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Role of the NF-κB pathway and nitric oxide in mammary gland involution after weaning. Implications in breast cancer.

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CERTIFICAN:

Que la presente tesis doctoral, titulada "ROLE OF THE NF-κB PATHWAY AND NITRIC OXIDE IN MAMMARY GLAND INVOLUTION AFTER WEANING. IMPLICATIONS IN BREAST CANCER" (PAPEL DE LA VÍA DEL NF-κB Y DEL ÓXIDO NÍTRICO EN LA INVOLUCIÓN DE LA GLÁNDULA MAMARIA TRAS EL DESTETE. IMPLICACIONES EN CÁNCER DE MAMA), que presenta Doña Ana Bosch Campos para optar al Grado de Doctor por la Universidad de Valencia, ha sido realizada bajo su dirección en el departamento de Medicina de la Facultad de Medicina dentro del programa de doctorado 260S así como en la Fundación para la Investigación del Hospital Clínico Universitario de Valencia (INCLIVA), y que se encuentra finalizada y lista para su presentación a fin de que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, firman la presente en Valencia, a 14 de marzo de 2012.

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¿Cómo que no puedes hacer más?
¿No será que no puedes hacer menos?

José María Escrivá de Balaguer

INDEX

TABLE INDEX

FIGURES INDEX

ABBREVIATIONS

A	Adenine
Aa	Amino acid
AKT	v-akt Murine Thymoma Viral Oncogene
Ala	Alanine
AMPK	Adenosine Monophosphate Kinase
AP	Aminopeptidase
Apaf-1	Apoptosis protease-activating factor-1
APP	Acute Phase Proteins
APR	Acute Phase Response
BAD	Bcl-2-associated death promoter
BAFF	B-cell activating factor of the TNF family
BAX	BCL2-associated X protein
BCL-2	B-cell CLL/lymphoma 2
BCL-X	BCL2-like 1
BH4	Tetrahydrobiopterin
BIM	BCL2-like 11
C	Cytosine
c-FLIP	Cellular Fas-associated death domain-like IL-1b-converting enzyme-inhibitory protein
C/Ebp	CCAAT/Enhancer Binding Protein
CAD	Caspase Activated Dnase
CD	Cathepsin D
CD40L	CD40 Ligand
CDC42	Cell Division Cycle 42
CNTF	Ciliary Neurotrophic Factor
COX-2	Cyclooxygenase-2
CP	Carboxypeptidase
DFF	DNA fragmentation factor
DISC	Death Inducing Signalling Complex
DR	Death Recpetor
E	Oestrogen

ECM	Extra-cellular Matrix
EDRF	Endothelium-Derived Relaxation Factor
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELF5	E74-like factor 5 (ets domain transcription factor)
eNOS/NOS3	Endothelial Nitric Oxide Synthase
EP	Endopeptidase
EPc	Cysteinyl Endopeptidase
EPO	Erythropoietin
ER	Oestrogen Receptor
ERK	Extracellular-signal Regulated Kinase (MAPK)
ESI	Electrospraylionization
FAD	Flavin Adenine Dinucleotide
Fas	TNF receptor superfamily member 6
FasL	Fas Ligand
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FMN	Flavin Mononucleotide
FOXO3a	Forkhead box O3
G	Guanine
G-CSF	Granulocyte Colony-Stimulating Factor
GAS	Gamma Activated Sequence
GH	Growth Hormone
GM-CSF	Granulocyte– Macrophage CSF
GRB2	Growth Factor Receptor-bound Protein 2
GSH	Glutathione (reduced)
GSK3	Glycogen Synthase Kinase 3
GSNO	S-nitrosoglutathione
GSSG	Glutathione (oxidized)
HER	Human Epidermal growth factor Receptor
HLA	Human Leukocyte Antigen
IAP	inhibitor of apoptosis proteins

IB	Immunoblot
ICAM-1	Intercellular Adhesion Molecule-1
IEF	Isoelectric Focusing
IGF	Insulin-like Growth Factor
IGFBP	IGF Binding Proteins
IGFR	Insulin-like Growth Factor Receptor
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
INF	Interferon
iNOS/NOS2	Inducible Nitric Oxide Syntase
IP	Immunoprecipitation
IRF1	Interferon Regulatory Factor 1
IRS1	insulin receptor substrate 1
JAK	Janus Kinase
LC	Liquid Chromatography
LIF	Leukaemia Inhibitory Factor
LKB1	Liver Kinase B1
LTβ-R	Lymphotoxin-β Receptor
M6P	Mannose-6-Phosphate
MALDI-TOF-MS	matrix-assisted laser-desorption ionization–time-of-flight MS
MAPK	Mitogen-Activated Protein Kinase
MDM2	Murine Double Minute 2
MHC	Major Histocompatibility Complex
MIP-1a	Macrophage Inflammatory Protein-1a
MMP	Matrix Metalloproteinase
MS	Mass Spectrometry
mTOR	Mammalian Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells

NLS	Nuclear Localization Signal
nNOS/NOS1	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NO-tyr	nitrotyrosine
NSAID	Non-Steroidal Anti-Inflammatory Drug
O2	Oxigen
$ONOO^-$ /PN	Peroxynitrite
OSM	Oncostatin M
OsmR	Oncostatin M Receptor
p-AKT	phosphorilated AKT
p-STAT3	phosphorilated STAT3
p-STAT5	phosphorilated STAT5
PDK1	3-phosphoinositide-dependent protein kinase 1
Pg	Progesterone
PgR	Progesterone Receptor
PHAP	putative HLA-DR-associated protein
PI	Propidium Iodide
PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol bisphosphate;
PIP3	Phosphatidylinositol triphosphate
PKA	cAMP dependent Protein Kinase
PLA2	Phospholipase A2
PRL	Prolactin
PRLR	Prolactin Receptor
PS	Signalling Peptide
PTEN	Phosphatase and Tensin Homologue Deleted on Chromosome Ten
PTH1R	Parathyroid Hormone Related Protein Receptor 1
PTHRP	Parathyroid Hormone Related Protein
PUMA	BCL2 binding component 3
RAGE	Receptor for Advanced Glycation End product
RANK-L	Receptor Activator of NF-κB Ligand

RAPTOR	regulatory associated protein of TOR.
RNS	Reactive Nitrogen Species
Rsmad	Receptor Substrate Smad
RTK	Receptor Tyrosine Kinase
S.E.M.	Standard Error Mean
SAA	Serum Amyloid A
SH2	Src-Homology 2
Slpi	Secretory Leukocyte Protease Inhibitor
Smac	Second Mitochondrial Activator of Caspases
Smad	Mothers Against Decapentaplegic Drosophila Homolog
STAT	Signal Transducer and Activator of Transcription
T	Thymine
TAM	Tumour Associated Macrophages
TDLU	Terminal Ductal-Lobular Unit
TEB	Terminal End Buds
TGFβ	Transforming Growth Factor beta
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand
Tsc-22	Tgfβ3-stimulated clone 22
TSC1	Tuberous Sclerosis 1
TWEAK	(TNF)-like weak inducer of apoptosis
Tyr	Tyrosine
uPA	urokinase-type Plasminogen Activator
UR	Unrelated
VCAM-1	Vascular Cell Adhesion Molecule-1;
VEGF	Vascular Endothelial Growth Factor.
WAP	Whey Acidic Protein
XOR	Xantine Oxidoreductase

INTRODUCTION

INTRODUCTION

1. PHYSIOLOGICAL ASPECTS OF MAMMARY GLAND DEVELOPMENT AND INVOLUTION

The mammary gland is a highly dynamic and complex tissue with a fundamental function: to provide nutrition and immunological protection to mammalian offspring by means of milk production (Ward & German, 2004). Considering the complex biochemical mechanisms of milk production and secretion, lactation appears to have evolved gradually, becoming a highly specialized skin gland that initially, like other skin glands, was part of the protective epithelial body barriers. Corroborating this hypothesis, milk is rich in enzymes such as xanthine oxidoreductase (XOR), lysozyme and other molecules with antimicrobial and protective effects. This composition strongly suggests that the mammary gland evolved from the innate immune system, as a mucus skin gland protecting the newly evolving mammalian skin from infectious disease (figure 1) (Vorbach et al, 2006). It has also been hypothesized that lactation, as it is known today, evolved as an inflammatory response to tissue damage and infection and that inflammatory molecules became key regulators of lactation. This way, the nutritional value of milk evolved subsequently from its immunological function (Shahani et al, 1973).

In fact, as it will be exposed further on, mammary gland evolutionary steps share many common mechanisms with inflammatory responses (Vorbach et al, 2006). These pro-inflammatory changes, more evident during post-lactational involution, are an adequate environment to allow tumour progression, given an oncogenic event (Lyons et al, 2011).

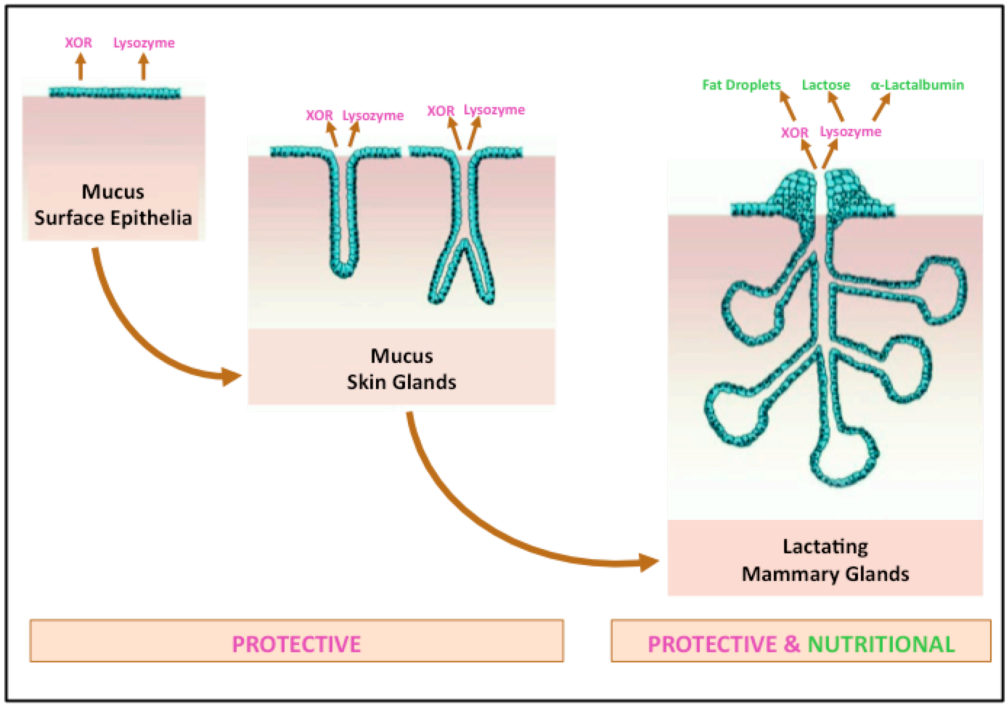

Figure 1. Proposed evolution of the mammary gland from a mucus secreting epithelial gland. Mammary glands presumably evolved as mucus-secreting skin glands. The evolution of additional functions resulted in the secretion of fat droplets, α-lactalbumin and lactose. Consequently, the mammary gland evolved from a protective immune organ into a reproductive organ unique to the class mammalia. Adapted from (Vorbach et al, 2006).

1.1 MAMMARY GLAND ANATOMY

Mammary gland structure consists of a compound tubulo-alveolar gland embedded within an irregular connective tissue that undergoes a series of changes from conception to senescence (Stingl et al, 2005). It consists of 15 to 25 lobes, each of which is drained by a collecting duct that terminates in the nipple. The collecting duct has several branches which end in the basic functional unit of the breast – named the terminal ductal-lobular unit (TDLU) (figure 2). The TDLU is composed of a small segment of terminal duct and a cluster of ductules (acini), which are the actual secretory units. The functional structures are surrounded by a varying amount of fat and collagenous tissue. The main arterial supply is from the internal mammary and lateral thoracic arteries. The venous drainage is mainly by branches of internal thoracic veins,

but anatomical variations have been described. The most important lymphatic drainage is to the axilla, while less of the lymph flow is drained via internal and posterior intercostal lymphatics.

As mentioned above, the breast is affected by physiologic changes in morphology and function throughout life-time from menarche to menopause, during each menstrual and pregnancy/lactation cycles. These changes are regulated by hormonal activity, mainly prolactin (PRL), oestrogen (E) and progesterone (Pg). At menarche, the main events include development and growth of ductal and lobular units. During pregnancy and lactation, a remarkable rise in hormone levels induces growth and secretory activity of the breast. Post-menopausally, the breast undergoes involution characterized by atrophy of the parenchymal structures (Tavassoli, 1992). The modifications observed with each menstrual cycle and pregnancy/lactation cycles have been the basis of studies that prove that mammary gland initially develops from multipotent embryonic progenitors which give rise to lineage-restricted stem cells with renewal potential that ensure the homeostasis the gland (Villadsen et al, 2007). Microscopically, the normal breast structure is classified into two tissue compartments: a stratified epithelium, derived from embrionary ectoderm, which consists of ducts and alveoli that contain milk-producing cells; and the mesodermally-derived stroma, also known as mammary fat pad which main components are adipocytes, although it also contains fibroblasts, cells from the haematopoietic system, extracellular matrix (ECM), blood vessels and neurons (Hennighausen & Robinson, 2005)A small part of the ducts at the nipple is lined by squamous epithelium (Tavassoli, 1992). In the normal breast, the stratified epithelium consists of two different cell populations, epithelial and myoepithelial, which origin is now believed to be the lineage restricted stem cells (Van Keymeulen et al, 2011). These cells can be differentiated with immunohistochemical staining using antibodies against cytokeratin (CK) and myosin respectively (figure 2 A).

The epithelial cells are luminal secretory and ductal cells, which undergo functional differentiation in pregnancy to produce milk. The myoepithelial cells encase the luminal cells, are contractile and participate in the delivery of milk

in response to oxytocin stimulation (Hennighausen & Robinson, 2005). It has been hypothesized that the generation of cellular heterogeneity in breast lesions depends on the underlying developmental program of the normal breast, and that breast carcinoma heterogeneity could arise from the neoplastic transformation of either an epithelial or myoepithelial cell, or even from a lineage restricted stem cell (Gusterson et al, 1982; Van Keymeulen et al, 2011).

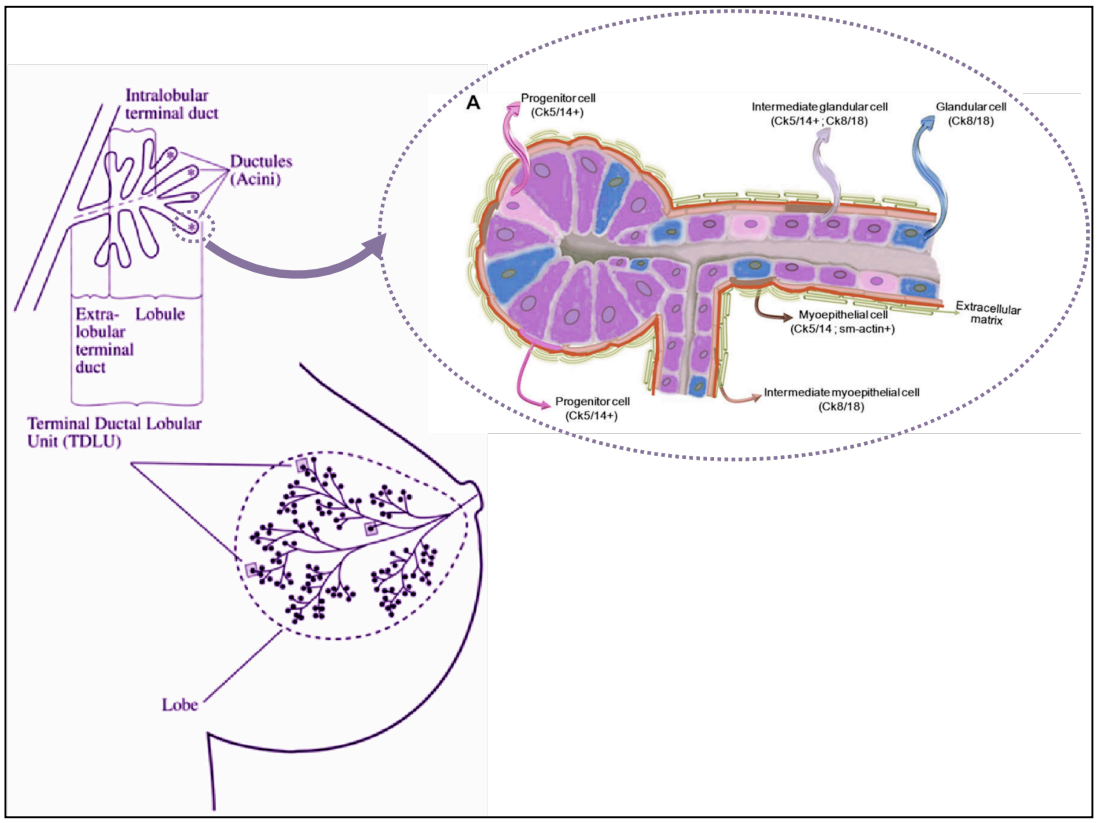

Figure 2. Mammary gland structure from a macroscopic and microscopic point of view (A). Adapted from (Bosch et al, 2010; L Tabar, 1998).

As for the changes that the mammary gland undergoes throughout the life spam of a mammal, it is important to differentiate distinct stages within normal development from embryonic stage to the final involution process during menopause. There are two characteristics that make this organ unique; the first is that most of its development takes place post-natally. While the primordia of the gland are formed early in embryogenesis as derivatives of the epidermis, growth of the gland proceeds rather slowly until puberty when the

parenchyma starts to extend and fill the mammary fat pad (Robinson et al, 1999). The second characteristic is that its structure changes periodically in a female's reproductive life with successive cycles of proliferation, differentiation and apoptosis. In fact, these changes take place in each menstrual cycle, but the terminal functional differentiation is reached only during pregnancy and lactation when the development of alveoli reaches its final differentiation stage and synthesis of specific milk proteins takes place. This is followed by subsequent massive apoptosis of epithelial tissue with weaning. These dramatic changes have placed the mammary gland one of the most important physiological models to study proliferation and apoptosis at a molecular level.

On the other hand, mammary gland developmental program shares striking similarities with breast carcinogenesis program. In fact, as it will be specified later on, pregnancy and post-partum related breast cancer is a disease with increasing incidence and worse prognosis, and preliminary data seem to point to the special physiologic environment in mammary gland during pregnancy and specially involution, as the main reason for this aggressiveness. Before going into detail on the parallelisms between mammary gland physiologic changes and carcinogenesis, it is important to lay out the different stages in mammary gland development and the molecular pathways underlying them. Focus will be placed on the mouse model, as it is the most studied and there is evidence that it is a good surrogate to human physiology.

1.2 STAGES IN MAMMARY GLAND DEVELOPMENT

Mammary development occurs in three distinct and differentially regulated stages: embryonic, pubertal and adult. The adult phase can be further subdivided in pregnancy/lactation development, involution post-weaning and involution during menopause.

1.2.1 Embryonic Stage

The first morphological indication of mammary development in most

mammalian embryos is the appearance of a localized thickening on the ectoderm or epidermis. In many mammals, the formation of the individual gland buds is preceded by the elevation of an epidermal mammary crest and a milk line that runs along most of the length of the trunk. This milk line then fragments and regresses except for the individual gland buds that may form (anteriorly, in the thoracic region as in primates and elephants, posteriorly in the inguinal region as in most ungulates, or all along the trunk as in pigs). In the mouse embryo, bilateral mammary lines are formed on the ectoderm between embryonic day 10 and 11. From a morphological point of view, epidermal cells become columnar and multilayered, defining a ridge that protrudes above and below the plane of the single-layered primitive epidermis (Hens & Wysolmerski, 2005). From a molecular point of view, the specification of the mammary line is dependent on the activation of the canonical Wnt/β-catenin and the fibroblast growth factor (Fgf) pathway, which acts in parallel to Wnt (Chu et al, 2004; Mailleux et al, 2002). The second step in mammary gland morphogenesis is the placode formation which can be observed by embryonic day 11.5. The same signalling pathways are implicated in this step (Wnt and Fgf) together with the transcription factor Gata3 (Asselin-Labat et al, 2007; Eblaghie et al, 2004; Hens & Wysolmerski, 2005; Mailleux et al, 2002). The third step is the bud formation. The size of the bud slowly increases until a circumscribed epithelial ball is formed within the epidermis by embryonic day 15. In this step Wnt signalling also seems to take an important role (Chu et al, 2004; Hens & Wysolmerski, 2005). During this period, the epithelial bud is surrounded by layered mesenchymal cells which constitute the primary mammary mesenchyma. The interaction between epithelial cells and the underlying mesenchyma is crucial for further development of the gland. Another factor that plays an important role in the epithelial-mesenchyma interaction, is the parathyroid hormone related protein (PthrP) (Foley et al, 2001) which is also necessary for the mammary mesenchyme to induce the nipple formation from the overlying epidermis (Foley et al, 2001; Wysolmerski et al, 1998). The final step is the formation of the rudimentary ductal tree. This initial branching is hormone independent (Hennighausen & Robinson, 2001). A primary sprout grows out on embryonic day 16. Continued proliferation until the end of gestation leads to formation of a small ductal tree that consists of

10 to 15 branches arising from the single duct that emanates from the nipple (Robinson et al, 1999). The genes that regulate this phase are PthrP and its down stream effectors, along with Wnt which seems to be crucial for the outgrowth of the initial ducts from the bud (Cowin & Wysolmerski, 2010; Hens & Wysolmerski, 2005). A graphical summary of the embryogenic stages of mammary gland development can be seen in figure 3.

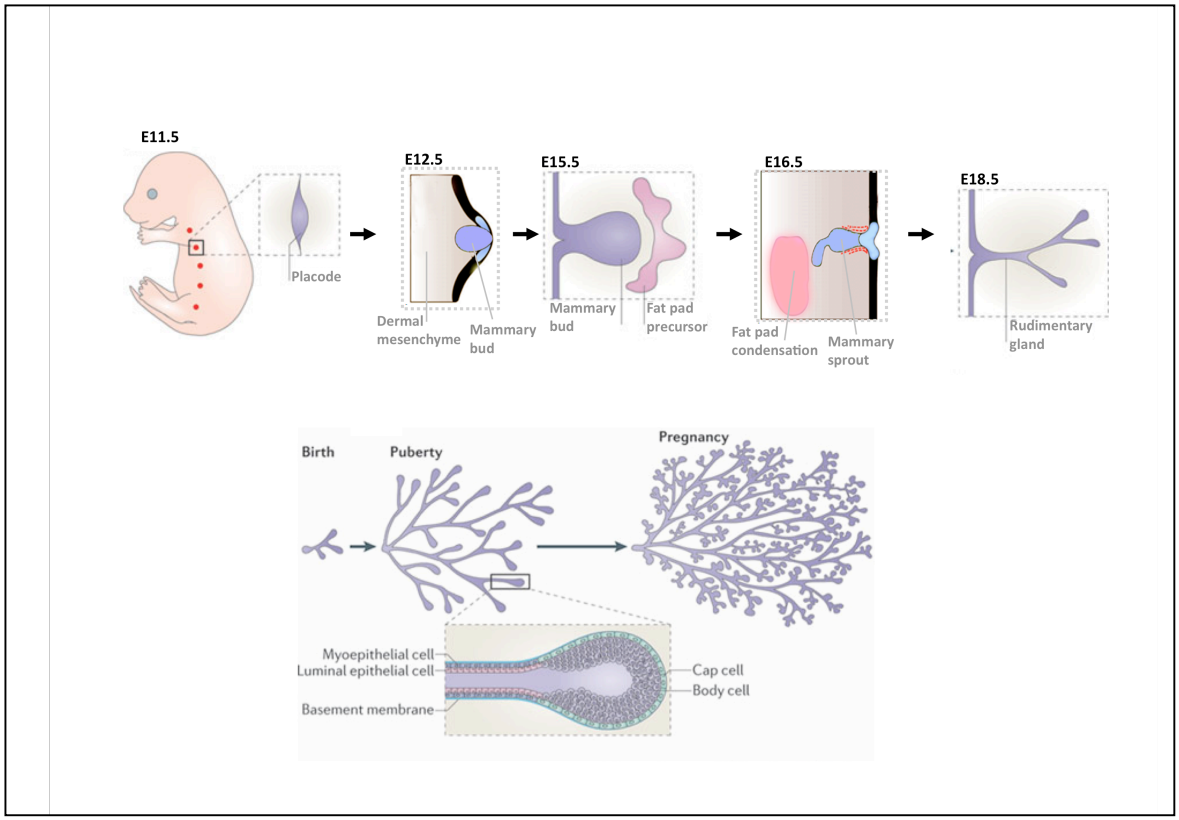

Figure 3. Mammary gland formation in the embryonic and adult phase. After placode appears, the mammary bud develops through cell proliferation. This bud is surrounded by mesenchymal cells and will grow into rudimentary ductal tree. Most of the develoment of the gland is done post-natally with puberty, reaching the final differentiation state with pregnancy and lactation E: Embryonic day. (Cowin & Wysolmerski, 2010; Gjorevski & Nelson, 2011).

