alveolar development occurs during pregnancy: during the first half, alveoli increase in number and size, and during the second half, the alveoli start to form larger and differentiated secretory lobules. Terminal differentiation of alveolar epithelial cells is completed at parturition, with the production and secretion of milk (see Fig. 1.10) (Zucchi I, 2002). This epithelial growth is sustained by the mammary epithelial SCs (MASCs), which generate proliferating progenitor cells, that differentiate into alveolar secretory cells (see Fig. 1.6) (Polyak K, 2007). Finally, the end of weaning suppresses lactation and leads to involution of the lobulo-alveolar compartment, returning the mammary gland to its non-pregnant state (Zucchi I, 2002).

Mammary gland functional differentiation occurs with distinct morphological and molecular changes of the epithelial cells and allows for the production and secretion of milk. The secretory

alveolar cells represent the final cellular state of the differentiation process within the mammary gland. These differentiation steps taking place during pregnancy and lactation are defined and characterized by the sequential activation of genes encoding for the milk proteins: WDNM1, β-casein, WAP and α-lactalbumin. In particular, early pregnancy stage is characterized by the expression of WDNM1 and β-casein genes, the expression of which increases during alveolar proliferation in the second half of pregnancy and during lactation. Late pregnancy and lactation are characterized by the additional expression of WAP and α-lactalbumin. LA7D cells, as previously described, correlate to early pregnancy stage, because express WDNM1 and β-casein genes, but not WAP and α-lactalbumin (Zucchi I, 2002). The LA7 cells represent a model for *in vitro* differentiation of mammary epithelial cells.

In LA7D cells we demonstrated that p53, p73 and Otx1 are involved in cancer SCs differentiation, with the formation of structures that correlate with lobulo-alveolar differentiation at the end of the first stage of pregnancy. To verify if this pathway plays a role during *in vivo* linear and cyclical mammary gland development, we evaluated the Trp53, Trp63, Trp73 (mouse homologues to human Tp53, Tp63 and Tp73) and Otx1 gene expression levels in pregnancy, lactation and involution, in mice. We showed an increase of Otx1, Trp73 and Trp63 gene expression levels in lactation (2 weeks) and in regression, with an increase of Trp53 in gestation (2 weeks) (see Fig. 4.9).

p53 is a potent mediator of pregnancy. In the normal mammary gland of the virgin mouse, the p53 protein is expressed at basal levels in the ductal epithelium of the quiescent gland. Under hormonal stimulation, which occurs during pregnancy, p53 is translocated in the nucleus of the epithelial cells and is activated (Sivaraman L, 2001). We found high Tp53 expression levels at half pregnancy, when the alveoli start to differentiate. We hypothesize that p53 could be involved in this process, by activating asymmetric divisions of MASCs, and inducing cell differentiation.

During the cyclical development of the mammary gland, in physiological conditions, Otx1 expression is not regulated by p53, but its expression is correlated with Trp73 and Trp63. To confirm this datum we evaluated the Otx1, Trp73 and Trp63 expression in the p53-deficient mouse in lactation on the 7^{th} day, and we showed high levels of gene expression (see Fig. 4.10). It has been reported that the lactation in p53-deficient mice is normal (Blackburn AC and Jerry DJ, 2002). It is known that homeobox genes are involved in the development of mammary gland,

regulating proliferation and differentiation of MASCs and progenitor cells (Chen H and Sukumar S, 2003). We found that Otx1 is involved in lactation, and we hypothesize that, through activation by p73 or p63, it could regulate the terminal differentiation of the MASCs.

Otx1, Trp63 and Trp73 are also involved in the involution of the mammary gland. This process is characterized both by apoptosis of mammary epithelial cells and by tissue remodeling of the gland to a duct system similar to that in the mature virgin (Furth PA, 1999). It has been reported that OTX1 is involved in the pruning of long distance axonal projections during the development of the nervous system. The development of precisely wired neuronal circuits requires that axons grow to appropriate targets and form specific patterns of synaptic connections. In many animals, during embryonic development, the emergence of the final adult pattern of connectivity is preceded by the formation of transient "exuberant" connections. The OTX1 protein is required for the elimination of exuberant projections from neurons in layer 5 of the cerebral cortex. (Zhang YA, 2002). We found that OTX1 could exert a similar function during the mammary gland remodeling.