1.2.2 Pubertal Development

Post-natally, the mammary gland grows isometrically with body growth until puberty, when the influence of E and Pg cause massive ductal elongation

and branching. In fact, the changes observed in the mammary gland during puberty, pregnancy, lactation, menopause, and each menstrual cycle are all correlated to physiologically varying amounts of circulating oestradiol in the blood stream (Petersen et al, 1987). Puberty is a striking stage of mammary morphogenesis. Prompted by elevated levels of ovarian hormones, the ends of the growing ducts form the terminal end buds (TEB) in rodent anatomy, or TDLU in the human equivalent, which are the functional units of the gland. It is important to highlight that these are the sites where most breast tumours arise and the postulated site for stem cell niche (Hilakivi-Clarke, 2007). TEB contain two main cell populations, the cap cells which will give rise to myoepithelial cells; and the inner body cells that give rise to luminal cells of the mammary epithelium. It was thought that mammary stem cells gave rise to a common progenitor that would split into the two lineages mentioned (Siegel & Muller, 2010) but a recent study points out to the existence of two different lineage-restricted progenitors (Van Keymeulen et al, 2011). The TEB invade the fat pad and bifurcate at regular intervals generating the ductal tree. In the mature mouse the entire fat pad is filled with a system of primary and secondary ducts with side branching that form and disappear with each menstrual cycle, and TEB are replaced by terminal end ducts.

As specified, E and Pg play a crucial role in postnatal mammary gland development. Their function is exerted by means of their receptors (oestrogen receptor, ER and progesterone receptor, PgR) that are expressed in most epithelial cells (Hennighausen & Robinson, 2005). With every menstrual cycle proliferation and apoptosis occur in response to E. The mechanisms by which steroid hormones stimulate mammary epithelium growth are controversial. Among the specific hormone receptors involved, two ER have been discovered, ERα and ERβ. The first is predominant in breast tissue and is required for normal ductal elongation and outgrowth during puberty (Bocchinfuso et al, 2000). ER acts as a transcription factor which becomes activated upon ligand binding; translocates to The nucleus where jt joins the specific DNA sequences known as Estrogen Regulatory Elements (ERE) and activates gene transcription (Eroles et al, 2010). Additionally, binding of E to the ER can be modulated by Pg. There are also two known isoforms of PgR,

A and B, which are encoded by two transcripts derived from the same gene, but it is PgR B that is required to carry out the proliferative effect of Pg on mammary epithelial cells. Pg is essential for the expansion of the alveolar compartment while its contribution to ductal elongation and branching is minor (Hennighausen & Robinson, 2005). There are other transcription factors that play an important role in mammary gland postnatal development, namely GATA-3 which expression is, at this point, restricted to the luminal epithelial cells and is absent from the myoepithelial compartment. Its function is essential for ductal elongation. (Kouros-Mehr et al, 2006; Siegel & Muller, 2010). It has been demonstrated that the numbers of proliferative and apoptotic events vary during the menstrual cycle and that these variation occur also in a cyclic manner, but these variations do not depend solely on steroid hormone fluctuations, since other factors such as several Bcl-2 family proteins change during the menstrual cycles (Navarrete et al, 2005).

1.2.3 Adult Stage: Preganancy and Lactation

The final step in proliferation and differentiation of the mammary gland is reached with pregnancy and lactation. During pregnancy, the branched ductal system, that expanded during puberty, further develops into a lobuloalveolar compartment. Among the transcription factors that regulate this process NF-κB is known to play a crucial role (Cao et al, 2001). The ducts branch into decreasingly smaller ductules, which terminate in lobules. Lobules are composed of alveoli, which consist of secretory epithelial cells that undergo functional differentiation with parturition. Hence, the number of epithelial cells increases by approximately 100 fold in response to several hormones and signalling factors. The hormones that play an essential role in this step of breast development are E, Pg, growth hormone (GH), glucocorticosteroids, insulin and PRL, being the most important Pg and PRL (Hennighausen & Robinson, 2005). ER has a critical role in ductal elongation and PgR directs lobuloalveolar development during pregnancy (Siegel & Muller, 2010). In early stages of pregnancy, PgR positive cells are lined closely to the proliferating cells, which implies that the proliferative effect of PgR is mediated in part in a paracrine fashion (Brisken et al, 1998; Pang & Hartmann, 2007). After

parturition, progesterone withdrawal is a potent lactogenic trigger (Pang & Hartmann, 2007). During pregnancy PRL is involved in the maintenance of the corpus luteum in the early stages, to ensure the secretion of E and Pg, and in mammary gland development. After this initial stage, both functions of PRL are carried out by placental lactogens, until birth, when PRL will take over again.

The most dramatic event in the lactation cycle, lactogenesis, occurs at parturition when there is a sudden increase in the secretory activity of the mammary gland. Lactation has been artificially subclassified in two stages in order to better understand the process: the first stage represents the stage of pregnancy when the mammary epithelial cells differentiate into lactocytes (secretory mammary epithelial cells) with the capacity to synthesize unique milk constituents such as lactose, casein, α-lactalbumin, fatty acids etc. The second stage is the initiation of copious milk secretion. These two stages are known as secretory differentiation and secretory activation, respectively (Anderson et al, 2007). It is believed that along with PRL secretion from the hypophysis, the Pg withdrawal after birth triggers secretory activation. Also, the glucocorticoid receptors that are present in the cytosol of mammary epithelial cells, when bound to glucocorticoids, translocate to the nucleus and act synergistically with PRL-activated transcription factors to enable the synthesis of milk proteins, such as, casein and α-lactalbumin. The main regulatory factors that account for this final functional differentiation are known to be GATA3, ELF-5, AKT and STAT5.

1.2.4 Post-lactational Mammary Gland Involution

Post-lactational involution is the process following weaning of the litter by which the mammary gland undergoes massive cell death and tissue remodelling in order to return to a virgin-like state. The physiology and genetics of apoptosis are easily studied in the mouse mammary gland where most of the epithelium is removed within 6 days of weaning. The study of the mechanisms underlying this process has been possible using a forced

weaning model in which the pups are removed in day 10-11 after birth, which is the peak of lactation. This precipitates a synchronous involution and allows the study of the molecular mechanisms that take place and their morphological counterpart (Watson, 2006). This model has permitted to elucidate that there are two phases in post-lactational involution: a reversible first phase that lasts 48h; and a second irreversible phase that initiates the remodelling program and returns the gland to a pre-pregnant state (Lund et al, 1996). Each phase is characterized by specific gene expression.

In the first phase of involution after weaning, milk stasis induces apoptosis. This phase is triggered by local factors and lasts approximately 48h. The influence of local factors on apoptosis initiation has been proved using a teat-sealing technique. Milk accumulation triggers increased expression of the death-inducing *bax* gene through a yet-undefined mechanism. The same stimuli mediate loss of STAT5a and 5b phosphorylation disrupting the principal pathway for prolactin signalling (Li et al, 1997b; Marti et al, 1997). It is thought that milk stasis promotes apoptosis through loss of polarization of luminal cells.

As for the regulatory pathways and the genes that are up-regulated during this first phase of involution, two studies (Clarkson et al, 2004; Stein et al, 2004) have revealed that there are four distinct gene expression profiles in the first 4 days of involution that partially overlap one another: a rapid but transient increase in gene expression 12h after weaning (group1); a rapid activation, which peaks at 12 h and is sustained for up to 4 days (group2); a delayed induction of gene expression that is not maximal until 24 h but is also sustained for at least 96h after weaning (group3); and a gradual increase in expression, that peaks at the last time point of the array analysis, 96 h (group4). It is noticeable from these microarray studies that there is a clear up-regulation of acute phase response (APR) genes (Pensa et al, 2009). Different genes are expressed in quite distinct patterns with some being expressed early (within 12h) and others later, during the second phase. Group 1 contains transcripts for the death receptor (DR) ligands and class II APR genes. This cluster also contains genes activated by NF-κB. Gene cluster 2 is

characterized by a profile of STAT3 regulated genes, and it contains transcripts for inflammatory (*il-1β*) and anti-inflammatory regulators (*slpi* - secretory leukocyte protease inhibitor) and also acute phase proteins (APP) such as orosomucoid 2. The CAAT/enhancer binding protein transcription factor (C/EBP) δ, that has been shown to regulate involution (Thangaraju et al, 2005) and the class I APR (Poli, 1998) are in this cluster also. The third group of transcripts has a similar profile to group 2 but peaks at 24h. Genes expressed in this profile include *cd14* and *C/ebpβ.* CD14 could have a dual role as a suppressor of bacterial infection and as a mediator of the phagocytosis of shed cells (Devitt et al, 1998). The fourth group has a gradual and sustained increase in expression level from 24h onwards. The predominant genes in this group are associated with innate immunity, antimicrobial defence and inflammation and include immunoglobulins, complement components, some class II APP that are regulated by STAT3 alone (Alonzi et al, 2001). The regulator of phagocytosis, *mfge8*, has also this expression profile. Loss of MFGE8 results in deregulated phagocytosis during involution, tissue scarring, and failed lactation in subsequent pregnancies (Atabai et al, 2005; Hanayama & Nagata, 2005) emphasizing the importance of efficient clearance of dead cells for tissue remodelling and function. In addition to immunoglobulins, a range of soluble innate defence factors are expressed at high levels during involution, including antimicrobial factors that may protect from mastitis (Clarkson et al, 2004; Sordillo & Streicher, 2002). The striking outcome of these studies is the association of involution with an inflammatory and acute phase response. Among the regulatory pathways that control mammary gland involution from the early phases, STAT3, the NF-κB, TGF-β and phosphatidil inositol 3 kinase (PI3K)/AKT pathways are the most important and will be explained later on in more detail (Clarkson et al, 2004).

1.2.5 Mammary Gland Involution During Menopause

The last phase in mammary gland development is the final involution that takes place during menopause. Lobular involution is a distinct process from post-lactational involution. Unlike the dramatic cell death and remodelling

following weaning, lobular involution is associated with a gradual decrease in the complexity and extent of ductal epithelium with age. This stage, that implies both lobular and ductal regression (Howard & Gusterson, 2000), appears to be an irreversible process in which the number and size of acini per lobule are reduced and the intralobular stroma is replaced with collagen (Hutson et al, 1985; Radisky & Hartmann, 2009) whereas the glandular epithelium and interlobular connective tissue regress to be replaced by fat (Howard & Gusterson, 2000). Hence, the mammary tissue is no longer functional. The timing and extent of lobular involution can vary considerably among individual women (Hutson et al, 1985).

A large study has noted that the extent of lobular involution is associated with a significantly reduced risk of breast cancer (Milanese et al, 2006). While this finding is consistent with the widespread understanding that lobules (or TDLUs) are the anatomic substructure that gives rise to breast cancer, this study points to the fact that progressive degrees of involution are associated with reduced cancer risk. Completion of involution around age 50 coincides with the well-recognized slowing in the rate of increase of breast cancer at that age, raising the possibility that lobular involution is contributing to the decrease breast cancer risk (Baer et al, 2009; Henson et al, 2006).

2. MOLECULAR MECHANISMS CONTROLING MAMMARY GLAND DEVELOPMENT IN THE LACTATION/INVOLUTION CYCLE

2.1 LACTATING MAMMARY GLAND

As it has been previously specified, the final step in mammary gland development and differentiation is reached with pregnancy and lactation. Lactation, being the most differentiated state, is mainly regulated by PRL, secreted by the hypophysis. PRL mediates its function via its receptor (PRLR), a transmembrane protein of the class I cytokine receptor family (Boutin et al, 1988). This family includes the GH receptor, interleukin (IL) receptors, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte– macrophage CSF (GM-CSF), leukaemia inhibitory factor

(LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and the more distantly related interferon (IFN) receptors (Watson & Burdon, 1996). PRLR is associated with a Janus kinase (JAK) and a Signal Transducer and Activator of Transcription (STAT) factor. The Stat family member that is activated down stream of PRLR during lactation is STAT5. Briefly, ligand binding (PRL) induces dimerization of one or more of the receptor chains, which brings together two JAK. The proximity of the receptor chains plus their associated JAKs results in tyrosine cross-phosphorylation of both JAKs and specific residues on the receptor. The phosphorylated tyrosine residues on the receptor recruit STAT5 protein, which in unstimulated cells exist as monomers in the cytosol. A transient association of the STAT with the receptor results in tyrosine phosphorylation of the STAT, which oligomerizes with at least another STAT protein through specific SH2 (Src-homology 2) domain-phosphotyrosyl interactions and becomes activated. Activated STAT5 translocates to the nucleus and binds to DNA specific Gamma Activated Sequence (GAS) sites (TTCCNGGAA) activating transcription. Activated JAK2 may also associate with the SH2 domain of SRC homology collagren protein (SHC), which then interacts with GRB2 (an adaptor protein) following tyrosine phosphorylation, thereby activating the mitogen activated protein kinase (MAPK) pathway. Figure 4 schematically depicts this molecular pathway.

STAT5 is a transcription factor that was originally identified by its role in activation of transcription of milk-protein genes in response to PRL activation (Wakao et al, 1994). Now, it is known to be encoded by two different genes, giving rise to STAT5a and STATb; and to participate in a plethora of signalling pathways (Ihle, 2001). *Stat5a* deficiency in mice results in the loss of mammary gland development and consequently in lactation failure (Liu et al, 1997a). The deletion of *Stat5b* alone results in a phenotype with similarities to that observed in GH receptor deficient mice (Udy et al, 1997). Deleting both *Stat5a/b* results in a phenotype that is identical to that observed in PRLR deficient mice and is consistent with the concept that all the physiological functions mediated by PRL require STAT5a/b (Teglund et al, 1998).

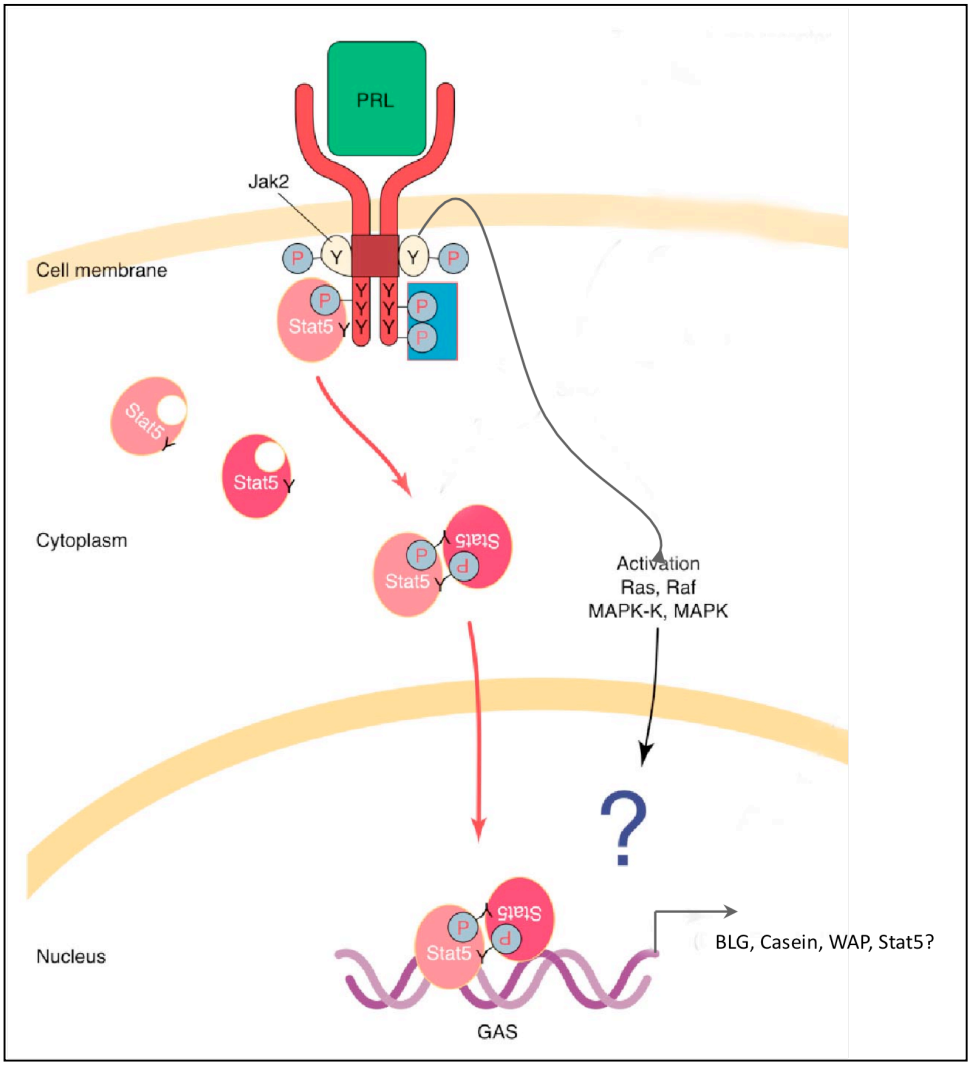

Figure 4. Prolactin signal transduction pathways. A schematic representation of the pathways that have been implicated in prolactin signal (PRL) transduction. GAS: TTCCNGGAA (Watson & Burdon, 1996).

Activation of STAT5 is a crucial switch in mammary gland development during pregnancy and lactation. It is essential for inducing proliferation and differentiation of the ductal and alveolar luminal cells, and it is also necessary to allow survival and function of these cells during lactation. These different functions are carried out by this transcription factor throughout pregnancy. A murine model in which the two *stat5* genes were deleted at different time points showed that loss of *stat5* prior to pregnancy prevented epithelial

proliferation and differentiation. Deletion of *stat5* during pregnancy, after mammary epithelium had entered Stat5-mediated differentiation, resulted in premature cell death, indicating that at this stage epithelial cell proliferation, differentiation, and survival require STAT5 (Cui et al, 2004).

PRL activates STAT5 via the PRLR-JAK2 tandem (see figure 4), hence PRL is essential for alveolar proliferation in early pregnancy (Ormandy et al, 1997). Still, it is not the only participating mechanism in mammary gland development during pregnancy. In fact, studies have identified human epidermal receptor (HER) 4 as another mediator in STAT5 activation that affects the functional differentiation of luminal cells after the proliferative phase mediated by PRLR (Long et al, 2003). STAT5 target genes have known roles in cell proliferation, survival, differentiation and, of course, milk synthesis. The following table summarizes the main target genes and their known function, inferred from mouse models:

Table 1. STAT5 target genes during lactation.

Target gene	Function
Receptor activator of nuclear factor κB (NF-κB)- ligand (Rank-L)	Alveolar development during pregnancy during later steps. Important for differentiation. It activates NF-κB activity, which in turn activates Cyclin D1 expression (Fata et al, 2000).
NF-κB	It is not a direct target of STAT5, but it is indirectly activated via Rank-L. Its abrogation allows for expansion of mammary epithelium but it is unable to differentiate. Hence, mice that lack NF-κB function in the mammary epithelium cannot lactate (Cao et al, 2001).
Cyclin D1	Both a target gene of STAT5 and NF-κB. Loss of this gene leads to under-proliferation of alveolar cells, which are also unable to differentiate (Fantl et al, 1995).

Connexin-26	Component of GAP junctions which are essential for polarization of the luminal cells and formation of junction between them. This process is crucial for the establishment of functional alveoli (Ormandy et al, 2003).
Whey Acidic Protein (WAP) β-casein	Milk proteins (Miyoshi et al, 2001).
Member of transcription factor family ETS (ELF5)	Participates in alveolar proliferation and differentiation. Its absence allows for alveolar bud formation but not for its proliferation and maturation (Zhou et al, 2005).

Another transcription factor that needs to be briefly mentioned for its implication in mammary gland development during pregnancy is GATA3. As in earlier developmental steps, GATA3 is a key regulatory factor that allows alveolar differentiation of mammary progenitor cells during pregnancy. Its deletion at this stage of mammary gland development results in impairment of lobuloalveolar development, resulting in a lactation-deficient phenotype (Asselin-Labat et al, 2007). ELF-5 has a pivotal role in alveolar differentiation and secretion (Choi et al, 2009). Still, the functional relationship between GATA3 and STAT5/ELF-5 has not yet been elucidated (Siegel & Muller, 2010).

2.2 MOLECULAR SIGNALLING DURING MAMMARY GLAND INVOLUTION AFTER WEANING

When lactation ends, the excess of epithelium that developed and differentiated to produce milk has no further function, hence, those cells are no longer needed and the mammary gland regresses to a pre-pregnant state in order to prepare for the next pregnancy. As referred to earlier, mammary gland involution occurs in two phases. In the first, the main mechanism by which the surplus epithelial cells disappear is apoptosis, and during the

second phase the mammary gland undergoes a profound remodelling, with degradation of basal membrane and extracellular matrix and a concomitant loss of the lobular-alveolar structure.

2.2.1 Mechanisms of Cell Death

Before explaining in more depth the mechanisms involved in this first phase of involution, it is important to explain briefly the mechanisms by which a cell can die. Dying cells enter a process that is reversible until a “point of no return” is trespassed, still, this point is yet to be specifically defined from a biochemical point of view. A number of morphological criteria can be used to define cell death: i) cell has lost plasma membrane integrity. This can be determined by the incorporation of dyes such as propidium iodide (PI) or trypan blue in *in vitro* models; ii) the cell has undergone fragmentation into what is referred to as apoptotic bodies; iii) the dead cell or its fragments are engulfed by adjacent cells *in vivo*.

Although there are many ways for a cell to die, only the main four will be referred to, and focus will be placed on apoptosis, since, it is the main event in this first phase of involution. For a more extensive review, see the recommendations of the Nomenclature Committee on Cell Death 2009 (Kroemer et al, 2009).

I) APOPTOSIS

The term was coined in the year 1972 by Kerr et al to describe a specific type of cell death that involved conversion of scattered cells into small round masses of cytoplasm that contained specks of condensed nuclear chromatin (Kerr et al, 1972). During apoptosis the morphological features that can be observed are: rounding-up of cells, retraction of pseudopodes, reduction of cellular and nuclear volume (pyknosis), nuclear fragmentation (karyorhexis), minor modifications of organelles, plasma membrane blebbing and engulfment by phagocytes *in vivo* (Baehrecke, 2002; Barkla & Gibson, 1999). As for the biochemical features, apoptosis can be defined through the determination of activation of proapoptotic Bcl-2 family proteins (such as BAX and BID), the activation of caspases, mitochondrial transmembrane potential

dissipation, mitochondrial membrane permeabilization, DNA fragmentation and plasma membrane rupture (Kroemer & Martin, 2005; Kumar, 2007; Lamkanfi et al, 2007). It is important to point out that none of these features are definitory per se of apoptosis and that there are different types of apoptosis that are triggered through different biochemical routes. Apoptotic cell death is activated by extrinsic, receptor mediated, or intrinsic, mitochondria mediated, signalling pathways both converging in the activation of caspase 3. However, it should be kept in mind that not all forms of apoptotic cell death induce activation of caspase 3 and not all forms of apoptotic cell death involve caspases (Orrenius et al, 2003).

Caspases are the main effectors of apoptosis. They are a family of proteases that depend on a cystein nucleophile to cleave motifs possessing aspartic acid (aspase) hence the term caspase (Thornberry & Lazebnik, 1998). Caspases are produced as inactive zymogens that have a large and a small subunit preceded by an N-terminal prodomain. Two aspartic cleavage sites are processed sequentially and the large and small subunits associate to provide the active site for the enzyme. The active caspase is a tetramer of two heterodimers that contains two active sites (Thornberry et al, 1997).

Besides, in the apoptosis intrinsic pathway there are various types of proteins essential to the process. One of these protein families is the BCL-2 family that constitutes a critical intracellular checkpoint. It includes both proapoptotic and antiapoptotic regulators that are classified according to the homology they share within four conserved regions named BCL-2 homology (BH). The antiapoptotic members share an homology in all four of the BH domains 1-4. This subgroups includes BCL-2, BCL-XL, BCL-W, A1 and MCL-1. The multidomain proapoptotic members (BAK, BAX and BOK) conserve BH1-3 and when activated they undergo a conformational change, oligomerize and allow the permeabilization of the mitochondrial outer membrane allowing the release of intermembrane space proteins such as cytochrome c that has an important role in apoptosis. The third subtype of BCL-2 family members (BAD, BID, BIK, BLK, HRK, BNPI3 and BIM-L) only contain homology in one domain, BH3. They are also proapoptotic and they

work upstream of the other BCL-2 members. They respond to specific signals and result in the activation of BAX and BAK and thereafter the rest of the apoptotic cascade. The function of antiapoptotic members is to bind and sequester the BH3 only molecules to prevent the activation of apoptosis effectors BAX and BAK (Danial & Korsmeyer, 2004). This cell death pathway has other control proteins such as inhibitor of apoptosis proteins (IAPs) and second mitochondrial activator of caspases (Smac). Once free, cytochrome c binds to Apoptosis Protease-Activating Factor-1 (APAF-1) which becomes competent to recruit procaspase-9 in the presence of ATP. This cytochrome c/Apaf-1/procaspase-9 complex is known as the apoptosome. Procaspase-9 bound to Apaf-1 is capable of self-processing into caspase-9, which leads to the activation of procaspase-3 (Danial & Korsmeyer, 2004; Orrenius et al, 2003). The apoptosome function is also regulated by the oncoprotein pro-thymosin-α (Pro-T) and the tumour suppressor putative HLA-DR-associated protein (PHAP).

The extrinsic pathway occurs through the activation of death receptors (DR) such as Fas/CD95, which, following ligand binding undergoes a conformational change and assemble on its cytoplasmic domain a signalling complex known as the DISC (Death Inducing Signalling Complex) (Muzio et al, 1996). Procaspase 8 is then recruited and it is believed that high local concentrations of this procaspase lead to autoproteolitic activation and subsequent activation of caspase-3 and 7. Fas-induced apoptosis can follow two pathways depending on the type of cell: in type I cells, which are refractory to BCL-2, caspase-8 activates procaspase-3, which cleaves target proteins, leading to apoptosis. In type II cells BCL-2 can block Fas-mediated cell death, therefore a mitochondrial amplification loop is needed; caspase-8 cleaves BID, which, in turn, induces the translocation, oligomerization and insertion of BAX and/or BAK into the mitochondrial outer membrane (see figure 5 for details) (Orrenius et al, 2003; Scaffidi et al, 1998).