5.5 The properties of mammary gland cancer SCs: involvement of p53/p63/p73/Otx1 pathway in breast cancer and metastasis formation

Breast cancer is the leading cause of cancer-related death in women world-wide. Despite significant advances in diagnosing and treating breast cancer, several major unresolved problems remain, such as tumor progression, recurrence, treatment and therapeutic resistance. Resolving these problems is complicated by the fact that breast cancer is not a single disease, but is highly heterogeneous at both the molecular and clinical levels (Polyak K, 2007). According to the "cancer SCs hypothesis", tumors are derived from mutated stem cells that have retained, or progenitors/differentiated cells that have regained, the SC property of cell-renewal. The cancer SCs possess the capacity for indefinite self-renewal, while the lineage of committed progenitor cells, have lost this capacity (see Fig. 1.12). Therefore, in tumors there is a heterogeneous and complex population of cancer SCs, progenitor cells, transit amplifying cells, and differentiated cells (Zucchi I, 2008).

Rat breast cancer SCs, LA7, can form heterogeneous tumors from a single cell when injected in mice. LA7-generated tumors contain at least three distinct cell types: polygonal, epithelial

elongated and mesenchyme-like cells, distinguishable by their morphology and expression markers. The polygonal and epithelial elongated cells have different properties in term of the ability to sustain sphere formation, and the capacity for 3D organotypic growth and in terms of tumor-development and tumor-sustaining capacity. The mesenchymal-like cells exhibit limited expansion capacity and no capacity to generate sphere and 3D organotypic growth. The polygonal cells are self-renewing cancer SCs, and the epithelial cells are lineage-committed progenitor cells (Zucchi I, 2008).

In our work we evaluated the role of Otx1, Trp53, Trp63 and Trp73 in the development of tumors and metastasis, after the injection of LA7 and LA7D in mice (see Fig. 4.12).

A single undifferentiated LA7 cell has the capacity to generate tumors. These tumors contain highly proliferating cells, expressing the proliferation marker Ki67, and terminally differentiated cells, expressing luminal and myoepithelial markers. They also show partial higher order of structural organization, reminiscent to the architecture of the tissue of origin. Cells derived from these tumors were cultured and established under selective conditions, obtained removing mouse cells and rat tumor differentiated cells (Zucchi I, 2007). Trp53 was over-expressed and ΔNp63 down-regulated in the established cultures (see Fig. 4.12), indicating the possible Tp53 activation to lead to the asymmetric divisions of breast cancer stem cells.

Tumors derived from LA7D cells retain only a sub-set of tumor-initiating cells. The differentiation of multipotent LA7 cells results in a cellular hierarchy in which most cells do not contribute to tumor initiation and growth. Cells derived from these tumors do not form mammospheres *in vitro*, and the sphere-forming ability is associated with stem cells (Zucchi I, 2007). In these committed cells derived from LA7D-tumors we found over-expression of Otx1, Trp53 and Trp73, confirming their role in differentiation. Instead, ΔNp63 and TAp63 are down-regulated (see Fig. 4.12).

LA7 and LA7D injected in mice are able to form metastasis. The cancer SCs or the progenitor committed cells can migrate from origin tumor, invade a new tissue and undergo metastatic growth. These processes allow the neoplastic cells to enter lymphatic and blood vessels for dissemination into circulation (see Fig. 1.14). There is diversity in tumor cell migration: the cells can migrate as a single cell or as collective cells. Collective movement of cells is a well described phenomenon that occurs during embryological development, such as during the development of glands and ducts of mammary tissue. In breast cancer highly differentiated,

collective cell invasions are described (Friedl P and Wolf K, 2003). Otx1 gene is over-expressed in metastasis induced by both LA7 and LA7D cells (see Fig. 4.12). Several experiments in zebrafish embryos give rise to the fact that OTX1 is able to induce cell aggregation in embryonic development (Bellipanni G, 2009). In the migration process OTX1 could induce cellular aggregation of neoplastic cells, and in metastasis OTX1 could activate cell differentiation, under p53 and p73 regulation.

5.6 p53/p73/p63/Otx1 network in human breast cancer

p53, p73 and p63 exhibit significant homology in the DBD and OD. They can bind and transactivate the same target genes and form homo/heterotetramers. The ratio between the different oncogenic (ΔN isoforms) and anti-oncogenic (TA isoforms) family members is critical to direct the balance between life or death following DNA damage (Moll UM and Slade N, 2004).