When the apoptotic cascade has been initiated and caspases activated, DNA degradation is one of the endpoints of the process. This phenomenom first occurs at A/T rich regions to produce 50 to 200kb DNA fragments. It has

been described that a caspase activated DNase (CAD) is normally kept inactive by its inhibitor DNA fragmentation factor-45 (DFF-45) that is cleaved by caspases 3 and 7, hence eliminated, leaving CAD to degrade DNA (Liu et al, 1997b).

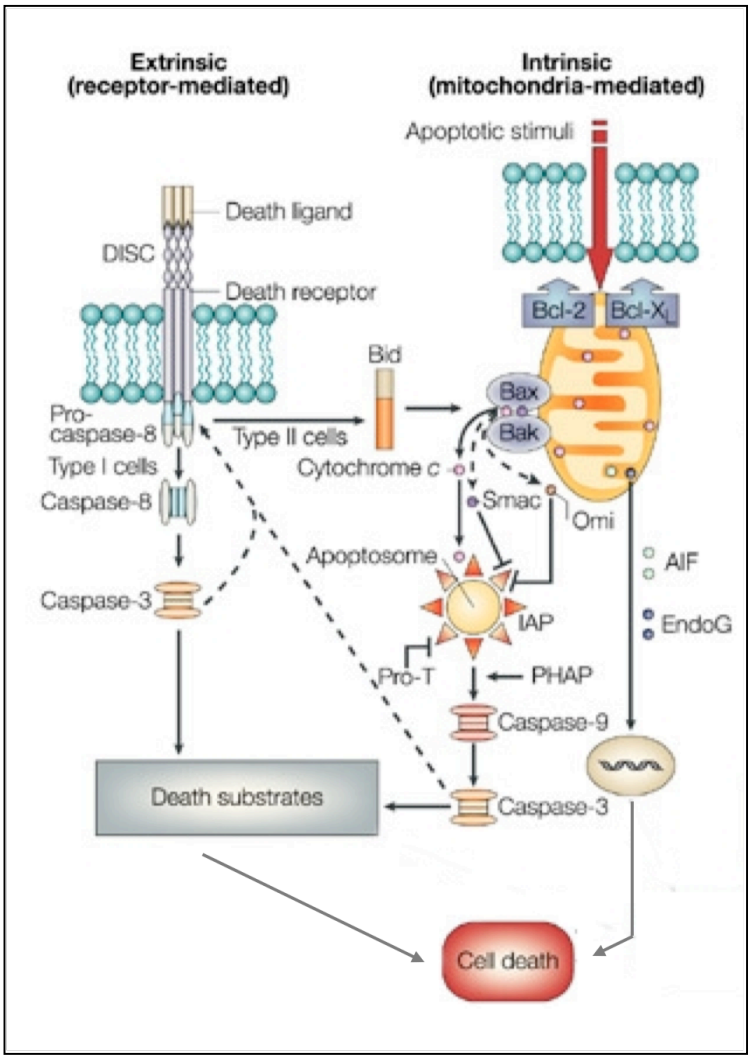

Figure 5. Graphical representation of the main apoptotic pathways. The extrinsic, receptor-mediated apoptosis pathway and the intrinsic, mitochondria-mediated pathway (Orrenius et al, 2003).

Apoptotic cell death terminates with the phagocytosis of the dead cell. The most important signal used by the apoptotic cell in order to activate phagocytosis is the exposure of phosphatidylserine on the outer cell surface (Savill & Fadok, 2000). This will stimulate phagocytic activity through several receptors, including CD91, CD14, CD36, $\alpha_v\beta_3$ integrin, phosphatidilserine

receptor and scavenger receptors (Fadok et al, 2000; Savill et al, 1992). Phagocytosis also favors DNA degradation via DNase II within the lysosomes of the phagocytes. In addition to engulfment of apoptotic cells, macrophages are important regulators of proinflammatory responses. It is important to achieve suppression of proinflammatory factors during apoptotic cell clearance as well as to abrogate the initiation of any immune reaction against self peptides. Defects in clearance of apoptotic cells may create a proinflammatory ambiance that may predispose to autoimmune disorders and facilitate cancer progression (Danial & Korsmeyer, 2004).

II) NECROSIS

Morphologically characterized by gain in cell volume, swelling of organells and plasma membrane rupture that leads to release of noxious cellular constituents. This process generates exudative inflammation in the surrounding tissue. The decision of the cell to die by necrosis or apoptosis is thought to depend on the nature and the severity of the insult and to day, there is no consensus on the biochemical changes that may identify unequivocally necrosis, which is still defined largely by the absence of apoptotic or autophagic markers, although some processes have been implicated in necrotic cell death. These phenomena include mitochondrial alterations, lysosomal changes, increased Ca^{2+} concentration in cytosol and activation of non-caspase proteases such as calpains and cathepsins (Kroemer et al, 2009; Orrenius et al, 2003). Recently it has been demonstrated that necrosis can also participate in a physiological process such as post-lactational mammary gland involution. It has been demonstrated that during this process there is increased lysosomal membrane permeability and an increase in the cytosolic concentration and activity of the cysteine peptidases cathepsins B and L. This new concept of physiological necrosis is starting to be known as necroptosis (Kreuzaler et al, 2011; Luke & Silverman, 2011).

III) AUTOPHAGY

Characterized by the sequestration of cytoplasmic material within autophagosomes for bulk degradation by lysosomes (Kroemer et al, 2009).

Autophagosomes are two-membraned and contain degenerating cytoplasmic organelles of cytosol (Levine & Klionsky, 2004; Levine & Kroemer, 2008). The fusion between an autophagosome and a lysosome to form autolysosomes marks the completion of the autophagic pathway. This type of cell death, unlike apoptosis, has little or no association with phagocytes (Baehrecke, 2005). It also lacks chromatin condensation and is accompanied by massive autophagic vacuolization of the cytoplasm (Kroemer et al, 2009).

IV) CORNIFICATION

This is a very specific form of programmed cell death that occurs in the epidermis. It leads to the formation of cornecytes (dead keratinocytes containing specific proteins such as keratin, loricrin, SPR and involucrin; and lipids). It is considered the terminal differentiation program of keratinocytes, necessary for skin function (mechanical resistance, elasticity, water repellence and structural stability).

2.2.2 Mechanisms Implicated in Post-Lactational Involution

2.2.2.1 Signalling Pathways Implicated in Mammary Gland Involution

I) LIF/STAT3

Apoptosis is induced in the mammary gland within 12h of the end of suckling and increases(Chapman et al, 1999) sharply during the first phase of involution (Lund et al, 1996). The initiation of involution is characterized by changes in the activity of two members of the STAT family of transcription factors. The phosphorylation status, and thereby the activity, of the transcription factors STAT5 and STAT3 changes rapidly at the onset of involution (Liu et al, 1996). STAT5a and STAT3 have reciprocal patterns of phosphorylation with levels of STAT5a decreasing and STAT3 increasing at the beginning of involution. These changes can be detected within 12h after weaning of the pups (Li et al, 1997b; Liu et al, 1996). In the mammary gland, STAT5 phosphorylation and activation can be induced by PRL, epidermal growth factor (EGF), and GH whereas it is known that, in vivo, the factor that activates STAT3 during involution is leukaemia inhibitory factor (LIF) which is

induced 30 fold during weaning (Gallego et al, 2001; Kritikou et al, 2003; Schere-Levy et al, 2003). STAT3 is critical for the initiation of apoptosis and involution and its absence reduces dramatically the number of apoptotic cells but it does not abolish apoptosis entirely (Chapman et al, 1999). Alterations in the levels of apoptosis have been demonstrated in *stat3* transgenic mouse models where the impairment of its function leads to a significant decrease in the level of apoptosis at day 2. However, there is not a complete absence of programmed cell death at either day 2 or 6 of involution, implying that the regulation of apoptosis is not entirely dependent on the presence of STAT3 (Chapman et al, 1999; Humphreys et al, 2002).

As already mentioned, STAT3 acts as a transcription factor, some of its target genes being already identified. The STAT3 target genes have diverse functions and many have been validated as important regulators of involution in their own right. Table 2 summarizes some of the principal STAT3 transcripts which are also proapoptotic or antiiflamatory mediators (Clarkson et al, 2006).

Table 2. Summary of STAT3 target genes in mammary gland during involution after weaning (Clarkson et al, 2006).

Gene title	Gene symbol	Pro-apoptotic	Inflammation/acute phase response
B-cell leukemia/lymphoma 3	*Bcl3*	*	*
CCAAT/enhancer binding protein (C/EBP), beta	*Cebpb*		*
CCAAT/enhancer binding protein (C/EBP), delta	*Cebpd*	*	*
FBJ osteosarcoma oncogene	*Fos*	*	
insulin degrading enzyme	*Ide*	*	
interferon activated gene 204	*Ifi204*	*	
oncostatin M receptor	*Osmr*		*
phosphatidylinositol 3-kinase, regulatory	*Pik3r1*	*	

subunit, polypeptide 1 (p85 alpha)			
purine-nucleoside phosphorylase	*Pnp*	*	
secretory leukocyte protease inhibitor	*Slpi*		*
MAD homolog 1 (Drosophila)	*Smad1*	*	
signal transducer and activator of transcription 3	*Stat3*	*	*
X-box binding protein 1	*Xbp1*	*	

Once STAT3 is phosphorylated, it translocates to the nucleus where it activates the transcription of oncostatin M receptor (OSMR). Oncostatin M (OSM) is a cytokine normally produced by macrophages, and the ligand for OSMR that, when active, will take over LIF's function and activate STAT3 (Watson & Brown, 2008), thereby establishing a positive feedback-loop. Conversely, OSMR activation results in STAT5 dephosphorilation (Tiffen et al, 2008). These data further confirm the importance of the STAT5/STAT3 switch in mammary gland involution. OSMR-KO mice exhibit a delayed second phase involution, which correlates with the involution trigger function of STAT3 (Tiffen et al, 2008).

Other STAT3 targets that are known to have a role in involution include CAAT/enhancer binding protein (*C/ebp*)δ, a transcription factor involved in the acute phase response and that when deleted, delays mammary gland involution (Thangaraju et al, 2005) and SOCS3, a negative regulator of STAT3 that when knocked down accelerates involution (Sutherland et al, 2006). Additional targets of interest include *slpi*; *Pi3kr1*, that encodes three regulatory subunits of the p85α gene which bind to the p110 catalytic subunits of class I PI3K (Abell et al, 2005); Insulin-like growth factor (IGF) binding protein 5 (*igfbp-5*) (Chapman et al, 1999); and *stat3* itself.

As explained, many of the transcriptional targets of STAT3 are inflammatory mediators and APR genes. This inflammatory response is required for efficient clearance of apoptotic mammary epithelium during involution. STAT3 functions in a non-cell autonomous manner to modulate the

mammary microenvironment during mammary gland involution. APR proteins display a range of functions related to acutely protecting the host from a variety of damages. Some of these proteins activate complement and phagocytic cell functions, others have anti-oxidant activity. Serum Amyloid A (SAA) has been implicated in inducing leukocyte migration (Urieli-Shoval et al, 2000) and Complement components impact on chemotaxis, opsonization, vascular permeability and dilation, while fibrinogens are important players in hemostasis, tissue repair and wound healing (Kushner, 1993). STAT3 plays a major role in the induction of both class I and class II APR genes, and with it, in the orchestration of inflammatory responses. Interestingly, it is also a key factor mediating anti-inflammatory responses (AIR) depending on the specific cell type (Poli V, 2003). The fact that STAT3 is up-regulating all these APR proteins strongly suggests that this transcription factor plays two distinct roles in mammary gland involution- promotes apoptosis on one side and on the other it protects the tissue from excessive damage, guiding remodelling.

II) NF-κB

Another key regulator in mammary gland involution process is NF-κB. NF-κB is a group of dimeric transcription factors with roles in immunity, inflammation, proliferation and apoptosis. These were first identified based on their interaction with the immunoglobulin light –chain enhancer in B cells and they are present in all cells (Sen & Baltimore, 1986). The group is formed by seven distinct proteins that can form a variety of dimers, not all of which are active (Barnes & Karin, 1997). These proteins include: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel.

In non-stimulated cells, most NF-κB dimers are retained in the cytoplasm by binding to inhibitory IκB proteins, except for the dimers formed by p105 and p100, which are inactive and contain intrinsic IκB-like moieties. In response to pro-inflammatory stimuli, such as tumour necrosis factor (TNF) or interleukin 1β (IL-1β), that interact with the death receptors (DR) such as CD95/Fas, TNF-related apoptosis-inducing ligand (TRAIL) receptors TRAIL-R1/-R2 and TNF-R1, the serine/threonine-specific IκB kinase (IKK) complex,

composed of the IKK1/α and IKK2/β catalytic subunits and the IKKγ/NEMO regulatory subunit, is activated. This results in IκB phosphorilation (p-IκB) and its eventual ubiquitin-mediated degradation in the proteasome. This leads to the unmasking of NF-κB nuclear localization signal (NLS) permitting the nuclear entry of NF-κB dimmers (Ghosh & Karin, 2002). Of the two catalytic subunits, IKK2/β is the most critical for the IκB degradation, forming the core of what is known as the classical activation pathway. On the other hand, IKK1/α activation by NF-κB inducing kinase (NIK) is required for the inducible processing of the inactive p100 protein to its active form p52, thus forming the core of the alternative pathway (Senftleben et al, 2001). Figure 6 shows schematically both activation pathways of NF-κB, the canonical and the alternative.

NF-κB is both expressed and active throughout different stages of mammary gland development. Its activity is high during pregnancy, when it regulates epithelial proliferation and branching (Baxter et al, 2007; Cao et al, 2001) but is found to be inactive during lactation, since it inhibits STAT5-mediated gene transcription, which is fundamental in this developmental stage (Clarkson et al, 2000; Geymayer & Doppler, 2000). However, during involution, NF-κB is reactivated as early as 1h after pup-removal, and its activity increases significantly at 24h, peaking at 72h (Clarkson et al, 2000). It regulates apoptosis and involution after weaning probably through the canonical pathway activated by DR ligands- TNFα and (TNF)-like weak inducer of apoptosis (TWEAK) (Watson, 2006). This means that its activation during involution is through the canonical pathway. NF-κB activation allows it to translocate to the nucleus where it binds to specific DNA sites, activating the transcription of target genes. As for the profile of the target genes of NF-κB, it correlates well with APR genes, (Clarkson & Watson, 2003) as it has been previously described for STAT3. Hence, it is possible that both transcription factors cooperate in the induction of an immune related response during involution.

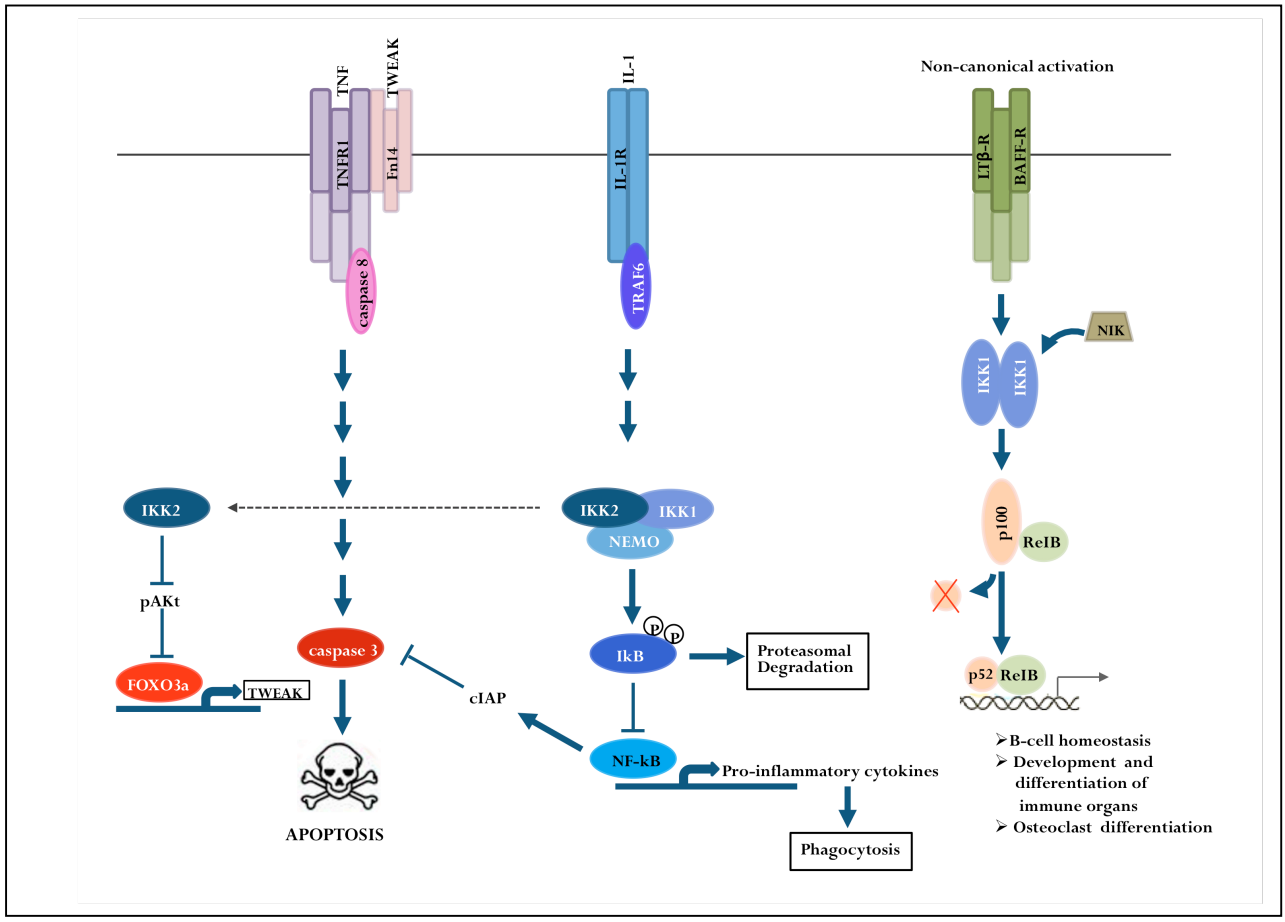

Figure 6. NF-κB signalling. On ligand interaction with surface receptors, one of two NF-κB activation pathways can be elicited. Canonical signalling depends on IKK2/β and IKKγ/NEMO and induces the transcription of genes that regulate inflammation and cell survival. The interaction of these ligands with the TNFR1 results in activation of caspase mediated apoptosis via caspases 8 and 3. Furthermore, IKK2/β is also required indirectly for TWEAK transcription.The non-canonical NF-κB activation is mostly involved in the regulation of B-cell development (Baxter et al, 2006; Baxter et al, 2007; Bollrath & Greten, 2009).

Among these NF-κB target genes, table 3 summarizes some of them, and it can be observed that many of these NF-κB regulated genes partake in pro-inflammatory responses.

Table 3. Transcriptional targets of NF-κB signalling. The biological outputs of NF-κB signalling are mediated by the transcriptional gene products it turns on. CD40L, CD40 ligand; c-FLIP, cellular Fas-associated death domain-like IL- (Wong & Tergaonkar, 2009).

Function	**Transcriptional target**
Cytokines/chemokines	IL-8, IL-6, TNF-α, IL-1b, GM-CSF, MIP-1a, MCP-1 and Rantes
Antimicrobial effectors	Defensins, NO and O2•
Enzymes	NOS2, COX-2 and PLA2
Immunoreceptors	B7.1 and MHC class I
Transcription factors	GATA3, STAT4 and IRF1
Growth factors/modulators	IL-2, GM-CSF, CD40L, Cyclin D1, VEGF, IL-6 and c-myc
Regulators of apoptosis	A1, c-IAPs, c-FLIP, Bcl-xl and Bcl-2
Cell adhesion molecules	ICAM-1, VCAM-1 and E-selectin
Invasion/angiogenesis	Maspin, VEGF and MMP-9

Since NF-κB is mostly related with anti-apoptotic signalling, its activation during the first phase of mammary gland involution might seem contradictory, given that the luminal epithelium undergoes massive apoptosis. In fact, initial *in vitro* studies with a mammary gland epithelial cell model showed that NF-κB suppressed cell apoptosis (Clarkson et al, 2000). Later studies have shown that NF-κB and its upstream regulator IKK2/β also promote apoptosis during involution (Connelly et al, 2010). Also, IKK2/β has been found to regulate FOXO3a and with it, TWEAK expression, via an Akt dependent process (figure 6) (Baxter et al, 2006). Thus, both IKK2/β and NF-κB are essential in early involution for induction of the extrinsic pathway of cell death. It is also noteworthy that DR ligands (TNFα, Fas Ligand and TRAIL) are NF-κB target genes (Baetu et al, 2001; Kasibhatla et al, 1998; Trede et al, 1995) which

have been demonstrated to be very important in early activation of apoptosis in mammary gland involution (Baxter et al, 2006).

III) TGFβ pathway

The Transforming Growth Factor beta (TGFβ) family is conformed by a group of regulatory cytokines that regulate the expanding systems of epithelial and neural tissues, the immune system, and wound repair. Most members of this cytokine family exist in variant forms (e.g., TGFβ1, β2, and β3). The bioactive cytokine molecule is a dimer composed of a polypeptide chain that is cleaved from a precursor by enzymes. The active dimer signals through the coupling of two pairs of receptor serine/threonine kinases known as the type I and type II receptors, respectively (Massague, 2008). Upon binding TGFβ, the type II receptors phosphorylate and activate the type I receptors that then propagate the signal by phosphorylating Smad transcription factors. Once activated, the receptor substrate Smads (RSmads) shuttle to the nucleus and form a complex with Smad4, a binding partner common to all RSmads (Shi & Massague, 2003). Smad4-RSmad complexes associate with additional DNA-binding cofactors in order to achieve binding with high affinity and selectivity to control the expression of hundreds of target genes in a given cell (Massague, 2008).

Currently, there are three known mammalian isoforms of TGFβ, namely TGFβ1, TGFβ2 and TGFβ3 (Pelton et al, 1990; Roberts & Sporn, 1992). These have been shown to be expressed at different stages of mammary gland development, although none of these transcripts is detected in lactating glands (Pelton et al, 1990; Robinson et al, 1991). TGFβ1 and 3 have been demonstrated to inhibit ductal outgrowth (Daniel et al, 1989; Silberstein & Daniel, 1987). Also, TGFβ3 has been proved to be a local mammary-derived signalling factor synthesized in response to milk stasis that induces apoptotic cell death during the first phase of involution. In fact, TGFβ3 expression increases as early as 8h after pup removal and its absence in mammary tissue results in normal development but delay in apoptosis during early involution (Nguyen & Pollard, 2000). As explained before, TGFβ3 signalling is

mediated by the Smad pathway. Indeed, Smad3-KO mice show reduced apoptosis upon forced weaning (Yang et al, 2002). It is worth noting that one of the transcriptional targets of TGFβ3 that shows increased abundance 12-24h after weaning is the ZIP-protein TGFβ3-stimulated clone 22 (Tsc-22) which has been associated with apoptosis and is down-regulated in various types of cancers (Kawamata et al, 2004; Shostak et al, 2003; Uchida et al, 2000).

IV) PI3K/AKT

IGFs and their signalling axis, the PI3K/AKT/mTOR pathway, have a significant role in cellular functions such as growth and cell survival, proliferation, division, metabolism, apoptosis and migration capabilities. It has also been studied as a major pathway involved in regulation of mammary gland involution. When IGF-1 interacts with its receptor (IGF-1R) it facilitates the phosphorylation of tyrosine residues in the intracellular domain of the receptor. This will allow for the phosphorylation of tyrosine and serine residues of the insulin receptor substrate (IRS) and Src. The consequence of this series of phosphorilations is the activation of the MAPK and PI3K/AKT/mTOR pathways (Baserga et al, 2003).

IGF pathway is regulated in many critical points, from ligand availability to negative feedback mechanisms exerted by mTOR (Wan et al, 2007) and is activated under normal conditions when the supply of nutrients is appropriate and it promotes the synthesis of lipids, proteins and glycogen. In case of energy shortages, however, the kinase pathways are activated by adenosine monophosphate kinase (AMPK) leading to the inhibition of earlier biosynthetic processes (Towler & Hardie, 2007). Figure 7 depicts graphically this pathway and its regulators.

The IGF/PI3K pathway is involved in mammary gland involution regulation, since it has been proved that when overexpressed, involution is delayed (Hadsell et al, 1996; LeRoith et al, 1995; Moorehead et al, 2001; Neuenschwander et al, 1996) and PTEN, a negative regulator of the pathway, enhances apoptosis in the mouse mammary gland epithelium (Dupont et al,

2002). The activation of this pathway converges into the activation of the critical survival kinase AKT.