Molecular interaction between TA/ΔN isoforms have been demonstrated both at the protein/DNA level and at the protein/protein level (Moll UM and Slade N, 2004). Mixed protein complexes are found between ΔNp63/TAp73-TAp63 and ΔNp73/TAp63-TAp73 isoforms, while complexes between wild-type p53 and p73 or p63 are not observed. In contrast, mutated p53 could interact with TAp63 and TAp73 proteins. p53 mutant, ΔNp63 and ΔNp73 isoforms generally exert a dominant-negative action toward wild-type p53, TAp63 and TAp73 isoforms (Moll UM and Slade N, 2004).

The interactions between p53 family members are under strict molecular regulation and lead to the cellular fate during development or under DNA damage. These interactions are critical to maintaining the genome integrity, regulating proliferation, differentiation, senescence or apoptosis. Oncogenic stress signals deregulate the cellular ratio between TA/ΔN isoforms in favor of the ΔN isoforms which inhibit apoptosis and lead to the proliferation and renewal of cancer SCs. On the contrary, differentiation signals shift the balance between TA and ΔN isoforms toward the TA isoforms, which regulates the asymmetric division of stem cells (Fig. 5.3) (De Young MP and Ellisen LW, 2007).

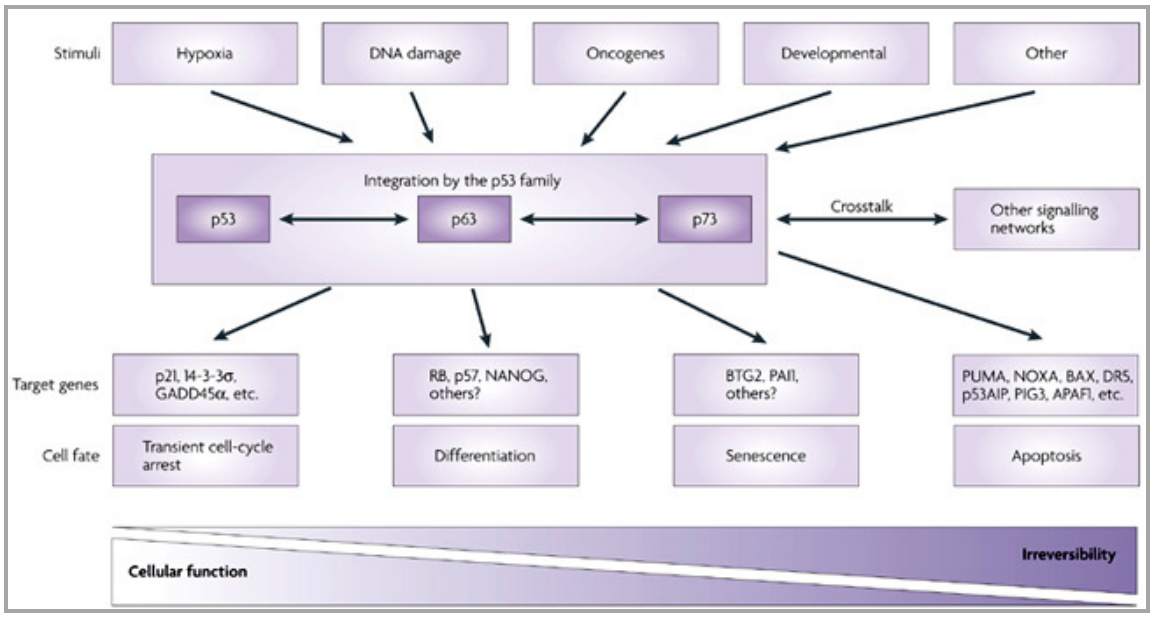


Figure 5.3. p53-family-based cell-fate decision. The p53 family is activated by cellular stress, hyper-proliferative signals and developmental stimuli. These inputs are integrated within the p53 family in crosstalk with other cellular signaling networks to reach an appropriate cell fate decision that is executed by the selective transactivation of distinct transcriptional programmes. The most common outcomes are reversible cell cycle arrest, irreversible cell cycle exit (differentiation or senescence) or apoptotic cell death.