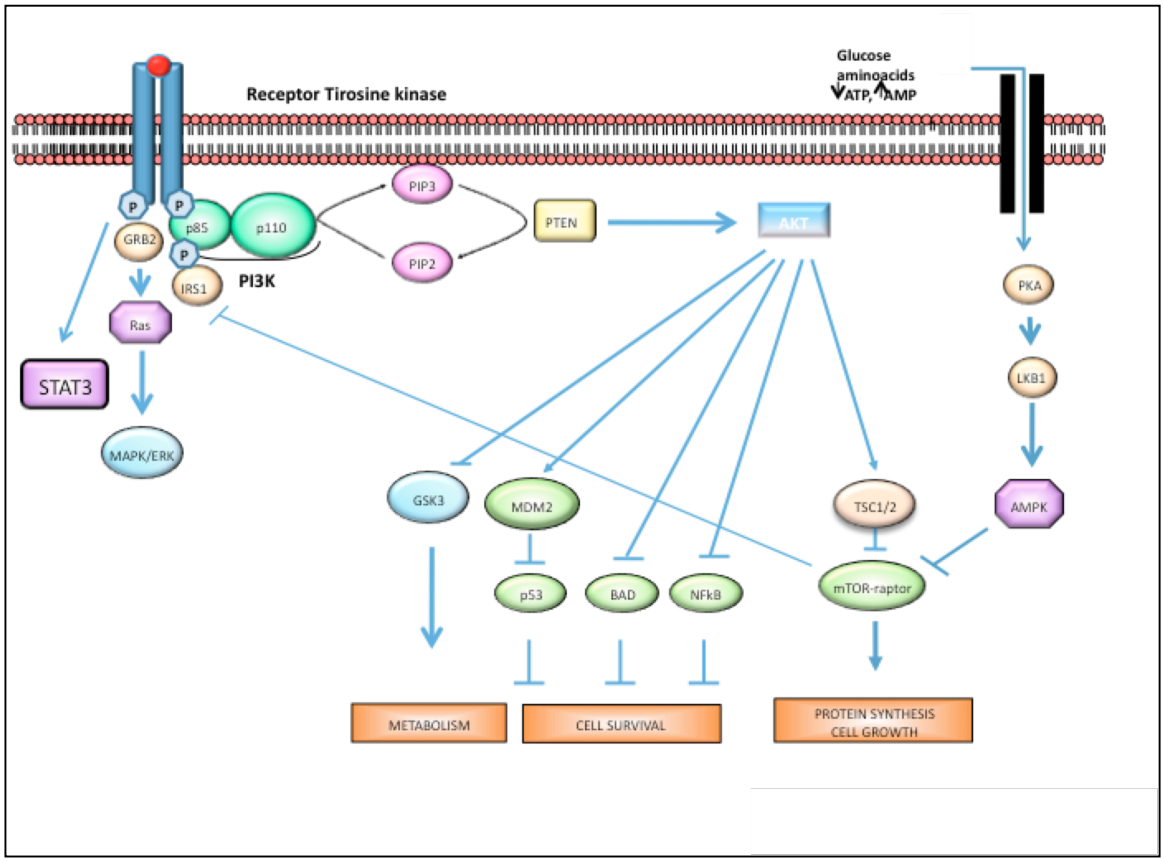

Figure 7. PI3K/AKT/mTOR and AMPK pathways. The activation of the membrane tyrosine kinase receptors (RTK) such as the IGF1R causes activation of intracytoplasmic effectors. AKT is activated via PI3K and has multiple targets, including mTOR, which promotes protein synthesis and cell growth. Cellular energy sensor AMPK and LKB1 exert the opposite effect, inhibiting mTOR. The RTK activation also leads to the activation of other routes such as the MAPK and STAT3. Adapted from (Baserga et al, 2003; Eroles et al, 2011).

In a mouse mammary context, levels of *akt* mRNA have been reported to increase slightly during pregnancy and more dramatically during lactation, decreasing sharply as the gland begins to involute (Schwertfeger et al, 2003). AKT protein levels also halve by 48h of involution and this decrease in total Akt corresponds to an even greater reduction in activated (phosphorylated)

AKT (Abell et al, 2005; Schwertfeger et al, 2001; Strange et al, 2001). This coincident downregulation of AKT survival signalling correlates with the initial apoptotic phase of involution. Beyond mere correlation, the importance of shutting down this pathway at involution has been demonstrated by mammary-specific overexpression of AKT in transgenic mice. Two independent studies expressing constitutively active AKT observed delays in post-lactational involution and delayed onset of apoptosis, consistent with AKT antiapoptotic role (Hutchinson et al, 2001; Schwertfeger et al, 2001).

The IGFR/PI3K/AKT pathway is down-regulated during the lactation-involution apoptotic transition by two mechanisms, the loss of cell-matrix integrity and the upregulation of IGFBPs. Indeed, as it has been mentioned before, IGFBP-5 is a transcriptional target of STAT3; therefore, with the activation of the latter, there is an increase of IGFBP-5 within the first 24h of involution (Gilmore et al, 2000; Strange et al, 2001; Tonner et al, 1997; Tonner et al, 1995). This binding protein sequesters IGF which cannot interact with the IGF-1R thus preventing IGF-mediated survival in the first phase of mammary gland involution. Furthermore, inactivation of the central player of this pathway, AKT, has many known effects that favor apoptosis. AKT exerts its effects through the phosphorylation of different substrates, which results in the activation or inhibition of its targets. Caspase 9, a crucial apoptosis initiation caspase, is inactivated by AKT mediated phosphorylation (Cardone et al, 1998). Also, phosphorylation of the fork-head transcription factor FOXO3a, prevents its translocation to the nucleus were it activates the transcription of pro-apoptotic factors such as FasL and Bim (Brunet et al, 1999; Dijkers et al, 2000). AKT also targets BAD, which, after phosphorylation, undergoes degradation so it cannot exert its function of sequestering anti-apoptotic members of its own family such as BCL-2 and BCL-X (Baxter et al, 2007). AKT also affects the NF-κB pathway via degradation of IκBα (Kane et al, 1999).

Beyond the first 48h after weaning, when mammary tissue starts the remodelling stage, AKT becomes activated again. This would be in line with

the intense activity observed in the mammary gland at this point in order to reassemble its structure to get ready for the next pregnancy. It is intriguing that the apoptotic process is triggered in certain alveolar cells yet the neighboring cells survive and integrity of the alveolar wall is maintained. It is possible that an over-riding survival signal is initiated in these surviving cells, most likely pAKT. Interestingly, AKT is a component of the NF-κB, PI3K, STAT3 and TGFβ pathways, suggesting AKT is a master sentinel that regulates early involution of the mammary gland. (Baxter et al, 2007)

Taken together, the four pathways described co-operate in a precisely orchestrated manner to allow for the apoptosis of the excess epithelial cells in the first phase of involution. This first phase will trigger the second, which is predominantly characterized by the extensive remodelling of the tissue. With it, mammary gland will be ready for a new pregnancy/lactation cycle once the process is terminated.

2.2.2.2 Effectors of Mammary Gland Tissue Remodelling in the Second Stage

I) Role of Metalloproteases

The second, irreversible stage of postlactational involution begins at approximately 48 h after weaning. A gradual reduction of circulating hormones during the first stage is necessary for progression to this stage (Feng et al, 1995; Li et al, 1997b). Here, there is glandular collapse, re-differentiation of adipocytes, and remodelling of the ductal epithelium. During this phase there is a substantial expression of serine and matrix metalloproteases (MMPs) which contribute to the degradation of the basement membrane, phenomenon that will also convey in further apoptosis of luminal cells and the overall involution of the mammary gland (Green & Lund, 2005). MMPs represent a continuously growing family of endopeptidases classified into subfamilies based on their substrate specificity and domain homology (Benaud et al, 1998).

During this second phase of involution, MMP-2, MMP-3, MMP-9 and MMP-11 are expressed, together with urokinase-type plasminogen activator (uPA) which is a serine protease (Li et al, 1994; Lund et al, 2000; Talhouk et al, 1992). The expression of these MMPs is localized to myoepithelial cells and stromal fibroblasts surrounding the degenerating ducts and alveoli. It is noteworthy that tissue inhibitors of MMPs (TIMPs) are overexpressed during the first phase of involution, and as involution progresses, their levels diminish, and hence, MMPs become increasingly activated (Talhouk et al, 1992; Talhouk et al, 1991). The patterns of expression of TIMPs and MMPs correlate with the loss of cellular differentiation, extensive tissue remodelling and cell death (Lund et al, 1996). Also, the critical balance between TIMPs and MMPs provides a mechanism to coordinate the transition between the first stage of mammary involution and the second, which, as already described, has to do with removal of matrix proteins and of the remaining cell debris generated in the first phase, adipocyte differentiation and remodelling of the epithelial ductal tree (Green & Streuli, 2004).

The various MMPs are expressed sequentially throughout involution. MMP-2 is the first to be both expressed and activated following weaning, this is followed by MMP-3, uPA and MMP-9 (Benaud et al, 1998; Lund et al, 2000; Talhouk et al, 1992). The sequential expression of MMPs suggests that these enzymes may have different functions, or, more likely, they may play a role in an activation cascade, whereby one MMP is first secreted and then activated, participating thereafter in the activating cleavage of the other MMPs.

II) Cathepsin D

Another family of proteases involved in the second phase of involution is the cathepsin family. This family of proteases is among the most studied lysosomal hydrolases that degrade proteins at an acidic pH. They can be divided into three subgroups according to their active-site amino acid. Cathepsins B, C, H, F, K, L, O, S, V, W have a cysteine in their active site. Cathepsins D and E have aspartic acid and cathepsin G has a serine. Cathepsins function primarily as endopeptidases within endolysosomal compartments, although some have exopeptidase activity. Apart from their

function in the protein turnover, they can also perform specific functions playing a role in processes such as neovascularisation, antigen presentation, cell growth and tissue homoeostasis (Liaudet-Coopman et al, 2006). Given the acidic pH found within the lysosomes, it is not surprising that all cathepsins are optimally active at acidic pH and, although they are still highly active at neutral pH, raising the pH usually results in the irreversible folding which reduces their lifetime in the cytosol. However, during cell death acidification of the cytosol is common and so loss of cathepsin function upon release from lysosomes is likely to be diminished resulting in cleavage of cytosolic substrates (Lagadic-Gossmann et al, 2004).

Cathepsins are thought to modulate involution through three mechanisms: i) lysosomal mediated cell death (necroptosis); ii) autophagy and iii) extracellular remodelling. And even though studies are still not conclusive about the specific roles that each member of the cathepsin family may play specifically during this physiological process, RNA profiles show a rise in the expression of cathepsins B, K, S, L, and D. Interestingly, although the expression of these endopeptidases can start rising as early as 12h after weaning, the expression is maximal after the 48h time point. It is also important to keep in mind that these proteases have to undergo specific post-translational modifications in order to become activated, therefore, it is plausible that the increased expression does not correlate exactly with an increased activity (Clarkson et al, 2004; Watson & Kreuzaler, 2009).

Human Cathepsin D is an endopeptidase that belongs to the aspartic peptidase family A1. It is found in lysosomes of most human cells and its fundamental function is to catalyse the hydrolysis of peptidic bonds in peptides and proteins (Fusek & Vetvicka, 2005). Its expression is regulated by steroid hormones, growth factors IGF-1, TNF-α, EGF and by retinoic acid (May et al, 1993; Scarborough et al, 1991; Sheikh et al, 1996; Wang et al, 2000). It is synthesized in the form of pre-pro-cathepsin (412 aminoacids Aa) and it is translocated to the endoplasmic reticulum via a signal sequence. In the endoplasmic reticulum, the pre-pro form is further processed and loses the signal sequence, thus leading to the generation of pro-cathepsin D. The

Aa sequence of pro-cathepsin D determines its post-translation modifications: particle rugosity, formation of disulphate bridges, N-glycosylation and phosphorylation.

It is within the endoplasmic reticulum that the pro-cathepsin D undergoes glycosilation mainly with mannose oligosaccharide chains (Gieselmann et al, 1983; Kussendrager et al, 1972). After synthesis and glycosilation, it is transported to the Golgi apparatus where the terminal mannose residues undergo phosphorylation to mannose-6-phosphate (M6P) (Cantor et al, 1992; Cantor & Kornfeld, 1992). By means of these M6P residues, pro-cathepsin D is transported to primary lysosomes (Sahagian, 1984). Once in the primary lysosome, with the acid environment (pH= 4.5-5.5), the pro-cathepsin undergoes further changes in its way to be a fully functional peptidase, cathepsin D. These changes consist in a series of proteolytic transformations and autocatalytic activation. The cleavage of the bond between the aminoacids in position 64 (Glu) and 65 (Gly) releases a 44 Aa propeptide and transform pro-cathepsin D into a 348 Aa active single-chain form. The ultimate form of cathepsin D consists in a mature two chain protein obtained after the cleavage of the Ala204-Tyr205 bond and other cleavages on the carboxyl and amino terminus of the heavy and light chains respectively. The heavy chain, of 196 Aa residues is bound to the light chain of 141 Aa through hydrophobic bonds. The maturation process is schematized in the figure 8 (Hasilik & Neufeld, 1980; Minarowska et al, 2008; Richo & Conner, 1991).

When active, cathepsin D cleaves non-terminal peptide bonds in a polypeptide chain, hence the term endopeptidase. The cleaved bonds are normally formed by carboxyl groups of hydrophobic amino acids, specially aromatic. The catalytic site of cathepsin D is constituted by two Asp acid residues in positions 32 and 231(Fusek & Vetvicka, 2005); and the proteolitic activity of both the mono-chain and the double-chain forms is the same.

As already mentioned, its main function is to carry out intracellular digestion within the lysosomal compartment. Acidic pH is required for the activity of cathepsin D to degrade proteins in lysosomes with an optimum pH

of 3.5-5.5 in contrast to other proteases such as serine proteases and MMPs. It has also been shown that it can activate precursors of biologically active proteins in pre-lysosomal compartments of specialized cells (Diment et al, 1989). It is apparently also involved in the processing of antigens, hormones and neuropeptides. Cathepsin D is also required in certain epithelial cells for tissue remodelling. Still, cathepsin D seems to play a role in a plethora of other physiological processes regardless of its proteolytic function. In fact, cathepsin D deficient mice die prematurely from massive destruction of lymphoid organs and progressive atrophy of the intestinal mucosa, suggesting essential functions of this protease in tissue homeostasis (Saftig et al, 1995).

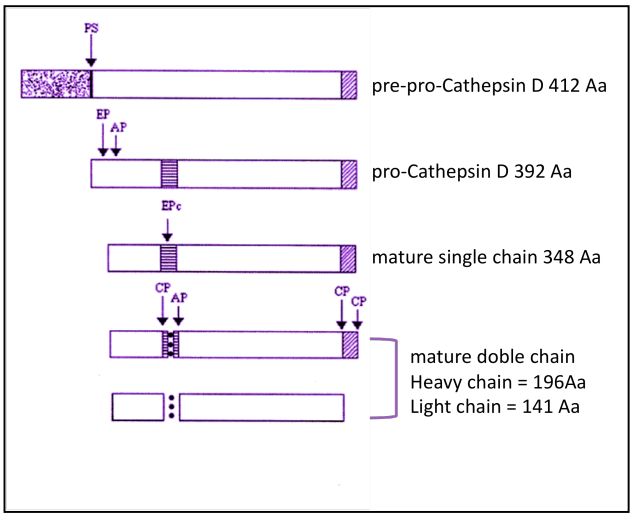

Figure 8. Proteolytic modification of cathepsin D from its pre-pro form to the mature double-chain form. The amino-terminal end is on the left hand side. . PS – signalling peptide, EP - endopeptidase, AP - aminopeptidase, EPc – cysteinyl endopeptidase, CP - carboxypeptidase, - hydrophobic bond (Minarowska et al, 2008)

Cathepsin D is constitutively expressed in nearly all cells, but, as it has been stated before, it is up-regulated in a hormonally dependent fashion (Cavailles et al, 1993). When cathepsin D is looked at in normal breast, in a quiescent state, mammary epithelial cells secrete minimal levels of pro-cathepsin D (Capony et al, 1989). But, since the physiological function of the mammary gland is lactation, when studying cathepsin D expression, post-translational modifications and functions during lactation and weaning, quite a

different picture is drawn. For example, pro-cathepsin D is secreted at relatively high levels into human, bovine and rat milk and it is thought to be activated by the low pH of the new-born digestive tract, playing a role in digestion (Benes et al, 2008; Margaryan et al, 2010). Also, the secretion of activated single-chain cathepsin D from the basal side of the epithelial cells toward the basement membrane has been identifed. There it is thought to process PRL to generate a bioactive fragment with anti-angiogeninc and pro-apoptotic properties (Castino et al, 2008; Hilfiker-Kleiner et al, 2007; Lkhider et al, 2004; Piwnica et al, 2006).

During weaning, its principal role is as an endopeptidase, and it is thought to have a role in the remodelling of the mammary gland stroma and also in the lysosomal mediated cell death, recently coined as necroptosis. It is of note that cathepsin D undergoes distinct protein processing at different stages of mammary gland development, and this customized processing results in differential enzymatic activity best fitting particular stages of development. In fact, at the onset of involution, the glycosilation pattern changes; and it reverts back to the original pattern by day 4 of involution (Margaryan et al, 2010). All in all, the complex post-translational modifications of this peptidase are far from being totally figured out, and changes can have an impact in its function throughout a dynamic process such as post-lactational involution.

2.2.2.3 Nitric Oxide and its implications in Cellular Signalling during Mammary Gland Involution

Nitric Oxide (NO) is a highly reactive molecule with low molecular weight, uncharged, soluble in both aqueous and hydrophobic environments and highly diffusible. It is the best example of a reactive molecule demonstrating both cytotoxic and cytoprotective properties (Wink et al, 1998). NO was first identified in the 1980's as Endothelium-Derived Relaxation Factor (EDRF), a substance generated by the endothelium that caused vascular relaxation and also the active component of nitorvasodilators (Ignarro, 1990). Conversely, NO was found to be generated by macrophages

participating in the anti-tumour and anti-pathogen response (Granger & Hibbs, 1996; Nathan & Hibbs, 1991). These initial observations led to an explosion of NO research, which has revealed the importance of this diatomic molecule in nearly every tissue in the body.

I) Formation

The chemical biology of NO divides its potential reactions into two categories: direct and indirect (Wink et al, 1993). The direct effects of NO are those chemical reactions that occur fast enough to allow NO to directly react with a biological target molecule. The indirect effects require that NO reacts with oxygen or superoxide anion to generate reactive nitrogen species (RNS) which subsequently react with the biological targets. The direct effects normally occur at low concentrations and the indirect effects occur at much higher concentrations. These indirect effects can be further categorized into oxidative stress (when the oxidation of the target molecule increases) and nitrosative stress (which implies the addition of a nitrosonium group NO^+) (Thomas et al, 2008). Figure 9 briefly summarizes the chemical reactions NO undergoes in order to become a reactive species.

Although there are alternative mechanisms to generate NO, such as acidification or reduction of nitrite, the vast majority of mammalian NO is derived enzymatically from NO synthase (NOS). This family of enzymes converts arginine to citrulline and NO in an NADPH and O^2 dependent process. There are three NOS isoforms that provide a wide range of concentration and temporal NO profiles. Two of these are constitutive (NOS1/nNOS neuronal and NOS3/eNOS endothelial) and one is inducible (NOS2/iNOS) (Stuehr et al, 2004). These isoforms are differentially regulated at numerous levels including transcription, translation, post-transcriptional and biochemical (Bredt, 1999; Fulton et al, 2001). There are two main differences between these isoforms, the duration of NO generation and the local concentration of NO that can be produced. Calmodulin dependency is important for the constitutive forms of the enzyme where calcium fluxes and phosphorylation through protein-protein interactions regulate their activity, providing a transient NO output (Mayer et al, 1989). In contrast, NOS2 has

calmodulin as a subunit, being thus permanently activated and capable of generating NO for long periods of time (Nathan & Xie, 1994). The enzymatic activity of NOS is tightly controlled depending on the substrate and co-factor availability, as well as the rate of electron transfer. Hence, in the presence of sufficient co-factors (NADPH, FMN, BH4, FAD), the NOS activity is dependent on the arginine and oxygen availability (Ghosh & Salerno, 2003). Furthermore, NOS enzymes are also known to form complexes with their targets, e.g. NOS2 regulates cyclooxigenase-2 (COX-2) activity in inflammatory settings (Kim et al, 2005).

$2NO+O2 \Rightarrow 2NO_2$ eq 1

$NO_2+NO \Leftrightarrow N_2O_3$ eq 2

$H2O+N_2O_3 \Rightarrow 2NO_2^-+2H^+$ eq 3

$O_2^-+NO \Rightarrow ONOO^-$ eq 4

$ONOO^- + 2NO \Rightarrow N_2O_3 + NO_2^-$ eq 5

Figure 9. Summary of the formation of RNS. The balance between oxidation and nitrosation chemistry depends on the flux of NO. In the case of the auto-oxidation in hydrophobic environments, NO_2 is first generated, but as levels of NO_2 increase there is rapid formation of N_2O_3 (equations 1 and 2), ultimately forming nitrite in water (equation 3). $ONOO^-$ is formed from the NO/O_2^- reaction (equation 4). Finally, the reaction rates of NO with these RNS are nearly diffusion controlled, facilitating the rapid conversion from an oxidative to a nitrosation profile (equation 5). eq=equation (Thomas et al, 2008).

II) Effects

Many *in vitro* studies using NO donors have demonstrated that different concentrations of NO exert different biological effects. For example, when cultured cells are exposed to NO for defined periods of time, various proteins were found to be sensitive to distinct concentrations of NO (Thomas et al, 2006). At sustained NO levels of 10-30nM, phosphorylation of ERK occurs. At 30-60nM NO, AKT is phosphorylated (Pervin et al, 2007; Prueitt et al, 2007; Ridnour et al, 2004; Thomas et al, 2004). When NO reaches a concentration of 100nM, HIF-1α is stabilized (Thomas et al, 2004) and at 400nM NO, p53 is

phosphorilated and acetylated, inducing cell cycle arrest and even an apoptotic response. When the concentration is above 1μM NO, mitochondria respiration is inhibited (Borutaite & Brown, 2006; Cleeter et al, 1994).

As for the achievable NO *in vivo* concentrations, it was very important to establish whether NO can achieve the higher concentrations *in vivo* that seem to mediate some biological effects on a *Petri* dish. Several studies have demonstrated that μM levels of NO can occur *in vivo* in an inflammatory microenvironment (Carcillo et al, 2003; Lee & Lee, 2005; Stadler et al, 1994). These high NO concentrations are achieved through production by macrophages which play an important part in the immune and inflammatory responses (Hanahan & Weinberg, 2011). Macrophages can generate different concentrations of NO that serve their different functions (Espey et al, 2000). Interestingly, the amount of NO generated by activated macrophages depends on how they are stimulated. And not only on animal models (the above are examples of septic models), there are also some examples in humans that μM NO levels may be achieved. Samples from ulcerative colitis found phospho-p53 co-localized with NOS-2. Also, in colorectal cancer there is a correlation between NOS2 and HIF-1α which indirectly suggests NO concentrations above 100nM (Yu et al, 2006).

In addition to concentration, temporal aspects of NO exposure are important. Certain proteins respond immediately to NO exposure, for example activated HIF-1α that is stabilized and accumulates as long as the minimum threshold of NO concentration is available, but when NO levels fall below this threshold, protein levels of HIF-1α disappear (Thomas et al, 2004). In contrast, phosphorylation of p53 by NO takes several hours, but it sustains long after the NO exposure has ceased. Therefore NO responders are categorized into various categories, namely immediate, delayed, transient and sustained (Thomas et al, 2008). Having established that endogenously generated NO provides a spectrum of biological responses based on concentration and duration of NO exposure, it is important to point out, once more, that endogenously generated NO ranges from basal levels in

endothelial cells (< 1nM) to that generated by fully activated macrophages (>1μM). This wide range of concentrations combined with its unique chemical reactivity, makes NO a very versatile signal transduction agent.

As said before, NO at high concentrations exerts its functions through the reaction with O_2 or superoxide to generate reactive nitrogen oxide species (RNS) that subsequently react with biological targets, inducing nitrosative modifications. Nitrosative modifications are selective processes that target precise molecular sites in proteins or lipids for gain or loss of function, in a manner somewhat analogous to the better-known phosphorylation or acetylation signal transduction mechanisms (White et al, 2010). In proteins, with the exception of heme-iron binding, these modifications manifest in two main forms, namely S-nitrosylation of cysteine thiols and nitration of tyrosine residues. S-nitrosylation occurs through the covalent attachment of a diatomic nitroso group to a reactive thiol sulfhydril in a redox-dependent fashion (Hess et al, 2005). Tyrosine nitration results from the covalent addition of a triatomic nitro group (NO_2) to the phenolic ring of tyrosine residues (Ischiropoulos, 2003). Both S-nitrosylation and tyrosine nitration may arise from the protein interactions with NO or RNS. Another important modification induced by NO is S-Glutathionylation. Briefly, the GSH/GSSG redox system maintains cellular proteins in a reduced state. Normaly, S-nitrosylated proteins are rapidly reduced by GSH (the reduced form of glutathione) and GSH can be S-nitrosylated to S-nitrosoglutathione (GSNO) which is also an effective agent for the direct transfer of NO to other thiols by transnitrosylation. In addition, glutathione could potentially replace the NO on S-nitrosylated proteins, converting them into an S-glutathionilated form (Singh & Gupta, 2011). Figure 10 provides an overview of the chemical reactions NO is involved in, from synthesis to biological effects.

Although non-enzymatic dependent, protein nitrosative modifications are very selective processes. This is corroborated by the relatively small number of tyrosine and cysteine residues that seem to be the target of nitrosative adducts (Stamler et al, 2001). It has also been established that the local

environment of the residue plays a major role on whether it will react with RNS (Souza et al, 1999; Stamler et al, 1997). Protein nitrosative modifications are negatively regulated by denitrosylalting and denitrating enzymes such as S-nitrosoglutathione reductase, thioredoxin, protein disulfide isomerase and xanthine oxidase (Liu et al, 2001; Nikitovic & Holmgren, 1996; Trujillo et al, 1998). Still, there is not so much information about possible denitrating enzymes.