In human ductal and lobular invasive breast cancer we found over-expression of Otx1, Tp53, TAp63α and TAp73α, with down-regulation of ΔNp63α and ΔNp73α (see Fig.4.14.A-F), and statistical correlation between Otx1/Tp53 (see Fig. 4.2), and ΔNp63α/ΔNp73α (see Fig. 4.14.G). These data suggest that Otx1, Tp53, TAp63α and TAp73α over-expression represent an attempt to force the neoplastic cells to differentiate, and this raises the hypothesis that Otx1 could be involved in the balance between symmetrical and asymmetrical cell division.

5.7 OTX1 cellular localization: role in the cell differentiation

In the human ductal and lobular invasive breast cancer we showed the OTX1 cytoplasmic localization in the most of samples, while in few samples and in non neoplastic tissue adjacent to carcinoma we detected nuclear localization (see Fig. 4.3). In LA7 cells we showed nuclear localization, while during the differentiation of LA7 cells we detected an increase of OTX1 cytoplasmic localization.

The OTX1 nuclear and/or cytoplasmic localization suggests different functions of this molecule in cell differentiation. During the development there are some examples. It has been reported that the subcellular localization of the OTX family members have a role in differentiation during embryo development. During the corticogenesis the OTX1 protein is required for the axon refinement of the neurons in the layer 5. OTX1 protein is initially located to the cytoplasm of the progenitor cells in the ventricular zone, and remains cytoplasmic up to neurons migrate and begin start to differentiate. During the first week of postnatal life, when layer 5 neurons begin pruning their exuberant axons, OTX1 is actively imported into the nuclei (Zhang YA, 2002). OTX2, a member of the OTX family, is involved in the differentiation of the rods and bipolar cells in fetal retina. The subcellular localization of OTX2 is important to determine the fates of rod photoreceptor and bipolar cells, during their generation, and initial differentiation in the neonatal retina. The segregation of postmitotic cells into immature rods and bipolar cells, under the influence of various extrinsic factors, such as retinoic acid, is accompanied by the OTX2 localization into cytoplasm and nucleus, respectively (Baas D, 2000).

We hypothesize that the subcellular localization of OTX1 could be involved in different pathways and stages of differentiation. Further studies are need to demonstrated the role of the OTX1 cellular localization in the tumors.

5.8 The retinoic acid and breast cancer therapy. Induction of breast cancer cells differentiation, through activation of p53, Otx1 pathway.

We demonstrated that retinoic acid (RA) induces transcription of both Tp53 and Otx1 in breast cancer cells.

RA, a biologically active metabolite of vitamin A, plays a critical role during normal development and in the establishment and maintenance of physiological process in adult tissues. Its function is induce cellular differentiation, through binding to the two classes of nuclear receptors, the retinoid acid receptors (RARs) and the retinoid X receptors (RXRs). RARs and RXRs are member of the steroid-thyroid hormone receptors super-family. Both types of retinoid receptors are encoded by three distinct genes, α, β, and γ. These receptors function as ligand activated transcription factors that bind to specific RA response elements (RAREs) on the target genes and regulate their expression. The ligand-binding domains (LBDs) of RARs and RXRs are

distinct, and can be pharmacologically targeted separately. The RARE sequence consists of AGGTCA or like core motifs arranged as a direct repeat with 2 or 5 bp spacing (Lee MO, 1995).

RARs can heterodimerize with RXRs, whereas RXRs also heterodimerize with other nuclear hormone receptors, including thyroid hormone receptors or vitamin D receptors. In absence of ligands for the RAR-RXR complex, the target genes of the receptors are repressed. This is due to the recruitment of complexes containing histone deacetylases (HDAC) and co-repressors. This process results in histone deacetylation, chromatin compaction and silencing of target gene promoter regions. After binding with ligands, such as RA, the retinoid receptors are activated. RA dissociates HDAC-containing co-repressor complex and recruit co-activator-histone acetyltransferase (HAT) complex. The final result is chromatin decondensation and transactivation of target gene promoter, involved in cell differentiation or apoptosis (Jimenez-Lara AM, 2004).

Data from animal models, epidemiology, preclinical and clinical trials have provided strong support for the use of retinoic acids as pharmacological agents in cancer therapy and prevention. Certain malignancies, such as acute promyelocytic leukemia (APL), juvenile chronic myelogenous leukemia, Kaposi's sarcoma and high-risk neuroblastoma, are already being treated with retinoid-based therapies. In addition, retinoids can be use in psoriasis and acne therapy, and prevent the development of non-melanoma skin cancer in patients with defects of DNA repair, such as xeroderma pigmentosus (Table 5.1) (Jimenez-Lara AM, 2004).