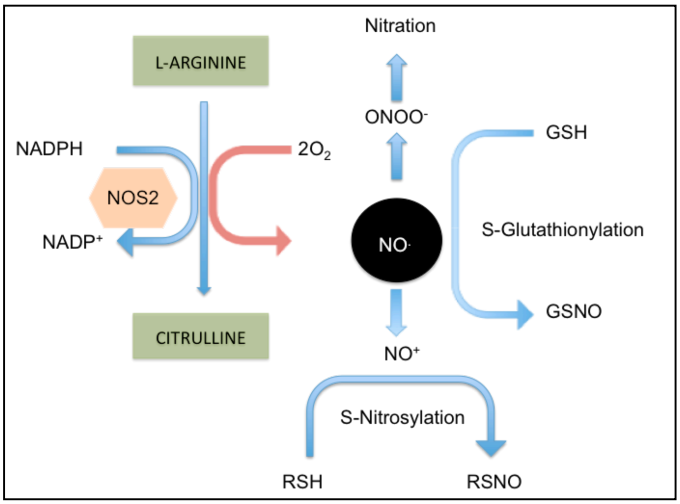

Figure 10. Chemistry of NO. NO is synthesized endogenously from L-arginine, NADPH and Oxigen. NO freely diffuses creating concentration gradients across subcellular compartments. Redox or additive reactions convert NO to NO species, which in turn, dictate the biological effects of NO. Adapted from (Singh & Gupta, 2011).

III) Nitrosative modifications in intracellular signalling

Nitrosative modifications play an important role in different signalling pathways. Generally speaking, and with respect to the main hubs that are activated during mammary gland involution, nitrosative modifications have been found to take an active part in regulation of cell signalling in the NF-κB and AKT pathways. Indeed, nitration of the 181 tyrosine residue in IκBα is consistent with the disruption of the interaction of the IκBα -p50 complex. Disrupting these interactions results in dissociation of the IκBα/NF-κB complex, and the latter becomes activated (Yakovlev et al, 2007). Furthermore, NF-κB has been proved to activate NOS2 transcription during mammary gland involution (Zaragoza et al, 2005), which could establish a feed-back loop where nitrosative modifications are required to activate this important hub in the involution process. Controversially, it has also been reported that RNS can negatively regulate NF-κB via S-nitrosylation of a

cysteine residue within IKKβ, which inhibits IκB phosphorylation preventing NF-κB nuclear translocation (Hess et al, 2005). Taken together, these data suggest that nitrosative stress may serve as both stimulatory and inhibitory effects on the NF-κB signalling pathway, depending on the prevailing posttranslational protein modifications. It is thus conceivable that, in acute inflammatory settings, S-nitrosylation of IKKβ could serve as a negative feedback control of the NF-κB dependent inflammatory response, but in a more persistent state of inflammation, tyrosine nitration could supersede the S-nitrosylation effect and exacerbate the inflammatory response.

In AKT pathway regulation, there has been an important amount of work covering the role of NOS2, nitrosative modifications and insulin resistance. For example, obese NOS2-KO mice are protected from developing insulin resistance (Perreault & Marette, 2001). Importantly, NOS2 is induced in skeletal muscle and adipose tissues of type 2 diabetic subjects, and its expression correlates with insulin resistance and obesity (Engeli et al, 2004). *In vitro* work has shown that exposure to $ONOO^-$ can induce tyrosine nitration of IRS-1, consequently impeding tyrosine phosphorylation and activation of downstream insulin-signalling intermediates (Nomiyama et al, 2004). Also, $ONOO^-$ can induce tyrosine nitration of AKT, which can blunt its kinase activity (Zhou & Huang, 2009).

All in all, NO has been proved to be a particularly interesting and rich signalling mediator, and since it is produced in pro-inflammatory settings, its specific role in mammary gland involution should be further characterized.

3. FROM MAMMARY GLAND INVOLUTION TO BREAST CANCER

Having reviewed all the different pathways involved, it is clear that mammary gland involution after weaning is a well orchestrated and complex process which is finely tuned by different signals. Following lactation and the weaning of pups, the mammary gland undergoes a sequential chain of events that end up with the gland returning to a pre-pregnant like state.

A deeper study into the molecular mechanisms involved in the regulation of mammary gland involution reveals different biological processes overlapping at the same time. Cell proliferation occurs not only during virgin development and pregnancy as thought at first, but also during mammary gland involution with a peak of DNA synthesis at 48h after weaning (Wiesen & Werb, 2000). On the other hand, the induction of inflammatory APR genes in mammary gland has been described as early as 24h after pups withdrawal (Stein et al, 2007). Concomitant induction of inflammatory, apoptotic and proliferative regulatory signals could represent conflicting messages for cells. However, under physiological conditions, a fine balance among these responses will condition tissue homeostasis, and therefore cell proliferation or apoptosis resolution will depend upon which side the balance is tilted.

In this sense, although the molecular mechanisms involved in the inclination to a particular biological process remain elusive, it is not unusual to find common regulatory proteins for the above mentioned processes (Aggarwal et al, 2009; Reuter et al, 2010; Shaulian, 2010). Most of these proteins have been identified as oncogenes or transcription factors persistently activated during neoplasic transformation, a circumstance that adds an increased interest for health purposes. Indeed, a deeper analysis of the main hubs that control mammary gland involution, namely STAT3 and NF-κB, shows that they not only modulate cell death and proliferation, but are also involved in tumour development and progression by the induction of an inflammatory environment in some way similar to that one observed in the involuting mammary gland of mice (Chariot, 2009; Torres et al, 2011). In fact, it has been suggested that deregulation of post-lactational involution may act to facilitate tumour formation (Lyons et al, 2011; Radisky & Hartmann, 2009). The elevated incidence of breast cancer associated with pregnancy has been suggested to be influenced by the tumour microenvironment developed in mammary gland after weaning (Lyons et al, 2011). Furthermore, in agreement with the tumour-promoting potential of the involuting mammary gland, it has

been described that up to 81% of the genes regulated by NF-κB during involution have been related with carcinogenic processes (Torres et al, 2011).

3.1 INVOLUTION AS A PRO-INFLAMMATORY PROCESS- THE CANCER LINK

As it has been explained earlier, the induction of pro-inflammatory cytokines, by both STAT3 and NF-κB, is an important event that takes place in the early stages of mammary gland involution. This might seem inexplicable, since involution after weaning is not a process associated clinically with inflammation (Stein et al, 2007). This could be partially explained because phagocytosis of apoptotic cells can activate a regulatory mechanism that down-regulates pro-inflammatory cytokine production (Fadok et al, 1998; Monks et al, 2005). It has been suggested that the chemokine/ cytokine pro-inflammatory signature is a natural consequence of the Stat3/NF-κB activation and that this would promote an unwanted cellular infiltrate that required sequestering through additional anti-inflammatory mechanisms regulated by physiological programs (Clarkson et al, 2004; Stein et al, 2004). In fact, in *stat3*-KO mice, mastitis is a common event and although this has not been associated with infection, it results in a massive infiltration of lymphocytes (Chapman et al, 1999).

Data from several studies have established that wound-healing programs are activated during mammary gland involution after lactation ends, and these programs entail macrophage activation (Clarkson et al, 2004; McDaniel et al, 2006; Schedin et al, 2004; Schedin et al, 2007; Schedin et al, 2000; Stein et al, 2004). Many genes involved in macrophage recruitment and activation are up-regulated at the RNA and protein levels during involution (Clarkson et al, 2004; Stein et al, 2004).

On the other hand, components of chronic inflammation are common in the microenvironment of many cancers, such as breast and colon (Balkwill et al, 2005). In cancer-related inflammation, immune cell infiltration is associated with high chemokine and cytokine signalling, protease mediated tissue remodelling and angiogenesis (Hanahan & Weinberg, 2011; Mantovani et al,

2008; Mantovani et al, 2007). Thus, physiologic gland regression after pregnancy can be a tumour promoting microenvironment, and a subset of breast cancer diagnosed within 5 years of a recent pregnancy is associated with a poor prognosis (Lyons et al, 2011; Schedin, 2006). This will be further explained in the discussion section.

Macrophages' presence in breast cancer pathology studies has been amply correlated with poor prognosis of this disease. In fact, tumour associated macrophages (TAM), have been linked to significant increased relapse rate and worse over-all survival with hazard ratios of 2.79 and 9.43 respectively (Leek et al, 1996). While the concept of tumour promoting immune cells might seem contradictory, many studies have looked into the role of TAMs as tumour favouring and it has been well established that macrophages can play a tumour suppressor and a promoter role, depending on the specific cytokine milieu (Goede et al, 1999; Lee et al, 1997; Leek et al, 1996).This dual role is due to macrophages' multifunctional nature since they perform diverse functions, such as the secretion of various pro-angiogenic factors, including various cytokines and matrix-degrading enzymes. Among the pro-tumorigenic factors and cytokines expressed by macrophages are: VEGF (Leek et al, 1994), urokinase (uPA) which is involved in ECM degradation, invasion and metastasis (Hildenbrand et al, 1999; Xu et al, 1997), MMP-9 (Davies et al, 1993; Naylor et al, 1994) and lysosomal proteases such as cathepsin D (Lipponen, 1996), although for the latter, it is the expression in tumour cells which has been correlated with metastasis, so the role of TAM-derived cathepsins in cancer progression remains unknown (Lah et al, 2000; Lipponen, 1996).

At a more specific level, macrophages are also a very important source of NO. It has been explained before that during inflammatory responses, the levels of NO can reach μM concentrations. In fact, generation of NO by NOS2 is a cardinal feature of inflamed tissues (Hussain et al, 2000; Jaiswal et al, 2000). As for the role of NO and nitrosative modifications in cancer development and cancer progression, contradictory reports exist (Ridnour et al, 2008). Earlier studies have described that NO is a critical component of the

immune response of macrophages (Hibbs, 1991). Other studies showed that lack of NOS2 could lead to tumour development (Hussain et al, 2004). Recent studies have shown a correlation between NOS2 and worse prognosis in breast cancer and melanoma (Ekmekcioglu et al, 2006; Prueitt et al, 2007). To date, there is substantial evidence implicating NO in cancer development as it can act as an endogenous mutagen (Wink et al, 1991), a pro-angiogenic factor (Jenkins et al, 1995), an enhancer of oncogene expression and an inhibitor of apoptosis (Ambs et al, 1998; Li et al, 1997a).

As it occurs in physiological processes, NO concentration and time of exposure play an important role in biological effects in pathological contexts. In MCF-7 cells, levels below 50nM NO increase cGMP-mediated ERK phosphorylation, intermediate levels (>100nM) lead to HIF-1α stabilization, and high NO (>300nM) are associated with p53 phosphorilation (Thomas et al, 2004). Hence, at NO levels of 100nM or lower, pro-survival responses are favoured in a neoplastic context. However, the pro-survival effects of NO are lost at concentrations above 400nM (Hofseth et al, 2003; Wang et al, 2003), this has been the biological rational for the development of NO donating drugs, since it is believed that high NO concentrations will result in cell death, but this issue will be discussed later. Figure 11 represents the mechanisms of action of NO on tumour cells. Given the capacity of macrophages to produce NO, it can be said that these immune system cells are responsible, at least in part, of NO output in tumour microenvironment (Ridnour et al, 2008). As referred to before, several studies have shown that patient survival decreases with enhanced NOS2 expression in the tumour (Ekmekcioglu et al, 2006; Glynn et al, 2010; Prueitt et al, 2007). These and other studies have established tumour development as persistent, non-healing wounds, with pro-inflammatory features and abundance of tumour associated macrophages (TAMs) implying worse prognosis (Chang et al, 2005; Leek et al, 1996; Whalen, 1990). All in all, data suggests that there is a link between inflammation and cancer progression (Lyons et al, 2011) and NO production seems to play a role in this setting.

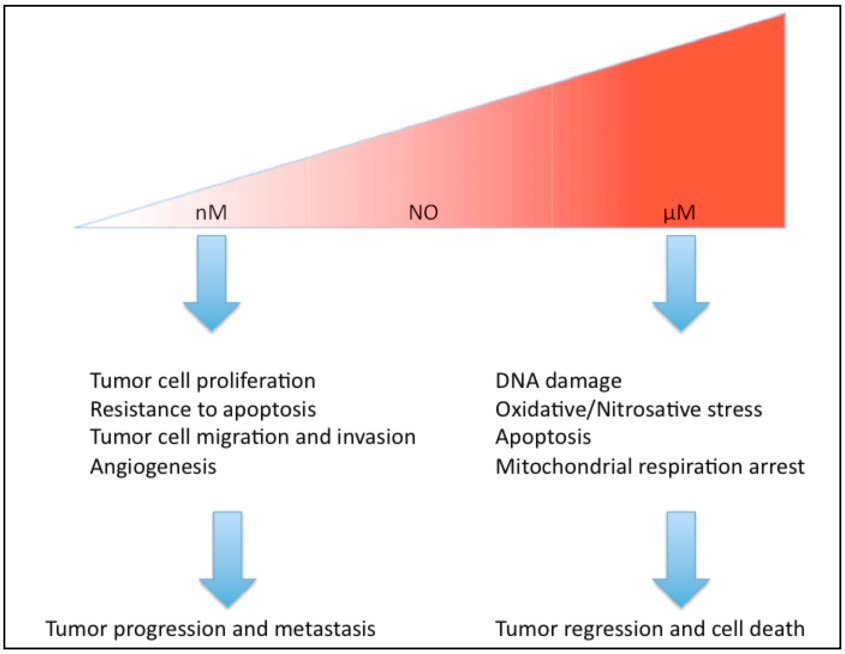

Figure 11. Diagram summarizing NO action on cancer cells. NO can influence tumour development in a time and concentration dependent manner. (Singh & Gupta, 2011)

3.2 STAT3 AS A BREAST CANCER-PROMOTING FACTOR

STAT3's role as the regulator of a pro-apoptotic program in normal mammary gland development is in apparent contradiction to its documented pro-survival functions in mammary tumour progression (Guo et al, 2006; Zhou et al, 2007). A potential explanation may lie in recent work demonstrating that the involuting mammary gland is a particularly good microenvironment for promoting mammary tumour metastasis (McDaniel et al, 2006; Schedin et al, 2007). Recently published data suggests that the proinflammatory functions of STAT3 during mammary gland involution may also have a critical role in mammary tumour progression. In vivo work with mice expressing the ErbB2 oncogene (HER2) has demonstrated that STAT3 dependent pro-inflammatory program is required for the metastatic phase of tumour progression. This work showed that STAT3 was not essential for tumour onset but STAT3 ablation implied significant decrease in metastasis occurrence. The analysis of gene expression profile of these *stat3*-defficient HER2 tumours revealed that the acute inflammatory response was dramatically impaired because the reduced

expression of STAT3 targets: C/Ebpδ, OsmR, SAA1, SAA2 (Ranger et al, 2009). What is intriguing is that STAT3 uses this same regulatory network for either promoting mammary gland involution and tumour progression. Hence, since STAT3 in involution modulates microenvironment, its role in tumour progression is probably to also alter the tumour microenvironment (Siegel & Muller, 2010).

3.3 NF-κB CANONICAL ACTIVATION AND MAMMARY TUMORIGENESIS

NF-κB plays a key role in regulating inflammatory response (Lin & Karin, 2007), and, as already explained, during mammary gland involution it can exert both pro-survival and pro-apoptotic signals in mammary epithelial cells. As for the role in carcinogenesis, NF-κB family members are over-expressed or activated in breast cancer cell lines, primary human breast tumours and other malignancies (Dejardin et al, 1995; Sovak et al, 1997). Still, many efforts are being made to elucidate the specific role of NF-κB in mammary epithelial cell vs inflammatory cell in tumour progression. In fact, a work published recently shows that NF-κB induced activation in epithelial mammary cells promotes tumour on-set, growth, angiogenesis and the infiltration of TAM. Also, its inhibition resulted in reduction of epithelial-mesenchymal transition, breast tumour stem cell expansion and heterotypic signalling in which epithelial cells recruited TAM (Liu et al, 2010).

Furthermore, *in vitro* investigations concerning activation of NF-κB in human breast cancer cells have established NF-κB as a provider of resistance to cytotoxic agents. It has been demonstrated that expression of the NF-κB inhibitor IκBα increases cancer cell sensitivity to paclitaxel, a key chemotherapeutic agent in breast cancer treatment (Patel et al, 2000). Also, doxorubicin increases NF-κB activity *in vitro* and inhibition of the pathway also increases cells sensitivity to this agent (Tapia et al, 2007). *In vivo* work in transgenic mice has proved that inhibition of NF-κB signalling leads to increased tumour latency and decreased tumour burden (Connelly et al, 2011). This data indicates that, in addition to the NF-κB role as an inducer of

the inflammatory response, its activation in epithelial cells contributes actively to tumour progression and it could be a potentially effective therapeutic target.

3.4 CATHEPSIN D AS A PROGNOSTIC FACTOR IN BREAST CANCER

The role of cathepsin D in cancer remains to be elucidated, but it has been published in numerous occasions that increased cathepsin D expression correlates with tumour aggressiveness, metastasis and poor survival (Liaudet-Coopman et al, 2006), and this is also true for breast cancer (Ferrandina et al, 1997; Rochefort, 1992; Westley & May, 1999). A meta-analysis of studies on node negative breast cancer and a complete study on 2810 patients have confirmed the value of high concentrations of cathepsin D as a marker of aggressiveness (Ferrandina et al, 1997; Foekens et al, 1999). As for the specific role this protease plays in carcinogenesis, many reports have established cathepsin D as a mitogenic factor, a stimulator of angiogenesis and a pro-metastatic factor.

Different mechanisms have been proposed as responsible for the mitogenicity of cathepsin D. On one hand, its catalytic activity has been implicated in the proteolytic activation of growth factors, rendering cathepsin D an indirect role in mitosis activation (Briozzo et al, 1991; Rifkin et al, 1997). Nevertheless, it is pro-cathepsin D that seems to play a major role in activation of mitosis. Various studies have demonstrated the growth stimulating effects of both pro-cathepsin D and its 44 Aa activation peptide on the proliferation of breast cancer cells (Fusek & Vetvicka, 1994; Vashishta et al, 2005; Vetvicka et al, 1999). In fact, this mitogenic function is thought to be mediated by the activation peptide of pro-cathepsin D through its interaction with a yet unknown cell surface receptor (Benes et al, 2006; Fusek & Vetvicka, 1994). It is hypothesized that the signal formed by interaction of the membrane receptor with the activation peptide is transmitted to the cell nucleus where it affects the expression of genes involved in cell proliferation, apoptosis and invasion. In fact, in *in vitro* studies with recombinant pro-cathepsin D activation peptide and breast cancer cell lines, a series of genes involved in these processes where found to be either up or down-regulated.

For example, NF-κB2 (p49/p100), BCL-2 binding component 3 (PUMA) and cell division cycle 42 (CDC42) were found to be up-regulated. These genes are implicated in cell cycle progression, survival, angiogenesis, invasion, anti-apoptotic activity and regulation of cell motility and adhesion. Also, treatment with activation peptide enhances the invasiveness of breast cancer cells (Benes et al, 2006). So it seems that cathepsin D's role in cancer progression is more related with a paracrine function, where either the pro-cathepsin D or its activating peptide interact with a yet unknown receptor. This interaction would activate a signalling pathway (possibly MAPK) and finally activate transcription of genes that regulate cell cycle progression, mitosis, angiogenesis and cell migration (Laurent-Matha et al, 2005). In fact, the direct involvement of enzymatic activity of the mature form of cathepsin D in growth and invasiveness of breast cancer cells has not been established (Liaudet-Coopman et al, 2006).

In line with the concept that every physiologic process is extremely well regulated, so is the complex processing of cathepsin D. As mentioned before, cathepsin D processing varies during the different stages of mammary gland development (Margaryan et al, 2010). These finely regulated changes could be deregulated during cancer progression, and render a plausible explanation for the apparent change in the role of cathepsin D in this pathological setting.

Having reviewed the major aspects of mammary gland involution, the genetic program that mammary cells recruit during the involution process after lactation, the pro-inflammatory environment created and the signalling pathways activated, it is clear that this physiological process has to be very well orchestrated. The pro-apoptotic and pro-survival signals are well modulated in order to not send equivocal messages that could favour an oncogenic event or its progression. Intriguingly, most of the mechanisms that partake in the post-lactational remodelling process have also been related in a way or another with tumour development and progression. This warrants the need for better understanding the mechanisms that regulate this process, in order to be able to also understand how they become de-regulated and allow for cell transformation and cancer development.

AIMS

AIMS

1. Our group has previously established that NOS2 expression is induced by NF-κB during weaning as early as 8 hours after litter removal (Zaragoza et al, 2005). NOS2 has been implicated in many pro-inflammatory processes, inducing higher and steadier levels of NO than those obtained with NOS1 and NOS3. This, added to the previous works that define mammary gland involution after weaning as a pro-inflammatory model, gave rise to question exactly which role did NO exert in this setting. Hence, our first objective is to establish the role of NO as a signalling molecule in the involution process, specifically in the regulation of two of the main pathways implicated in post-lactational involution, STAT3 and NF-κB.

2. NO has been characterized as a highly reactive molecule that produces reactive nitrosative species. NO can play contradictory signalling roles depending on concentration and temporal exposure. In general, lower NO concentrations promote cell survival and proliferation, while higher levels trigger cell cycle arrest, apoptosis and senescence (Thomas et al, 2008). More specifically, it has been shown that NO can induce a series of nitrosative post-translational modifications on proteins and lipids that play a key signalling mechanism in cell physiology, with a clear role in pathophysiology (White et al, 2010). In proteins, these modifications manifest in two main forms, either as S-nitrosylation of cystein thiols or as nitration of tyrosine residues. The second objective is to further explore the possible role of specific protein nitrosative modifications in mammary gland involution and how these post-translational modifications affect protein function.

3. Pregnancy is associated with a reduction in a woman's lifetime risk of developing breast cancer (Albrektsen et al, 1994; MacMahon et al, 1973; Rosner et al, 1994). However, studies in breast cancer incidence

have demonstrated a transient increase in breast cancer risk in the years immediately following pregnancy (Albrektsen et al, 2005; Chie et al, 2000; Lambe et al, 1994). Hence, pregnancy might both suppress and promote breast cancer, since, the initial effect of any completed pregnancy is an increased risk, but a crossover to long-term protection is seen in women of younger maternal age, and high parity (Lord et al, 2008). One of the hypotheses to explain this increased risk in breast cancer associated with pregnancy postulates that mammary gland involution, which goes hand-in-hand with a terminated pregnancy implies the activation of certain mechanisms that resemble pro-tumorigenic, wound-healing and pro-inflammatory micro-environment (Schedin, 2006). NO is one of the molecules induced in pro-inflammatory environments, and it has been proved to have deleterious or pro-survival effects in different cancer cell lines depending on dose and time of exposure (Thomas et al, 2008). The third objective is to explore in an *in vitro* luminal breast cancer model the specific pathway activation that NO can induce at high concentrations. The main aim of the third objective is to find which physiological mechanisms studied in the previous two objectives are recruited by transformed mammary epithelial cells when exposed to high NO concentrations.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. ANIMAL HUSBANDRY AND TISSUE EXTRACTION

Pregnant Wistar rats (Harlan), wild-type (WT) control mice and knock out NOS2 (NOS2-KO) mice (Taconic, Ejby, Denmark) were used. Mice were C57BL/6 and the genotype of the NOS2-KO strain was verified by PCR, using DNA obtained from tail samples taken at the end of the experiments. Animals were kept in individual cages in a controlled environment (12 hour light/12 hour dark cycle) and they received water and food *ad libitum*. They were cared for and handled in conformance with the NIH (National Institutes of Health) guidelines and the Guiding Principles for Research Involving Animals and Humans approved by the Council of the American Physiological Society. The Research Committee of the School of Medicine (University of Valencia) approved the study protocol. After parturition, the litters were maintained with at least ten pups in the case of rats and seven pups in the case of mice.

At the peak of lactation (days 9–11), mice were divided into groups as follows: control or weaned animals from which the litter was removed 6, 24, 48 and 72h before sacrifice. The rats were divided into different groups at the peak of lactation (days 12-14): control lactating rats (n=8) or weaned rats from which pups were removed 12 days after delivery to initiate involution. Mammary tissue samples were collected at the indicated times after weaning (at least three rats for each condition were used). . The animals were anaesthetized with sodium pentobarbital (60mg/kg body weight in 0.9% NaCl intraperitoneal; Braun Medical, Rubi, Spain) and killed immediately after removing the inguinal mammary glands that were snap-frozen in liquid nitrogen and stored at −80ºC for further analysis.

2. CELL CULTURE

The cell line used for the *in vitro* assays was the MCF-7 cell line, initially obtained from a metastatic pleural effussion of an invasive ductal

carcinoma oestrogen and progesteron receptor positve, HER-2 negative, was obtained from the ATCC.

Cells were grown in 6cm diameter plates with DMEM media supplemented with 2nM glutamine, 100U/mL of penicilin and 100µg/mL streptomycin and 10% fetal bovine serum. The *in vitro* experiments were carried out when the plates were at 70% confluence.

The nitration experiments were carried out using 3-Morpholinosydnonimine hydrochloride SIN-1 (sigma-aldrich) in different concentrations and in a time-dependent manner.

2.1 CELL VIABILITY ASSAY WITH MTT

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. The media was removed from the 6mm cell plates (control and SIN-1 treated MCF-7 cells) and the plates were washed twice with warm (37°C) phosphate buffer saline (PBS). Then, 3mL of MTT (1mg/mL of DMEM media, filtered through 0.2µm) were added and plates were incubated for 1h. The solution was mixed once during incubation time. The MTT solution was aspirated and the plates were washed once with PBS 1x. 2mL of N-propanol were added and the plates were incubated for 30 minutes under mild agitation and protected from light. 1mL of the N-propanol was collected onto a microcentrifuge tube and centrifuged for 2min at 12000*g.* Finally, the centrifuged N-propanol was diluted 1:4 and absorbance was read in a Shimadzu UV-160 spectrophotometer at 560nm.

3. MILK PRODUCTION

Milk production was measured as previously described by Sampson and Jansen with a simple mathematic method that allows estimation of daily milk yield in the well-nourished dam from pup weight and weight gain (Sampson & Jansen, 1984). Briefly, the following equation relating pup milk yield to pup weight and weight gain was used to estimate milk production:

$$\text{yield} = 0.0322 + 0.0667\ (\text{weight}) + 0.877\ (\text{gain})$$

Where yield is daily yield per pup ($g \cdot day^{-1} \cdot pup^{-1}$), weight is pup weight (g), and gain is daily pup weight gain (g/day).

4. TISSUE PREPARATION AND HISTOPATHOLOGY

Inguinal mammary glands from lactation and weaned wild-type and NOS2-KO mice were embedded in paraffin after removal and fixation in 4% (w/v) paraformaldehyde. Tissue sections of 5-7μm were cut and haematoxilin and eosin stained for histological studies.

5. QUANTIFICATION OF SERUM PROLACTIN LEVELS

On day 10 of lactation, blood from WT NOS2-KO (n=3 for each condition) was collected and centrifuged at room temperature at 3500 rpm during 20min. The supernatant (plasma) was collected and kept at -20°C until use. Measurement of prolactin levels in plasma was conducted using an enzyme immune assay (EIA) commercial kit (SPIbio). The EIA is based on the competition for limited specific rabbit anti-prolactin sites between sample prolactin and prolactin labelled with acetylcholinesterase AChE (tracer). Once the sample prolactin and the tracer are bound to the rabbit anti-prolactin antibody, the mixture is placed in a well that contains mouse monoclonal anti-rabbit antibody attached. The plate is then washed and Ellman's Reagent (enzymatic substrate for AChE plus chromogen) is added. The AChE tracer prolactin reacts with the Ellman's reagent and forms a yellow compound. The intensity of colour is determined with spectrophotometry (reading between 405 and 414nm) and it is proportional to the amount of tracer bound to the well and inversely proportional to the sample prolactin present in the well during the immunological incubation.

In this experiment, the plasma collected and kept at -20°C was thawed and centrifuged at 1600*g* and room temperature for 20min to eliminate any fibrin remaining. Following the manufacturer's instructions, the plate was

washed 5 times with the provided specific wash buffer. For prolactin determination a standard curve was used with prolactin concentrations ranging from 0.39 ng/μL to 25 ng/μL. In each well either 50μL of standard or 50μL of sample were pipetted followed by 50μL of prolactin tracer. Finally, 50μL of prolactin antiserum was added to every well and the plate was incubated at room temperature for 20 hours. After incubation, the plate was washed 5 times with the wash buffer and 200μL of Ellman's reagent pipetted to each well and incubated once more in the dark, at room temperature and shaking. The reading was performed using the Multiskan FC plate reader (Thermo Scientific) between 405 and 414nm.

6. COLORIMETRIC AND FLUOROMETRIC DETERMINATION OF NITRITE CONCENTRATION

Nitrite levels where measured with a colorimetric assay kit (Cayman Chemical Company) following the instructions of the manufacturer. Briefly, 100μL of cell culture media from cells treated with different concentrations of SIN-1 were collected and plated in a 96 well plate. 50μL of Griess Reagent R1, followed by 50μL of Griess Reagent R2 were added to each well. The plate was incubated for 10min at room temperature for color-development. Then, absorbance was measured with a Multiskan FC plate reader (Thermo Scientific) at 540nm.

Since this colorimetric method was not sensible enough to measure nitrite concentration *in vivo* on mammary tissue, a fluorimetric method was used. The method is based on the reaction of 2,3-diaminonaphthalene (Sigma-Aldrich) with nitrite under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent product. Samples from control lactating and 24h weaned mice were prepared by homogenizing 0.1g of tissue in 500μL of 50 mM Tris/HCl pH7.5 followed by a centrifugation at 12000*g* for 20min to avoid proteins interfering with the determination. The supernatant obtained was filtered using a 30KDa molecular mass cut-off filter (Microcon® YM-30; Millipore). An aliquot of 10μL of freshly prepared 2,3-diaminonaphthalene

(0.05mg/mL in 0.62M HCl) was added to 100µL of the supernatant obtained in the previous step and mixed immediately. After a 10 minute incubation at 20°C protected from light, the reaction was stopped with 5µL of 2.8M NaOH. Formation of the 2,3-diaminonaphthotriazole was measured using the SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Molecular Devices, Inc. Sunnyvale, CA. USA), with excitation wavelength at 365nm and emission read at 450nm. White opaque 96-well plates (Bibby Sterilin Ltd.) were used for optimal measurement of fluorescence intensity.

7. MOUSE GENOTYPING

In order to determine the mice genotype, tails were cut and incubated over night at 65°C in TESNA buffer (100mM Tris/HCl pH7.5; 10mM EDTA; 2% SDS and 10mM NaCl) together with 0.5mg/mL proteinase K. The product was then transferred onto a new micro-centrifuge tube and 1 volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) was added. The mixture was centrifuged for 15min at 12000*g* at room temperature using a MIKRO 200 centrifuge (Andreas Hettich GmbH & Co.KG; Tuttlingen, Germany). The aqueous phase was transferred into another tube and 500µL of isopropanol where added followed by a second centrifugation at 12000*g* for 15 more minutes. The pellet obtained was washed with 750µL of 80% ethanol and centrifuged again at 12000*g* for 15min. The pellet was finally resuspended in 65µL of preheated (65°C) H_20.

The PCR was carried out as follows:

The previously obtained DNA was diluted 1:10 in H_20. The PCR master-mix was made for a final reaction volume of 30µL: DNA (2µL of the dilution), 1x Buffer PCR (Invitrogen, Carlsbad, USA), 2mM $MgCl_2$, 0.2mM dNTP mixture, 0.8µL of oligonucleotide A for NOS2, 0.4µL of oligonucleotide B for NOS2 and 0.4µL of oligonucleotide D for NOS2, 0.15µL of Taq polymerase (Invitrogen, Carlsbad, USA) and Diethyl Pyrocarbonate (DEPC) water. The use of three oligonucleotides was due to the fact that NOS2-KO

mice did have the gene, but in a truncated form, hence the primer forward for WT and KO was different.

Table 4. Oligonucleotides for NOS2 amplification:

NOS A	ATCAGCCTTTCTCTGTCTCC
NOS B	GGCTTTCTGTCTGTTCTCTC
NOS D-28	GCCTGAAGAACGAGATCAGCAGCCTCTG

The PCR was performed with the Gene Amp PCR system 2700 (Applied Biosystems) and the PCR conditions were as follows: 95°C (1min); [95°C (30s); 63°C (30s); 72°C (30s)] repeat for 40 cycles; 72°C (10min); 4°C(∝).

The amplification product was then loaded onto a 1.5% agarose/TAE (40mM Tris, 20mM Acetic acid, 1mM EDTA) gel with DNA loading buffer (30% glycerol, 0.25% bromophenol blue) and ethidium bromide (Sigma-Aldrich). The reading was performed using an image analyzer system UVIdoc (UVITEC Limited, Cambridge, UK).

8. RNA EXTRACTION

Total RNA from mammary tissue or cell pellets was extracted using TRIzol reagent (Invitrogen Life Technologies). Briefly, 50-100mg of tissue or cell pellets obtained from 6cm diameter plates at 70% confluence at were homogenized at 4°C with 1mL of TRIzol reagent using either a 29G syringe in the case of cells or the Ultra-Turrax® T25 basic (IKA®-Werke GmbH & Co. KG. Germany) in the case of the tissue samples. The mixture was centrifuged at 12000*g* and 4°C during 10min in order to eliminate debris, and the supernatant kept. After a 5min incubation at room temperature, 200μL of chloroform per mL of TRIzol used were added. Incubation for 2-3min on bench top was followed by a new centrifugation a 12000*g* and 4°C for 15min. The aqueous phase was transferred to a new ultracentrifuge tube and 500μL of isopropyl-alcohol per mL of TRIzol were added, incubating for 10min at

room temperature. Then a new centrifugation at 12000*g* and 4°C was performed and the pellet obtained was washed with ethanol 75% (1mL per mL of TRIzol used) and centrifuged, this time at 7500*g* for 5min. The ethanol was eliminated and the RNA isolated resuspended in 200µL of Ultrapure RNAse free water. After this isolation, an additional column purification (RNeasy, Qiagen) was performed following the intructions of the manufacturer. RNA quantity and purity were determined using the NanoDrop ND-2000 (NanoDrop Technologies), and RNA integrity was assessed by determining the RNA 28S/18S ratio using RNA 6000 Nano Labchips in an Agilent 2100 Bioanalyzer (Agilent Technologies).

9. REAL-TIME RT-PCR

RNA (500ng) was reverse-transcribed into cDNA at 25ºC for 10min and 37ºC for 2h using a high capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA products were amplified by qPCR using the GeneAmp Fast PCR Master Mix (Applied Biosystems). All reactions were carried out in triplicate. Quantitative real-time PCR was run in the 7900HT Fast Real-Time PCR System. Pre-developed Taqman primers were purchased from Applied Biosystems (see table 5 below).

Table 5. Summary of the Taqman probes purchased from Applied Biosystems.

GENE NAME	TaqMan PROBE
Human 18s rRNA	4319413E
Mouse NOS2	Mm01309902_m1
Mouse Whey Acidic Protein (wap)	Mm00839913_m1
Mouse Bcl-3	Mm00504306_m1
Mouse Metallo-Protease 2 (mmp-2)	Mm00439506_m1
Mouse Metallo-Protease 9 (mmp-9)	Mm00442991_m1
Mouse beta casein (Csnb)	Mm04207885_m1

Mouse Leukemia inhibitory factor (lif)	Mm00434762_g1
Mouse Cathepsin D (ctsd)	Mm00515586_m1
Rat Cathepsin D (ctsd)	Rn00592528_m1
Human Metallo-Protease 9 (MMP-9)	Hs00234579_m1

Results were normalized according to 18s rRNA quantification in the same sample reaction. The threshold cycle (C_T) was determined, and then the relative gene expression was expressed as follows:

Fold change (relative amount) = $2^{-\Delta(\Delta Ct)}$

Where ΔC_t = C_t target – C_t housekeeping, and $\Delta(\Delta C_t)$ = ΔC_t treated – ΔC_t control

10. RT-PCR ARRAY FOR NF-κB SIGNALLING PATHWAY

The effect of weaning on the expression of 84 genes associated with NF-κB-mediated signal transduction were examined using mouse NF-κB signalling pathway RT^2 Profiler PCR array (SuperArray Bioscience) according to the manufacturer's instructions. In brief, 1µg of total RNA of control and 48 hour weaned mammary glands from WT and *NOS2*-KO mice was converted into cDNA using the RT^2 First Strand Kit (SABiosciences). This cDNA was then added to the RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences). Next, each sample was added to Mouse NF-κB Signalling Pathway PCR Arrays (catalogue number PAMM-025). All steps were carried out according to the manufacturer's protocol for the 7900 HT Fast Real-Time PCR System. PCR amplification was conducted with an initial 10 minutes step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1min. The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold (C_t). Data normalization was based on correcting all C_t values for the average C_t values of several constantly expressed housekeeping genes present on the array. The relative gene expression was expressed as follows: Fold change (relative amount) =

$2^{-\Delta(\Delta Ct)}$

Where ΔC_t = C_t target – C_t housekeeping, and $\Delta(\Delta C_t)$ = ΔC_t treated – ΔC_t control

11. ChIP (CHROMATIN IMMUNOPRECIPITATION) ASSAY

Chromatin from mammary gland extracts was fixed and immunoprecipitated as previously described (Borras et al, 2003). Briefly, mammary tissue samples were excised and immersed in PBS, pH7.4, and 1% (v/v) formaldehyde for 10min to cross-link the chromatin, and the reaction was stopped by adding glycine to a final concentration of 0.125M. After centrifugation at 1500*g* for 5min, the cell pellet was resuspended in cell lysis buffer (85mM KCl, 0.5% Nonidet P40, 5mM Hepes, pH8.0) supplemented with a protease inhibitor cocktail (Sigma), incubated on ice for 15 min and centrifuged at 3500*g* for 5min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10mM EDTA, 1%SDS, 50mMTris/HCl, pH8.1) at a ratio of 1:1 (v/w) relative to the initial tissue weight, incubated on ice for 10min, aliquoted in 1mL fractions and stored at −80 ºC until use for ChIP assay. Cross-linked chromatin (1mL of each sample) was sonicated on ice with 15 pulses of 10s at 38% amplitude with 50s stops, in a Vibra-Cell VCX-500 sonicator. The sonified chromatin was centrifuged at 14000*g* for 10min and the supernatants, containing soluble chromatin fragments were kept. Chromatin concentration was determined diluting 5µL of the chromatin samples 1:200 (v/v) in 1% SDS. The measurement was performed in quartz cuvets using the UV-160 UV-visible recording spectrophotometer (Shimadzu, Minnesota, USA) at 260nm.
The units of chromatin were calculated using the formula:

$$Absorbance_{260} \times 1000/5 = Units\ of\ chromatin/mL$$

In order to determine if sonicated chromatin was properly size-fractioned, 4.5U of the sonified chromatin were diluted in H_2O up to a final volume of 100µL, treated with RNase (Sigma-Aldrich) and incubated for

30min at room temperature. 0.4µg/µL Proteinase K (Roche) and 0.01% SDS were added followed by over night incubation at room temperature. The chromatin was then purified using the PCR purification kit QIAquick® DNA (QIAGEN, Hilden, Germany) following the instructions by the manufacturer. The purified DNA was stained with ethidium bromide and then loaded onto a 1.5% agarose/TAE (40mM Tris, 20mM Acetic acid, 1mM EDTA) gel with DNA loading buffer (30% glycerol, 0.25% bromophenol blue). The reading was performed using an image analyzer system UVIdoc (UVITEC Limited, Cambridge, UK). The average size of the chromatin fragments obtained was ~500bp.

The chromatin fragments were diluted 10-fold in dilution buffer (165mM NaCl, 0.01%SDS, 1.1%Triton X-100, 1.2mM EDTA, 16.7mM Tris/HCl, pH8.0) supplemented with protease inhibitor cocktail. The diluted chromatin fractions were precleared by adding 30µL/mL Protein A/G–agarose (Amersham Biosciences; previously blocked for 1h with 500*µ*g/mL tRNA and 1mg/mL BSA) and incubated for 4h at 4°C on a rotating plate. The suspensions were then centrifuged at 14000*g* for 30s to remove non-specifically bound chromatin fragments. Aliquots from the supernatant (equivalent to 50µg of DNA) were taken, incubated with 2µg of specific antibodies against the NF-κB p65 and p50 subunits (both from Santa Cruz Biotechnology), and left overnight at 4°C under rotation. The samples were then incubated with 50µL of blocked ProteinA/G–agarose under rotation for an additional period of 4h. The immunocomplex was recovered by centrifugation at 14000*g* for 30s and washed twice with low-salt buffer (150mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1mM EDTA, 50mM Tris–HCl), twice with high-salt buffer (500mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1mM EDTA, 50mM Tris–HCl), twice with LiCl buffer (250mM LiCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1mM EDTA, 50mM Tris–HCl) and finally twice with TE buffer (0.25mM EDTA, 10mM Tris–HCl). (Sandoval et al, 2004).

An aliquot of the cross-linked chromatin was treated as above, but in the presence of an unrelated antibody (IgG); the first supernatant, after preclearing with ProteinA/G–agarose, was saved as the Input fraction. The immunoselected chromatin was eluted from the ProteinA/G–agarose in two consecutive steps by adding 50µL of elution buffer (1%SDS, 100mM $NaHSO_3$) each time, with 30s of vigorous vortexing. The two supernatants were combined (IP (immunoprecipitated) fraction) and incubated at 65°C overnight to reverse formaldehyde cross-links. The DNA from all samples was purified with a PCR purification kit QIAquick® DNA (QIAGEN, Hilden, Germany) and used for PCR analysis of the target genes.
After DNA purification, input, immunoprecipitated, and unrelated antibody fractions were analyzed by PCR with the appropriate primer pairs to amplify the promoter of the target gene MMP-9 (Primer forward for MMP-9 promoter region: 5'- GTGAACACGGTGGCTGAAA-3'. Primer reverse for MMP-9 promoter region: 5'-CAGGCTCTTTGAAGCAGGATT-3').

The PCR was performed with the NETZYME® DNA Polymerase using the Gene Amp PCR system 2700 (Applied Biosystems) and the PCR conditions were as follows:

93°C (2min); [93°C (30s); 58°C (30s); 72°C (30s)] repeat 37 cycles; 72°C (10min); 4°C(∝).

The amplification product was then loaded onto a 1.5% agarose/TAE (40mM Tris, 20mM Acetic acid, 1mM EDTA) gel with DNA loading buffer (30% glycerol, 0.25% bromophenol blue) and ethidium bromide (Sigma-Aldrich). The reading was performed using an image analyzer system UVIdoc (UVITEC Limited, Cambridge, UK).
PCR fragments were size fractionated by 1.5% agarose/TAE gel electrophoresis and stained with ethidium bromide. The reading was performed using an image analyzer system UVIdoc (UVITEC Limited, Cambridge, UK).

12. PROTEIN EXTRACTION

Cell pellets collected from 6cm diameter plates at 70% confluence or freeze-clamped tissue (0.1g) were homogenized in 1mL of RIPA buffer (1.8mM NaH_2PO_4, 8.4mM Na_2HPO_4, 0.1 % (w/v) SDS, 1.0% (v/v) TritonX 100, 0.1M NaCl, 0.5% sodium deoxycholate, 1mM PMFS) supplemented with 2µL/mL of protease inhibitor cocktail and 5µL/mL of phosphatase inhibitor cocktail (both from Sigma) at 4ºC using a 29G syringe in the case of cells and an Ultra-Turrax® T25 basic (IKA®-Werke GmbH & Co. KG. Germany) for tissue. After homogenization, the sample was incubated for 1h at 4ºC in a rotating device followed by centrifugation at 4°C and maximum speed (14000*g*) for 30min.

The supernatant containing the total protein extract was collected and normalized for protein concentration using a Bradford-based assay (Bio-Rad protein assay) following instructions from the manufacturer. This assay involves the addition of an acidic dye (Coomassie® Brilliant Blue G-250 dye) to protein solution, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. Comparison to a standard curve provides a relative measurement of protein concentration. The absorbance maximum for the acidic solution shifts from 465nm to 595nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine, and a differential color change of the dye occurs in response to various concentrations of protein. The BSA protein standard was prepared with a range of 0.05µg/µL to 0.5µg/µL dilutions. 2µL of each standard and sample was pipetted into separate microtiter plate wells and 200µl of diluted dye reagent was added to each well. The sample and reagent were mixed using a microplate mixer followed by 5min incubation at room temperature. Absorbance was measured with a Multiskan FC plate reader (Thermo Scientific) at 595 nm.

13. WESTERN BLOTTING

Same amounts of protein (15µg) were denatured with Laemli buffer 5x (0.3M Tris/HCl pH6.8, 50% glycerol, 10% SDS, 0.05% bromophenol blue, 25% β-mercaptoethanol) at 95°C for 5min and loaded in SDS-PAGE gels for electrophoresis (concentration of bis-tris acrylamide varied from 8% to 15% depending on the molecular weight of the protein to be determined. After electrophoresis (electrophoresis buffer 0.25M Tris/HCl pH8.6, 1.92M glycine and 1.0% SDS), the proteins were eletroblotted onto a nitrocellulose membrane (Protran®, Whatman, Kent, UK) in transfer buffer (0.25M Tris pH8.6, 1.92 M glycine and 20% methanol). The membranes with the transferred proteins were blocked for one hour with 5% BSA in TBS-T (0.25M TrisHCl pH 7.5, 0.15M NaCl, 0.1% (v/v) Tween 20) followed by addition of a primary antibody. For all antibodies, the membranes were incubated over-night at 4°C with shaking; following four washes with TBS-T.

The blots where then incubated with the secondary antibody conjugated to horseradish peroxidase for 60min at room temperature. Finally, the blots where washed with TBS-T and detection was carried out using the chemiluminescent luminol reagent (Amersham™ ECL™ Western Blotting Detection Reagent. GE Healthcare UK Limited). The processor Curix60 (AGFA) was used for film development. The intensity of the bands was measured by densitometry using Image J, a public domain Java image processing program. Equal loading was confirmed by reprobing the blot against either α-tubulin or β-actin and by Ponceau staining. The antibodies and their source, as well as the concentration used for blotting are specified in the table below.

Table 6. List of primary and secondary antibodies for western blotting.

Protein	ORIGIN	DILUTION	
β-actin	Rabbit monoclonal	1:2500	Abcam (ab8227)
α-tubulin	Mouse monclonal	1:1000	SantaCruz Biotechnology (sc-5286)
ER-alpha	Mouse monoclonal	1:1000	SantaCruz Biotechnology (sc-73479)
Cathepsin D	Rabbit polyclonal	1:1000	SantaCruz Biotechnology (sc-10725)
Cathepsin D	Rat monoclonal	1:1000	R&D Systems clone 204712 (MAB1029)
Cathepsin D	Mouse monoclonal	1:1000	Calbiochem clone BC011 (IM03)
Anti-nitrotyrosine	Mouse monoclonal	1:1000	Upstate biotechnology clone 1A6 (#05-233)
STAT3	Rabbit polyclonal	1:1000	Upstate Biotechnology (#06-596)
p-STAT3 (Tyr 705)	Rabbit polyclonal	1:1000	Cell Signalling Technology (#9131)
STAT-5	Rabbit polyclonal	1:1000	SantaCruz Biotechnology (sc-1081)
p-STAT5 (Tyr694)	Rabbit monoclonal	1:1000	Cell Signalling Technology (#9314)
AKT	Rabbit polyclonal	1:1000	Cell Signalling (#9272)
p-AKT (Ser 473) (D9E)	Mouse monoclonal	1:1000	Cell Signalling (#4060)
IκB-β	Rabbit polyclonal	1:1000	SantaCruz Biotechnology (sc-945)
IκB-α	Rabbit polyclonal	1:1000	SantaCruz Biotechnology (sc-371)
Anti-Rabbit Immunoglobulins/ HRP	Polyclonal Goat	1:5000	DAKO Cytomation P0448
Anti-Mouse Immunoglobulins/ HRP	Polyclonal Goat	1:5000	DAKO Cytomation P0447

14. TWO-DIMENSIONAL ELECTROPHORESIS

Mammary gland tissue from lactating controls and 72h weaned rats was homogenized in rehydration buffer from Bio-Rad Laboratories (8M urea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), 50mM dithiothreitol (DTT), 0.2% Bio-Lyte® 3/10 ampholyte and 0.001% Bromophenol Blue). After cleaning up the samples with a ReadyPrep 2-D

Cleanup kit (Bio-Rad Laboratories), 500µg of tissue lysates were loaded on to precast immobilized pH gradient strips (IPG strips; pH5–8; Bio-Rad Laboratories) and subjected to isoelectric focusing (IEF) two-dimensional PAGE. The IEF conditions were those recommended by the manufacturer for the type of strip used. Second-dimension gels contained 8% acrylamide. Simultaneously, proteins were resolved by two-dimensional PAGE and then electrophoretically transferred on to nitrocellulose membranes (Protran®) or visualized using silver or Coomassie Blue staining. Membranes were probed with a 1:1000 dilution of anti-nitrotyrosine antibody as described above. Spots corresponding to nitrated proteins were excised manually from Coomassie Blue-stained gels and identified by MS.

15. PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

Protein preparation and tryptic digestion:

Protein spots corresponding to weaned mammary gland lysates recovered from two-dimensional gels were precipitated with 20% trichloroacetic acid (TCA) on ice and, after centrifugation at 20000*g* for 30min, the pellet was washed with cold acetone. The same procedure was performed in the case of human cathepsin D (25µg) treated with 20µM $ONOO^-$ or with 1.2M NaOH, in order to identify the nitration site of the enzyme. Precipitated proteins were resuspended in 25mM ammonium bicarbonate and digested with 12.5ng/µl trypsin for 12h at 37 °C.

LC (liquid chromatography)–ESI (electrospray ionization)-MS/MS (tandem MS) analysis:

MS/MS analysis was performed as previously described (Munoz et al, 2008). Microcapillary reversed-phase LC was performed with a CapLC™ (Waters) capillary system. Reversed-phase separation of tryptic digests was performed with an Atlantis, C_{18}, 3µm, 75µm×10cm Nano Ease™ fused silica capillary column (Waters) equilibrated in 5% acetonitrile and 0.2% formic acid. After injection of 6µL of sample, the column was washed for 5 min with the same buffer and the peptides were eluted using a linear gradient of 5–50% acetonitrile in 45min at a constant flow rate of 0.2µL/min. The column was

coupled online to a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80°C and the spray voltage was 1.8–2.2kV. MS/MS data were collected in an automated data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by CID (collision-induced dissociation) using an isolation width of 2.0 and a relative collision energy of 35V. Data processing was performed with MassLynx 4.1. Database searching was carried out with ProteinLynx Global Server 2.1 (Waters) and Phenyx 2.2 (GeneBio) against Uniprot knowledgebase release 12.3 consisting of UniprotKB/Swiss-Prot release 54.3 and UniprotKB/TrEMBL release 37.3 with 285335 and 4932421 entries respectively. The search was enzymatically constrained for trypsin and allowed for one missed cleavage site. Data were then manually inspected and the results were only accepted when the molecular mass and pI of the identified protein were coincident with the electrophoretic mobility of the corresponding spot in two dimensions.