Table 5.1. The clinical use of retinoids in cancer therapy and chemoprevention

Trade name	Retinoid	Activity	Therapeutic application
Tretinoin	All-*trans* retinoic acid	Pan-RAR	APL, leukoplakia (prevention), actinic keratosis, (prevention)
Alitretinoin, panretin	*9-cis* retinoic acid	Pan-RAR	Kaposi's sarcoma
Isotretinoin	*13-cis* retinoic acid	Pan-RAR	Oral leukoplakia, skin cancer, head and neck (in combination with IFNα-2a), neuroblastoma
Bexarotene	LDG1069	RXR (residual RAR activity)	Cutaneous T-cell lymphoma (stage IA-IB, IIA)
Fenretidine, 4- HRP	4-hydroxy-phenylretinamide	RARγ, RARβ, additional unknown activities	Second breast cancer
Acyclic retinoid	polyprenoic acid	RAR, RXR, PPAR activities	Hepatocellular carcinoma (prevention)
Adapalene, CD271		RARγ, weak RARβ	Cervical intraepithelial neoplasia (prevention)

[a]Abbreviations: 4-HRP, 4-hydroxy-phenylretinamide; APL, promyelocytic leukemia; IFN, interferon; PPAR, peroxisome proliferator activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor.

The pleiotropic actions of retinoids and their ability to force cells into differentiation and death, offer great promise for cancer therapy and chemoprevention. Following the success of RA in differentiation therapy for APL, interest has increased in the potential use of retinoids for the

prevention and treatment of human breast cancer and other solid tumors (Stephen R and Darbre PD, 2000). The problem is that long-exposure to RA can induce cell-resistance, so the clinical applications of RA have shown limited effects. A possible solution is combine retinoids with other therapies, such as chemoterapy, interferons, or anti-estrogens in estrogen (E)+/RAR+ patients (Hua S, 2009).

Expression of RARα receptors have been found at high levels in ER positive breast cancer cells. There are several reports that RARE are coincident with E receptor responsive elements (EREs) in the promoter of target genes (Stephen R and Darbre PD, 2000). Estrogen receptors (ERs) are a member of the steroid-thyroid hormone receptors super-family, like RARs. Many of the genomic regions bound by RARs overlapped with ERs (Fig. 5.2). Transcriptional analysis demonstrated that RARs and ERs have agonistic effects in the transcription of target genes. While RA induces cell differentiation and apoptosis, E signaling drives proliferation and promotes survival in cell lines (Hua S, 2009). Furthermore, it has been demonstrated a feed-back between RAR/ER transcription. E can up-regulate expression of RAR, while RA can down-regulate ER expression, suggesting a two-way interactive regulatory pathway. This highly coordinated intersection between RAR/ER signaling pathway, provides a global mechanism for balancing gene expression (Stephen R and Darbre PD, 2000).

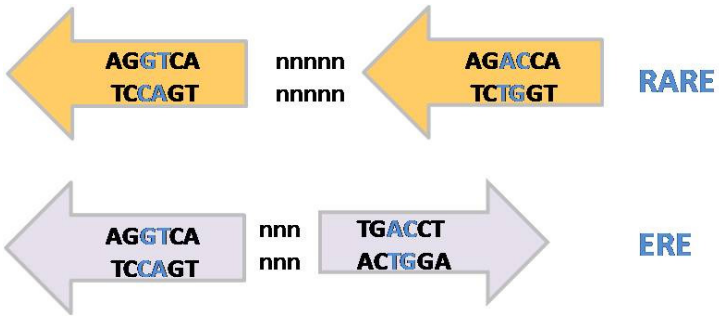


Figure 5.4. Schematic representation of the retinoic acid receptor responsive element (RARE) and estrogen receptor RE (ERE). Retinoic acid receptors and estrogen receptors are members of the steroid-thyroid hormone nuclear receptors super-family. They bind to the specific REs on the target genes. The ERE is a perfect inverted hexanucleotide repeat, while RARE is an imperfect hexanucleotide repeat. All hexanucleotides have the general sequence AGNNCA. The central two nucleotides confer the binding specificity.

In breast cancer cells, the RA has been shown to be associated with down-regulation of several genes essential for proliferation and survival, and with up-regulation of genes involved in cell

differentiation and apoptosis, such as Tp53. While RAR increases the Tp53 expression (Mrass P, 2004), ER inhibits the p53 functions (Konduri SD, 2010). Several lines of evidence suggest that p53 and ERα can influence each other activities through different mechanisms. Complexes between p53 and ERα proteins have been described, with inhibitions of p53 activity. ERα can also control p53 mRNA expression, and vice versa, and p53 can down-regulate ERα responsive genes by interfering with the binding of ERα to its EREs (Fernandez-Cuesta L, 2010).

The identification of the genes regulated by RARs in breast cancer cells, and in particularly the discovery of their extensive crosstalk with estrogen signaling, may benefit breast cancer diagnostics and therapeutic intervention. Specifically, RARs and ERs can diagnostically differentiate tumor subtypes and patient outcome. The Luminal type breast tumors show high expression of direct target genes of ERs and RARs, indicating that their antagonistic affects may be relevant for primary ER-positive tumors. The expression of RAR targets genes, predicts a positive clinical outcome (Hua S, 2009).

5.9 Conclusions

In our work we demonstrated that the Otx1 homeogene is over-expressed in human ductal and lobular invasive breast cancer. The p53 and TAp73α tumor suppressor proteins transactivate the Otx1 gene in the tumors. This pathway is involved in the differentiation of LA7, leading to the asymmetric division of the breast cancer stem cells in a spatial and temporal manner.

LA7 cells are a model for the study of mammary gland development. LA7 differentiated with DMSO form dome-structures that corresponds to a specific stage in the lobulo-alveolar development of the mammary gland occurring during pregnancy.

During the adult linear and cyclical (pregnancy, lactation, involution) mammary gland development the Otx1 expression is not regulated by p53, but is correlated with Tp73 and Tp63 gene expression in lactation and in regression. This suggests that Otx1 gene is regulated by p73 in physiological conditions, but in the tumor is involved p53. Needs to clarify the role of p63.

5.10 *Perspectives*

The relation between p63 and Otx1 is unclear. The Luc assay has shown that the p63 isoforms are not able to transactivate the Otx1 expression, but during physiological mammary gland development the Tp63 expression increases whit Otx1. The question is which is the target gene. The p63 protein regulates the Otx1 gene, or the OTX1 protein regulates the Tp63 gene?

Preliminary studies to identify down-streams of OTX1 were carried out using a computational approach based on the alignment of the conserved OTX1 binding sites between mammalian species (Orso F, 2010). The analysis found an OTX1RE in the Tp63 promoter in a non-canonical region in the intron 4. To confirm the molecular interaction between OTX1 and Tp63 promoter, Chip and Luc assay will be performed.

The same approach will be used to found retinoic acid responsive elements and estrogen responsive elements on the Tp53 promoter. The molecular mechanisms by which RARs/ERs regulate Tp53 expression are still unclear. It has been reported that these receptors regulate indirectly the Tp53 expression (Fernandez-Cuesta L, 2010)(Mrass P, 2004), but the presence of RAREs/EREs in the promoter of Tp53 has not been found yet. We demonstrated that RA increases the expression of both Tp53 and Otx1 genes, with differentiation of breast cancer cells as result, but the molecular activation of these pathway will be demonstrated. Furthermore we will look for RAREs and EREs in the other members of the p53 family.

p53 is hormonally responsive, activated by estrogen and progesterone during pregnancy (Sivaraman L, 2001). We will study the effects of the estrogen and progesterone on the mammary stem cells and LA7 breast cancer stem cells, evaluating if these hormones activate the Otx1, p53, p63, p73 pathway, at level of both mRNA and protein.

Simeone A. demonstrated that Otx1 directly activates the growth hormone (GH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) transcription in the anterior lobe of the pituitary gland (Acampora D, 1998). Furthermore p53 nuclear accumulation was found in corticotroph adenomas (Kontogeorgos G, 1999). We will evaluate the correlation between Otx1, Tp53,Tp63 and Tp73 in pituitary adenomas and in normal adenohypophysis to demonstrate the role of the hormones on the Otx1, p53, p63, p73 pathway.

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