16. IMMUNOPRECIPITATION

16.1 IMMUNOPRECIPITATION OF CATHEPSIN D USING SEPHAROSE BEADS

Protein A–Sepharose and Protein G–Sepharose beads(GE Healthcare UK Limited) (50:50 v/v) were blocked with bovine serum albumin 1µg/µL for 4h. 1mg of proteins obtained from tissue lysates from lactating and weaned mammary glands were precleared with the blocked sepharose beads during 1h at 4°C. The samples with the beads were centrifuged at 12000*g* for 5min. The supernatant was incubated first with either cathepsin D antibody from Santa Cruz Biotechnology or with an unrelated antibody also from Santa Cruz Biotechnology overnight at 4°C, and then with Protein A–Sepharose and Protein G–Sepharose (50:50v/v) beads for 4h at 4°C on a rotating device. Pellets were collected by centrifugation at 14000*g* for 1min at 4°C. The supernatants from the samples incubated with the unrelated antibody were taken to be used as INPUT, and each pellet was subsequently washed three times with 50mM Tris/HCl pH8.0. After washing the sepharose beads, the

immunocomplexes were eluted by boiling in the Laemli buffer for 5min and then subjected to SDS/PAGE immunoblotting as described previously.

16.2 PEPSTATIN A AFFINITY PURIFICATION OF CATHEPSIN D

Homogenates from weaned mammary gland (500µg of whole extract proteins) were incubated for 15h at 4°C with pepstatin A–agarose-coated beads (Sigma) previously washed with in 20mM citrate phosphate buffer pH7.0. Then the mixture was centrifuged at 20000*g* for 5min and the pellet was washed with the citrate phosphate buffer. Finally, the pellet was resuspended and denatured in 30µL of Laemli buffer prior to Western blot analysis.

17. *IN VITRO* PROTEIN NITRATION

Peroxynitrite ($ONOO^-$) was purchased from Calbiochem (La Jolla, USA), diluted in 1.2M NaOH and stored in an oxygen-free atmosphere at −80°C until use. Prior to experimentation, the concentration of $ONOO^-$ was measured by the increase in molar extinction coefficient (ε) at 302nm (ε_{302} $1.670 M^{-1} \cdot cm^{-1}$) in 1.2M NaOH (Radi et al, 1991). Recombinant cathepsin D (20µg) from human liver (Sigma) or 50µg of protein from control lactating mammary gland homogenates were incubated with 0–500µM $ONOO^-$ in assay buffer (pH3.5; from the cathepsin D assay kit). The incubation was carried out in the presence or absence of 30µM epicatechin (Sigma-Aldrich, Steinheim, Germany) and in the presence and absence of pepstatin A (Sigma). Epicatechin is used as a nitration inhibitor due to its action as a potent scavenger of $ONOO^-$. Pepstatin A is a specific inhibitor of cathepsin D. The reaction was performed by placing a small aliquot of $ONOO^-$ into a test tube containing the sample, immediately followed by a 20min incubation. In control test tubes, the samples were treated with equal volumes of 1.2M NaOH (vehicle). Aliquots were taken for Western blot analysis and determination of cathepsin D enzymatic activity.

As for the *in vitro* protein nitration in cell culture, 3-Morpholinosydnonimine hydrochloride (SIN-1. Sigma-Aldrich, Steinheim, Germany) was used. It was resuspended in DMEM up to a concentration of 100mM. MCF-7 cells grown in 6cm diameter plates at 70% confluence, were treated with different concentrations of SIN-1 in a time dependent manner. The final concentrations of SIN-1 were 1mM, 2mM, 3mM for 24h, to determine the appropriate SIN-1 concentration. Once the appropriate concentration of SIN-1 was determined, incubation times were reduced to 5, 10, 30min, 1 and 4h in order to determine early biological activity.

18. CATHEPSIN D ENZYMATIC ASSAY

Cathepsin D protease activity was measured using the commercially available cathepsin D assay kit (Sigma-Aldrich) following the method of Yasuda et al (Yasuda et al, 1999). Briefly, 5µg of protein obtained from mammary gland tissue of lactating rats and mice, at different times of weaning, were added to the assay buffer (pH3.5), followed by the addition of the quenched fluorimetric substrate 7-methoxycoumarin-4-acetic acid at a final concentration of 20 µM. The fluorescence released by the action of the enzyme was measured at 37°C in the SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Molecular Devices, Inc. Sunnyvale, CA. USA) during 60min of incubation in a kinetic reaction. To prove that the substrate was hydrolysed by cathepsin D only, the specific inhibitor pepstatin A (Sigma) was added to parallel samples at a final concentration of 0.2mg/mL to inhibit the cathepsin D activity. Samples (at least n=3) were assayed in triplicate. The assay was also performed for 2.4µg/mL human cathepsin D (Sigma-Aldrich) treated with $ONOO^-$ to induce tyrosine nitration, in order to determine whether this post-translational modification could affect the protease activity.

19. NUCLEAR EXTRACT ISOLATION

Isolation of nuclei from mouse mammary gland (WT and NOS2-KO) and different weaning conditions was carried out with the Nuclear Extract Kit from Active Motif (Belgium) following instructions by manufacturer. Briefly, 0,3g of

mammary gland tissue was treated with 750µL of hypotonic buffer supplemented with 1µL/mL DTT 1M, 1µL/ml detergent and 50µL/mL of phosphatase inhibitor (Sigma) and homogenized in a pre-chilled loose Dounce homogenizer. After incubation on ice for 15min the samples were centrifuged for 10min at 850*g* at 4ºC. The pellet was re-suspended in 500µL of 1x hypotonic buffer (supplemented with 1mM DTT, 10µL/mL protease and 50µL/mL phosphatase inhibitors from Sigma) and incubated on ice for 30 minutes with gentle shaking. The samples were then centrifuged at 14000*g* for 10min at 4ºC.The supernatant fraction (the nuclear protein extract) was stored at -80ºC or used immediately.

20. DETERMINATION STAT3 AND NF-KB NUCLEAR BINDING ACTIVITY

After obtaining the nuclear extract, the determination of NF-κB and STATs DNA-binding activity was carried out using TransAM™ STAT family or NF-κB p65 chemi kits both from Active Motif, also following the instructions from manufacturer. The protocol is based on ELISA-assay to detect and quantify transcription factor activation. The assay is performed on a 96-well plate on which an oligonucleotide containing an NF-KB or STAT3 consensus site has been immobilized. The active form of these transcription factors contained in the nuclear extract will bind specifically to the oligonucleotide after 1h incubation at room temperature. After three washes with the specific buffer provided, 50µL of NF-κB or STAT3 primary antibody; that detects the activated transcription factor bound to its target (dilution 1:1000 in antibody biding buffer); is added and the plate incubated for 1h. Then, a series of washes follows and a Horse Radish Peroxidase (HRP)-conjugated secondary antibody is added, providing a sensitive chemiluminiscent read out that can be quantified. Assays were performed using 5µg of nuclear extracts, and to check specificity, incubation was carried out in absence or presence of 20pmol of competitor oligonucleotide that contains either a WT or mutated STAT3 or NF-κB consensus binding site. The WT oligonucleotide prevents the NF-κB or STAT3 binding to the probe immobilized on the plate, acting as a negative control, while the mutated consensus oligonucleotide has no effect

on the transcription factor binding. The plate was read using a microplate luminometer (SPECTRAmax Plus 384, Molecular Devices).

21. MEASUREMENT OF CASPASE 3 ACTIVITY

Caspase 3 activity was measured in mammary tissue lysates using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision). Mammary gland was homogenized in lysis buffer, incubated on ice for 10min and centrifuged at 10000*g* for 15min. Supernatants from tissue homogenates were used to measure caspase 3 activity following the manufacturer's instructions. First, total protein concentration was measured and 200µg of protein were diluted in 50µL of Cell Lysis Buffer provided in the assay. 50µL of 2X Reaction Buffer (containing 10mM of DTT) were added to each well, followed by the addition of 5µl of the DEVD-pNa substrate (200µM final concentration). This mixture was incubated at 37°C for 1 hour before reading the plate with a Multiskan FC plate reader (Thermo Scientific) at 405nm.

22. GELATINE ZYMOGRAPHY (MMP9 AND MMP2 ACTIVITY)

Protein lysates (150µg) were electrophoresed on SDS/PAGE 10% mini-gels containing 1mg/mL gelatin (Bio-Rad Laboratories) using a non-reducing SDS sample buffer (62.5mM Tris/HCl, pH6.8, 25% glycerol, 4% SDS and 0.001% Bromophenol Blue). After electrophoresis, the gels were washed for 30min at room temperature (25°C) in re-naturing buffer (10mM Tris/HCl, pH7.5, and 2.5% Triton X-100) followed by 36 hour incubation in enzyme reaction buffer (50mM Tris/HCl, pH7.6, 200mM NaCl, 5mM $CaCl_2$ and 0.02% Brij-35) for 36h at 37°C. Gels were stained with Coomassie Brilliant Blue R-250 and gelatinolytic activities were detected after destaining by clear bands, indicating lysis of the substrate.

23. STATISTICAL ANALYSIS

Results are reported as means± S.E.M. The control and weaned rat data, as well as WT and NOS2-KO mice experiments data within the same

genotype were analysed by a one-way ANOVA. The homogeneity of the variances was analyzed by the Levene test; in those cases where the variances were unequal, the data were adequately transformed before ANOVA. Significant differences were determined by a Tukey-Kramer test. The letter ‘a’ always represents the lowest value within the group. Differences were considered significant at $p<0.05$.

A two-tailed Student’s *t* test with the Bonferroni correction was used for comparison of two groups. Differences were also considered significant at * $p<0.05$. Independent studies were conducted with a minimum of three replicates per condition to allow for statistical comparison.

RESULTS

RESULTS

1. GLOBAL ROLE OF NITRIC OXIDE DURING INVOLUTION OF MAMMARY GLAND AFTER WEANING.CHARACTERIZATION OF MAMMARY GLAND INVOLUTION IN NOS2-KO MICE

1.1 MOUSE GENOTYPING

Wild-type and NOS2 knock-out mice were purchased from Taconic with a C57BL/6 background. The genotype of the NOS2-KO strain was verified by PCR as explained in the Materials and Methods section. Figure 12 shows the PCR amplification product for both WT and NOS2-KO mice. It can be observed that there was an amplification product for WT and KO, but for NOS2-KO the product was smaller, around 300 base pairs (bp) while the WT showed a ≈ 400bp product. This is due to the fact that the NOS2-KO mice did have the NOS2 gene, but in a truncated form, and hence it resulted in a truncated non-functional protein.

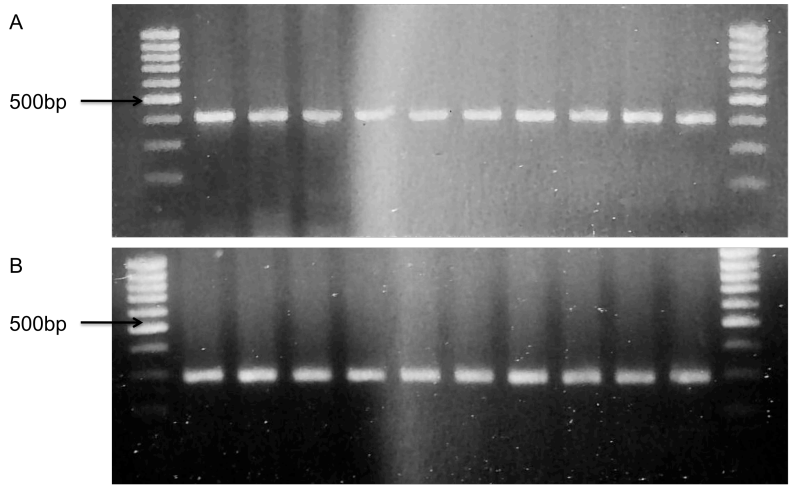

Figure 12. Genotyping of WT and NOS2-KO mice. Agarose gel electrophoresis showing the amplification product for NOS2 gene (410 bp) in the WT mice (A) or NOS2-KO animals (≈300 bp) (B).

1.2 NITRITE DETERMINATION IN THE WEANED MAMMARY GLAND

Our group had previously established that NOS2 expression is induced in weaned rats as early as 8h after litter removal (Zaragoza et al, 2005). NOS2 has been implicated in many pro-inflammatory processes, inducing higher and more steady levels of NO than those obtained with NOS1 and NOS3. This, added to the previous works that define mammary gland involution after weaning as a pro-inflammatory model, gave rise to question exactly which role did NO exert in this setting. To answer this question we used a NOS2-KO mouse model since, if during mammary gland involution the major source of NO is NOS2, the lack of this enzyme would result in lower NO levels during the physiological process of involution. In this sense, the first step to take was to confirm the increase in NO levels in the involuting mammary gland compared to lactation, and if in fact, NOS2-KO had lower levels of NO after weaning.

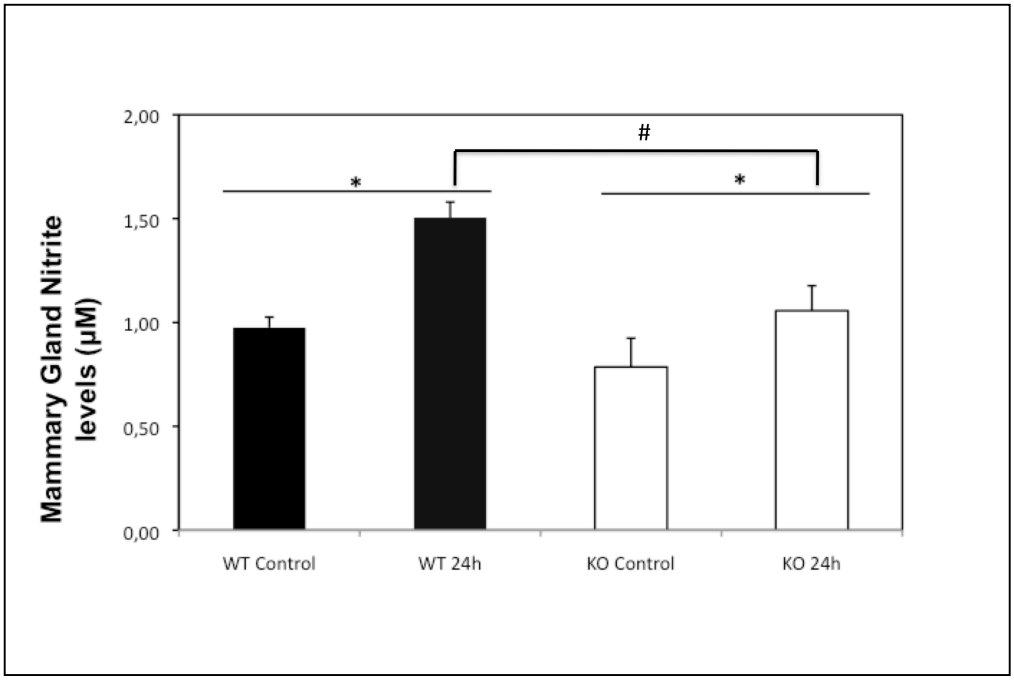

Figure 13. Nitrite levels measured in control and weaned mammary glands of WT and NOS2-KO mice. Results are displayed as means ± S.E.M. * and # indicate statistically significant differences ($p<0.05$).

Nitrite levels as an end-product of NO were measured in the mammary gland of control lactating and 24h weaned mice both in WT and NOS2-KO strains. It can be observed in figure 13 that NO production was increased significantly in the involuting mammary gland after 24h of pup removal compared with control lactating glands in both WT (0.98±0.02 and 1.5±0.03μM respectively; n=5) and NOS2-KO (0.79±0.06 and 1.06±0.05μM respectively; n=5) mice. However, nitrite levels in NOS2-KO mice were statistically lower than in WT littermates either in control lactating glands or in weaned glands (#).

In order to explain the increase in nitrite levels in the mammary gland of NOS2-KO mice, endothelial NOS (eNOS/NOS3) levels was determined by western-blotting in mammary gland extracts in NOS2-KO at the peak of lactation and at different times during weaning. Figure 14 demonstrates that, after an initial decrease in the early times of weaning, NOS3 levels increase from 24h onwards. This augmentation in NOS3 levels during weaning could explain the increase in nitrite levels observed in NOS2-KO mice. In fact, other works have already demonstrated that both NOS3 and NOS1 expression is increased during mammary gland involution after weaning (Islam et al, 2009).

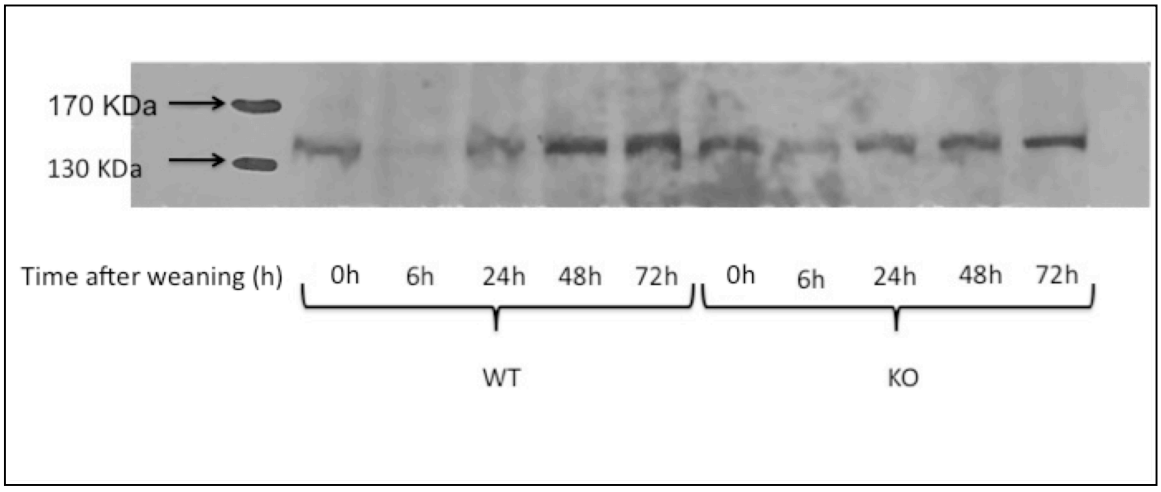

Figure 14. Western Blot to determine the expression of NOS3 in WT and NOS2-KO mice at the peak of lactation (0h) and during weaning (6h, 24h, 48h and 72h).

1.3 HISTOLOGICAL CHANGES IN THE MAMMARY GLAND

The mammary gland undergoes a series of changes during involution that have two clear stages, a reversible phase that lasts up to 48h and an irreversible phase that will remodel the structure of the mammary gland into a pre-pregnant like state. Since it was clear that NO levels (inferred by nitrite levels) were lower in the NOS2-KO mice, this model is optimal to study the possible role of NO in both stages of involution. The first approach was to determine if there were any structural differences between NOS2-KO and WT mice by means of histological studies. The inguinal mammary glands of these mice were blind-analyzed by an experienced pathologist at day 10 of lactation, and at 6, 24, 48 and 72h of involution. Histological analysis of tissue sections from WT mice (figures 15 and 16 panels I, iii, v, vii, and ix) showed a loss of epithelial cells that were shed into the alveolar lumen with concomitant loss of the glandular structure together with alveoli collapse and reappearance of adipose tissue from 24h of involution onward.

When comparing KO with WT mice, it was observed that on day 10 of lactation, NOS2-KO mice showed no significant differences in mammary gland development, and, as in the WT, they showed prominent secretory alveoli (Figure 15 and 16 panels i and ii). However, histological analysis of NOS2-KO mice during involution revealed a clear delay in mammary gland regression. After 24h weaning (figures 15 and 16 panel vi), KO mice showed the same condensed lobulo-alveolar structures that WT mice presented earlier, at 6h (figures 16 and 17 panel iii). Further on, at 48 h weaning, the mammary gland from WT mice showed and evident regression that was not present at the same time point in KO mice (panels vii and viii). In fact, the pattern of involution that the KO mice presented at 48h was similar to that of the 24h weaned WT mice (figures 15 and 16 panels v and viii). Surprisingly, at 72h weaning, both WT and KO mice presented similar levels of regression, hence it could be concluded that lack of NOS2 delayed but did not impair mammary gland involution after weaning. In fact, panels ix and x show a similar image, with adipocytes already differentiated and replacing the mammary gland alveolar structures in both WT and NOS2-KO mice.

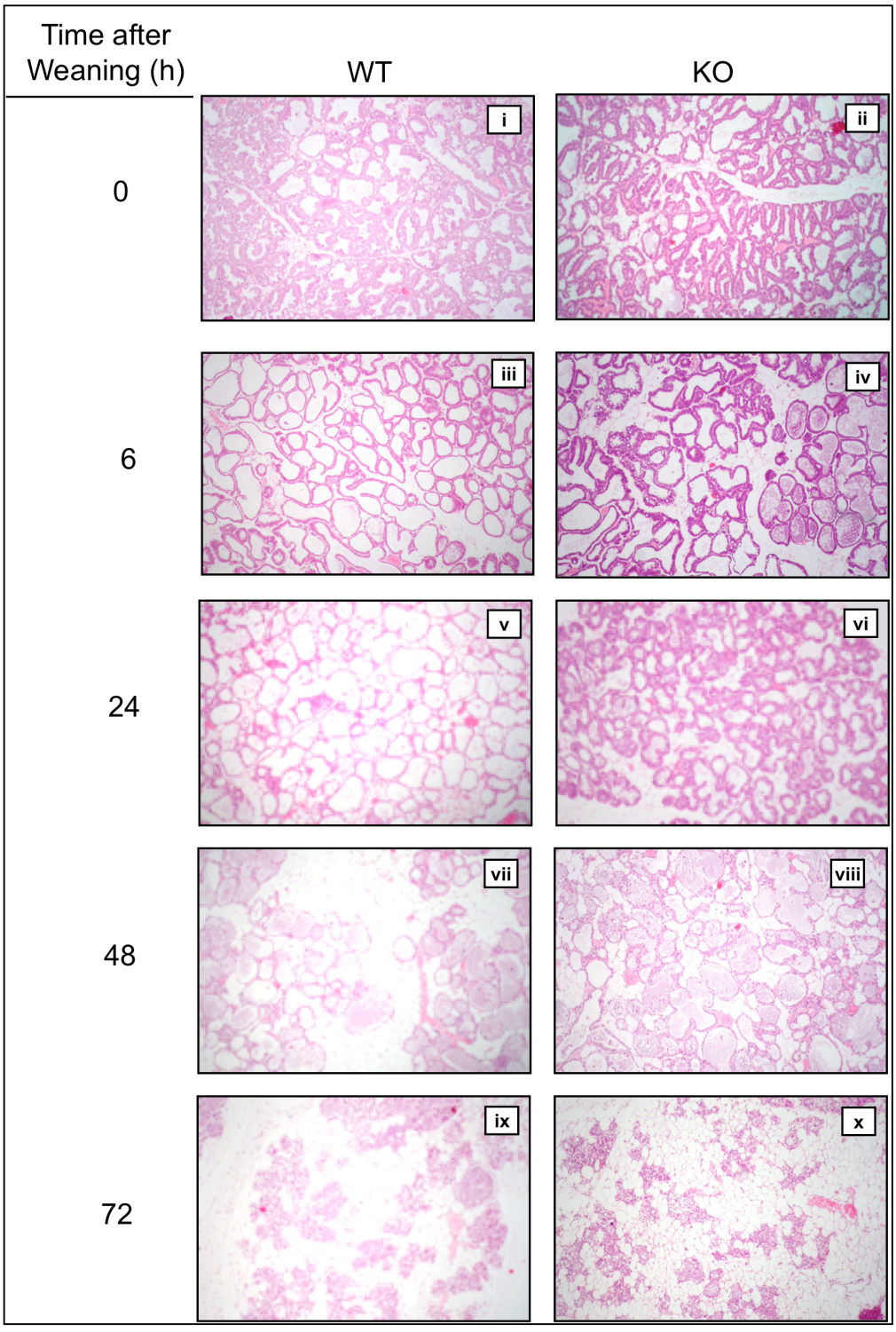

Figure 15. Histological study of mammary gland in WT and NOS2-KO mice. Haematoxylin and eosin-stained sections showing mammary gland morphology from WT (panels i, iii, v, vii and ix) and NOS2-KO (panels ii, iv, vi, viii and x) mice at the peak of lactation (panels i and ii) and 6h (panels iii and iv), 24h (panels v, vi), 48h (panels vii, viii) and 72h (panels ix and x) after induced involution as described in the methods section. Magnification 10X.

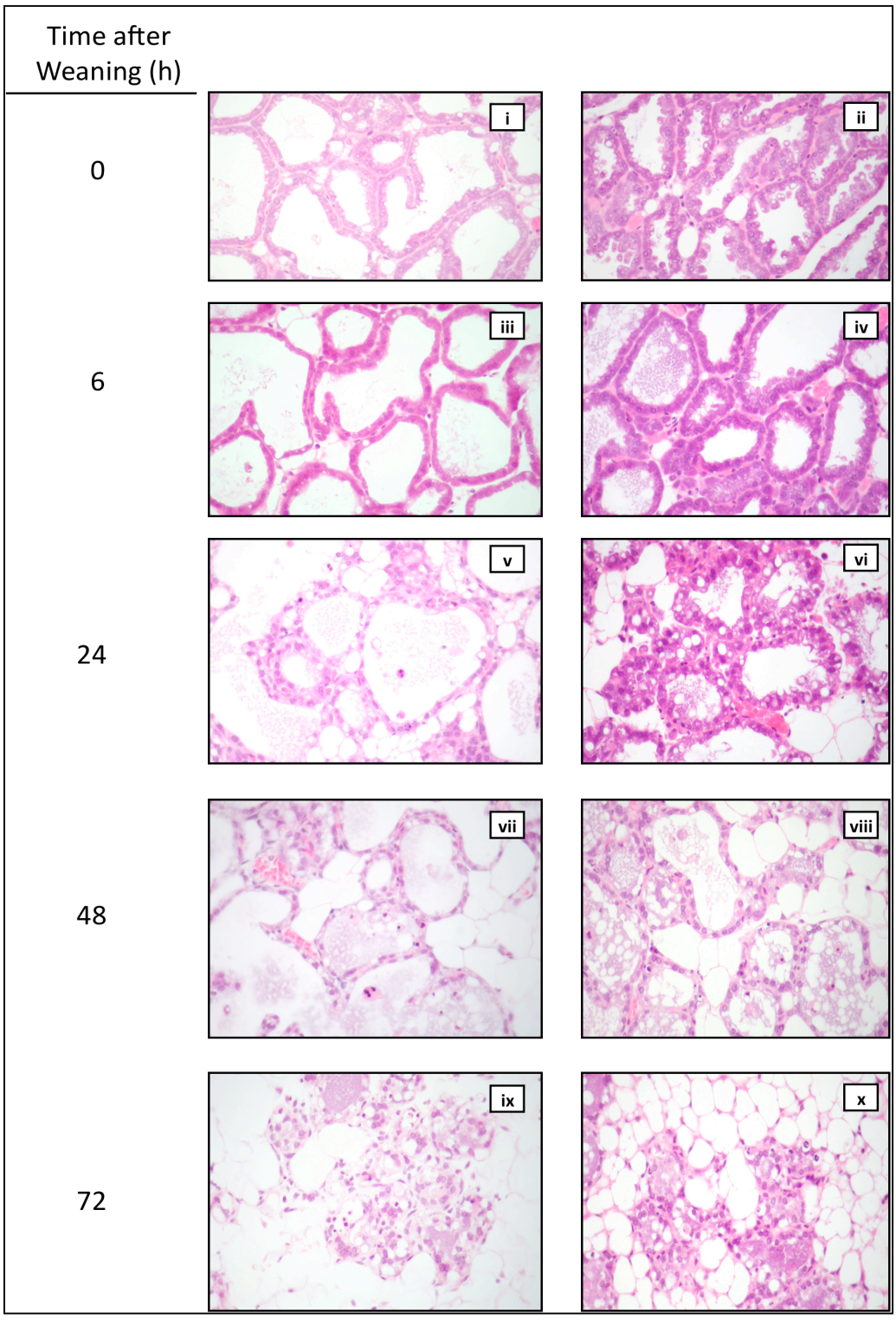

Figure 16. Histological study of mammary gland in WT and NOS2-KO mice. Haematoxylin and eosin-stained sections of mammary gland tissue from WT (panels i, iii, v, vii and ix) and NOS2-KO (panels ii, iv, vi, viii and x) mice at the peak of lactation (panels i and ii) and 6h (panels iii and iv), 24h (panels v, vi), 48h (panels vii, viii) and 72h (panels ix and x) after induced involution as described in the methods section. Magnification 40X.

1.4 RATIO MAMMARY GLAND/MOUSE WEIGHT

To establish if the differences between WT and KO mice were already present during lactation, and to further confirm the delay in involution observed in the pathology studies, the ratio between inguinal mammary gland weight and total mice weight was determined. In figure 17 it can be observed that this ratio was increased in the first 24h for both WT and NOS2-KO mice. After 24h there is a rapid loss of gland weight. The explanation for this is that regression does not occur immediately. It has been noted in other works (Monks et al, 2008) that milk secretion continues for a period of time and that, as it has been explained in the introduction, involution in the mouse is reversible for up to 24-48h after weaning. In the KO mice, this ratio was significantly higher after 6 and 24h of weaning when compared with WT mice. This effect could be either the result of an increased milk secretion in the NOS2-KO mice due to a hormonal factor, or the result of a delay in mammary gland regression, which would support the hypothesis that NO deficiency can influence mammary gland involution.

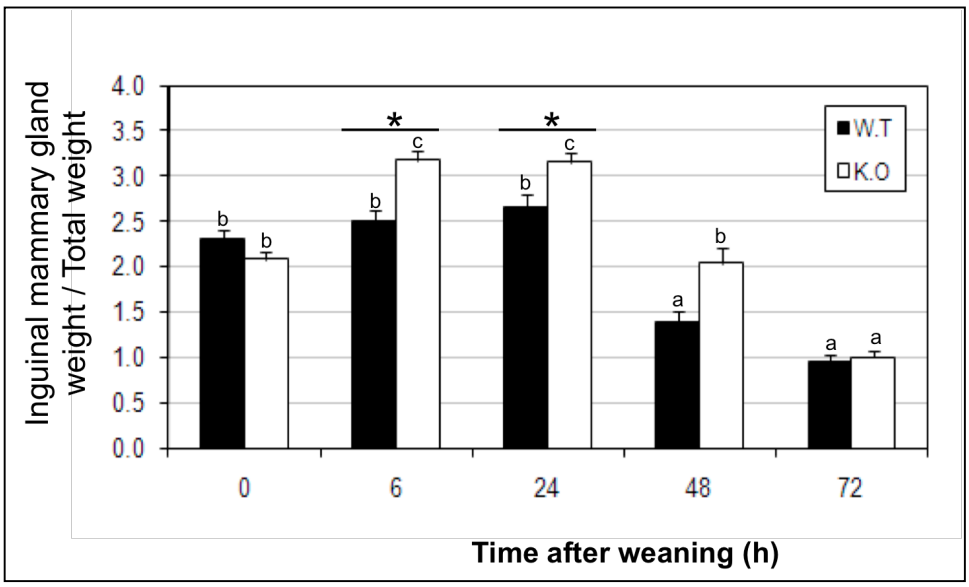

Figure 17. Ratio between inguinal mammary gland wet weight and total body weight in WT and NOS2-KO mice, at the peak of lactation and after different times of litter removal. Results are means ± S.E.M (n=6). To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant

differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. A Student's t test with the Bonferroni correction was used for comparison between WT and KO at each experimental time point; *$p < 0.05$.

1.5 SERUM PROLACTIN LEVELS

As it has been pointed out in the previous section, the variation found in the tissue slides as well as the statistical difference found in the mouse/mammary gland weight ratio could be due, not only to the decreased NO levels during weaning, but also in the lactating period (see figure 13 where nitrite levels are lower in the KO controls when compared to the WT mice). Given this possibility, we characterized other regulatory pathways that influence lactation and mammary gland involution, namely, serum PRL levels, since PRL is the main regulatory hormone in the lactating phase. This was an important point too because NO has been described to exercise an inhibitory control on calcium (Ca^{2+})-dependent PRL release in the adenohypophyis (Andric et al, 2003; Duvilanski et al, 1995).

Thus, serum prolactin was determined in six WT and NOS2-KO mice at day 10 of lactation. For the WT mice the plasma concentration of PRL was 165±27ng/mL and for the KO mice it was 213±16ng/mL, this difference being statistically significant. The higher PRL should translate into higher milk production in the NOS2-KO mice, hence, milk yield (in mL/pup; litters where normalized to seven-eight pups) was calculated according to the equation from Sampson and Jansen specified in the methods section. This equation takes into account pup weight and weight gain. Figure 18 shows that, in correlation with higher PRL levels, the milk production was slightly higher in the NOS2-KO mice, being this difference statistically significant around the peak of lactation (days 8 to 11).

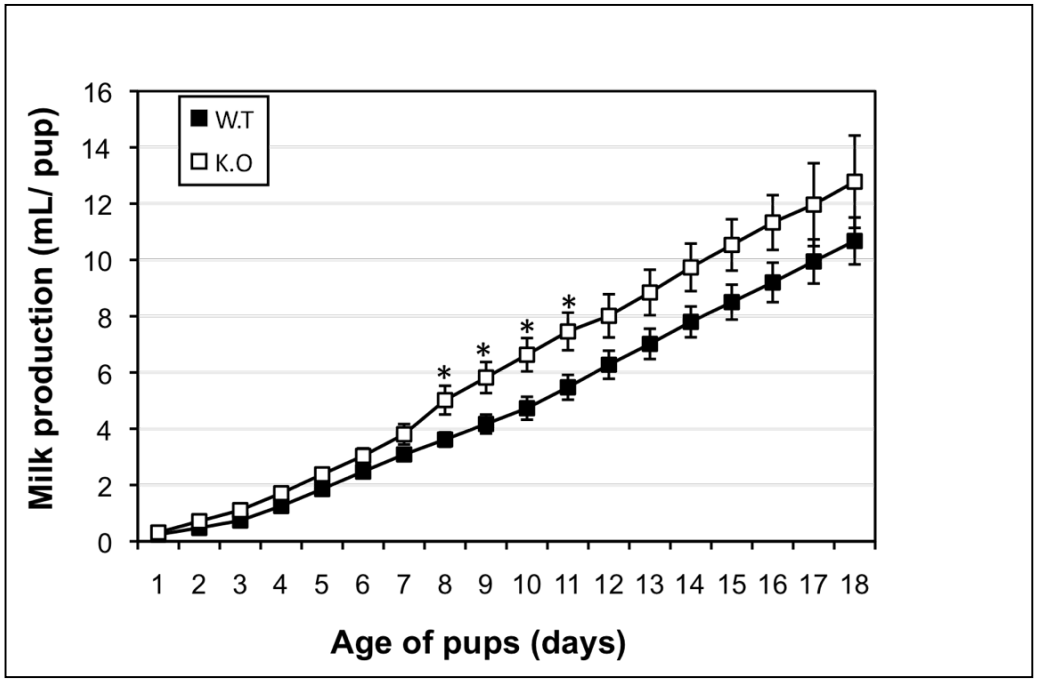

Figure 18. Milk production graph. NOS2-KO mice compared with WT mice. The start of the graph represents day 1 of lactation (first day post-partum). Results are means ± S.E.M (n=6). *p<0.05 when comparing WT and NOS2-KO mice at the same time point.

To see if this difference in milk production had any repercussion in pup nourishment, the daily weight gain by pups was also registered and a comparison between the WT and the NOS2-KO was performed. Figure 19 shows the graph comparing the weight gain between pups nourished by a WT or a NOS2-KO mother. The pups fed by KO mice increased weight during the lactating period significantly more that those fed by WT mice, although there were no weight differences at birth. This is also in line with the increased milk production in the KO mice, which is plausible with higher PRL levels and higher mammary gland weight/ total weight ratio.

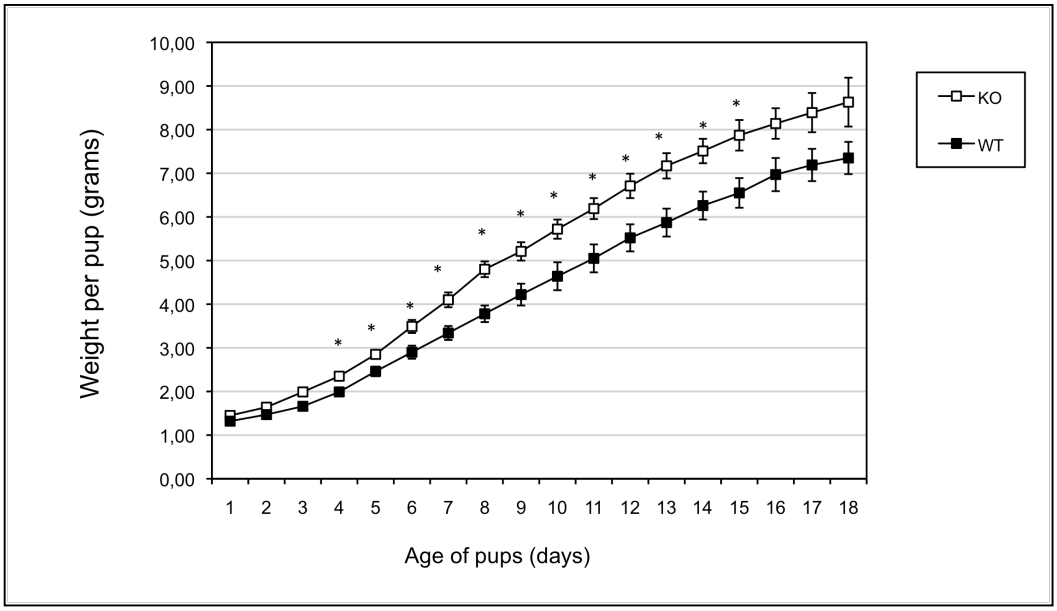

Figure 19. Graph representing the day by day weight gain of pups nourished by WT or NOS2-KO mice. The start of the graph represents day 1 of lactation (first day post-partum). Results are means± S.E.M. (n=6). *p<0.05 when comparing WT and NOS2-KO mice at the same time point.

1.6 STAT5 PATHWAY ACTIVATION IN WT AND NOS2-KO MICE

After establishing that NOS2-KO mice have a delay of around 48h in mammary gland involution, with higher PRL levels and higher milk output, the first pathway to be explored for regulatory effects was the STAT5 pathway, since it is the downstream effector of PRL during lactation. PRL exerts its function on the mammary gland epithelial cells via STAT5, which is a transcriptional factor down-stream of PRL receptor and JAK2. *Stat5* is encoded by two different genes, giving rise to STAT5a and STAT5b. STAT5 has been demonstrated to be crucial for regulation of milk protein gene expression. For example, the transcription of milk components such as whey acidic protein (WAP) and β-casein is regulated by STAT5 (Happ & Groner, 1993; Li & Rosen, 1995).

The next step was to determine whether there was a difference in the STAT5 activation. STAT5 activated state (phosphorylated) was evaluated by means of western blotting. Figure 20 shows a comparison between the STAT5 phosphorylation (P-STAT5 a/b) status at different time points of lactation and weaning for WT and NOS2-KO mice. Total STAT5 (STAT5a) was used as a control. As involution progresses total STAT5 is degraded, but its active form disappears much earlier after weaning. When comparing WT with NOS2-KO animals, it is clear that phosphorylated STAT5 levels diminish at an earlier stage in WT mice, whereas in NOS2-KO mice, p-STAT5 is still observed up to 48h involution. Considering that activated STAT5 is key during lactation, these findings are in agreement with the involution delay observed in the KO mice.

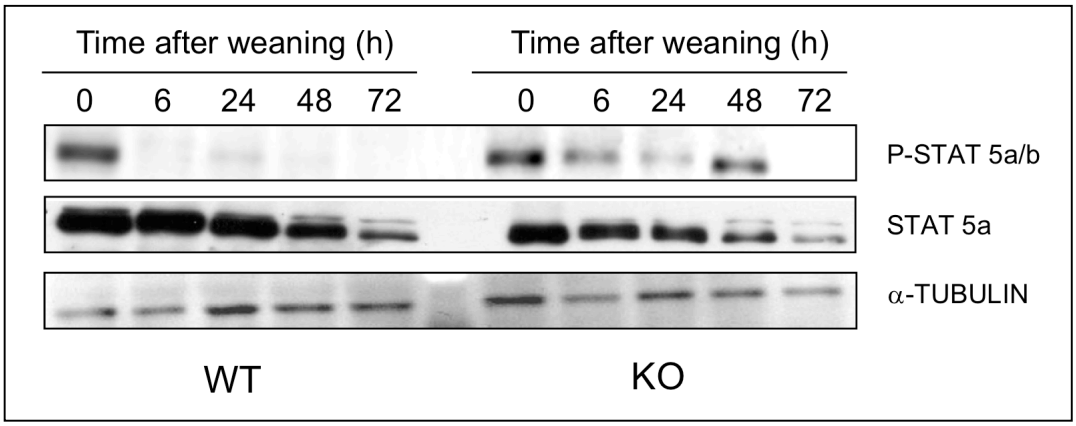

Figure 20. Western blot to determine STAT5 (STAT5a) and phosphorylated STAT5 (P-STAT5 a/b). α-Tubulin was used as a loading control.

When phosphorylated, STAT5 acts as a transcriptional factor during lactation inducing milk protein gene expression Hence, its activation was corroborated indirectly through the evaluation of β-casein gene transcription (see figure 21). As expected, β-casein mRNA levels drop dramatically with involution. STAT5 is no longer functional and hence it cannot activate milk protein gene transcription, this translates into the decline of this milk protein mRNA levels. Still, this decline is slightly delayed in NOS2-KO mice (see 24h and 48h time points) since at these time points p-STAT5 was still present in the KO mice (seen in the previous figure 20).

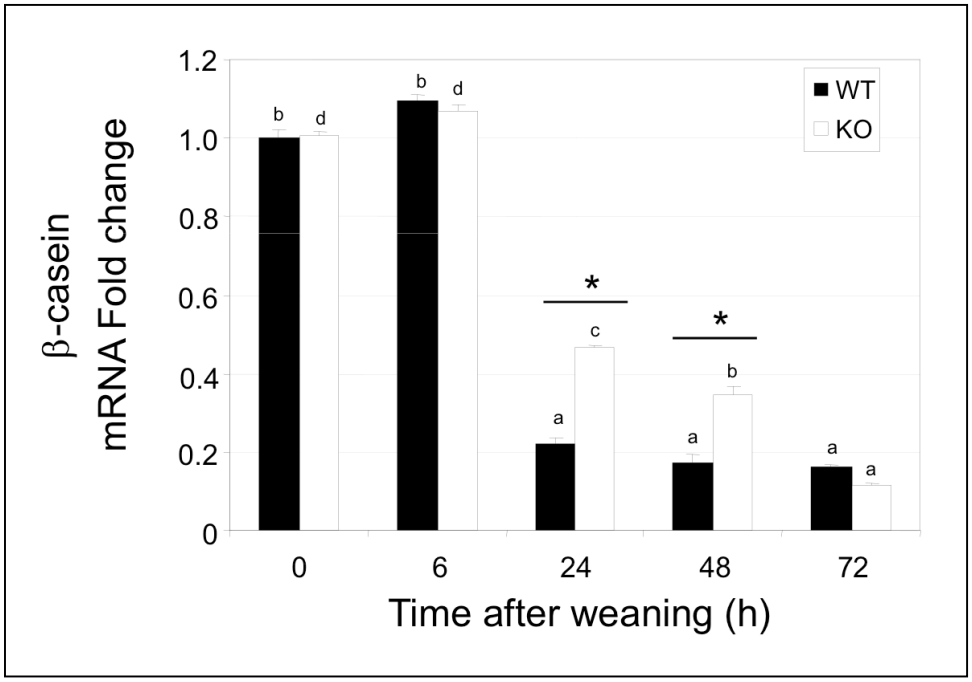

Figure 21. Real-time qPCR for β-casein gene expression. β-casein mRNA levels were normalized to 18S for each sample. Results are mean ± S.E.M relative amounts of the replicate tissue samples. ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. * $p<0.05$ when comparing WT and NOS2-KO mice at the same time point.

1.7 STAT3 ACTIVATION DURING WEANING IN WT AND NOS2-KO MICE

During weaning, milk stasis and the decrease in lactogenic hormones induce mammary gland involution. On a molecular level, this concept is determined, at least in part, from a change of the governing STAT transcriptional factor. STAT5 was crucial for lactation, but with weaning there is an "involution switch" where STAT3 takes over and triggers apoptosis as well as the transcription of pro-inflammatory factors. Therefore, STAT3 is essential for the initiation of apoptosis and remodelling following forced weaning. In fact, in vivo experiments with conditional deletion of *stat3* have proved that *stat3* inactivation results in delay of at least 3 days in mammary gland involution (Chapman et al, 1999). Given this crucial role in weaning, STAT3

phosphorylation at the peak of lactation and during involution was analyzed by Western blotting. Figure 22 shows that p-STAT3 levels were induced after 24h of weaning and declined thereafter in WT mice. STAT3 phosphorylation was not delayed in NOS2-KO mice, with a peak also at 24h, but clearly of lower magnitude. Total STAT3 protein levels were constant during lactation and involution in both WT and NOS2-KO mice, as shown in figure 22 (middle panel).

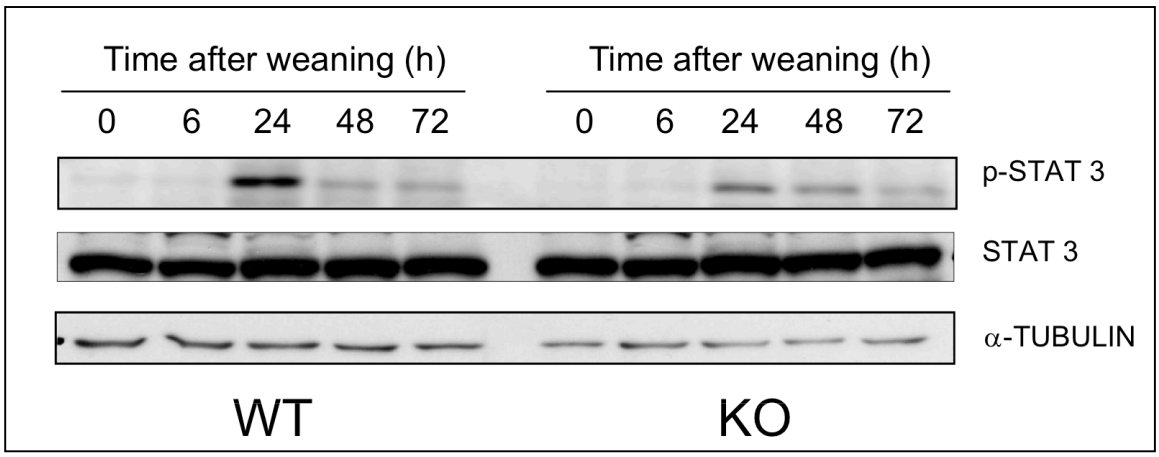

Figure 22. Western blot analysis of phospho-Tyr^{705}-STAT3 (p-STAT3) and total STAT3 (STAT3) in lysates derived from WT and NOS2-KO mice mammary glands at day 10 of lactation (0h) and at 6, 24, 48 and 72h of involution. α-tubulin was used as loading control.

Once seen that the levels of phosphorylated STAT3 were increased at 24h weaning, the STAT3 DNA-binding activity was tested by means of an ELISA-assay described in the materials and methods section. Figure 23 confirms that STAT3 was activated to a considerably lesser extent in NOS2-KO mice (5 ± 0.5 fold increase in WT compared with 3 ± 0.4 fold increase for KO after 24h of weaning).

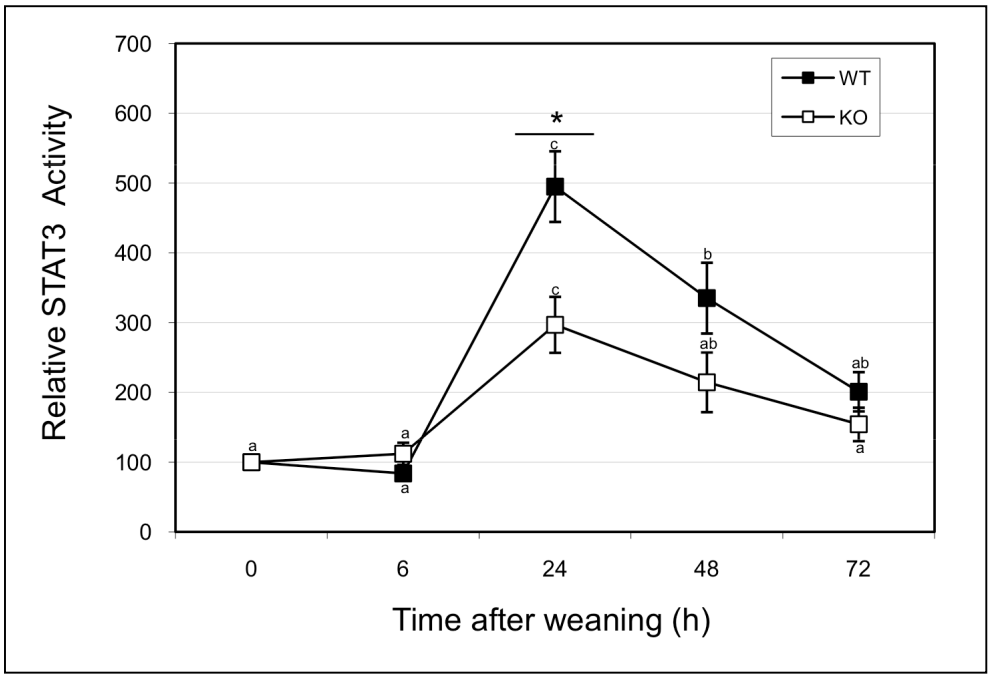

Figure 23. Graph showing STAT3 activity in nuclear extracts from WT and NOS2-KO mice at the peak of lactation (time point 0) and at different times of weaning. To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. *$p<0.05$ when comparing WT and NOS2-KO mice at the same time point. (n=4 for each genotype and time point).

Having found this difference in STAT3 phosphorylation and activation in WT and NOS2-KO animals, the next logical step was to determine at which point NO might play a regulatory role in STAT3 functionality, hence the upstream regulation was furthered studied to seek for differences between WT and NOS2-KO mice. The identified *in vivo* upstream regulator for STAT3 is the leukemia inhibitory factor (LIF). LIF binds to the specific LIF receptor (LIFR-α) which forms a heterodimer with a specific subunit common to all members of that family of receptors, the GP130 signal transducing subunit. This leads to activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription). After observing the activation of STAT3 at 24h weaning, the levels of LIF were quantified to explore any differences that could explain the divergence between STAT3 activity in WT and NOS2-KO mice. LIF mRNA

levels were examined using real-time PCR in lactating and 24h weaned glands, when STAT3 activation is maximal, in NOS2-KO and WT mice. LIF mRNA levels were not quantified at different time points because it has been described that once activated, Stat3 promotes the expression of the receptor for oncostatin M (OSMR) that substitutes LIF in its role of STAT3 activation, hence, levels of LIF decline from that point onward (Watson & Brown, 2008). As it is displayed in figure 24, LIF was substantially up-regulated in involution compared with lactation in WT mice. In NOS2-KO mice, LIF was also statistically increased when compared with control lactating, but the fold increase was significantly smaller than in WT littermates at the same time of weaning.

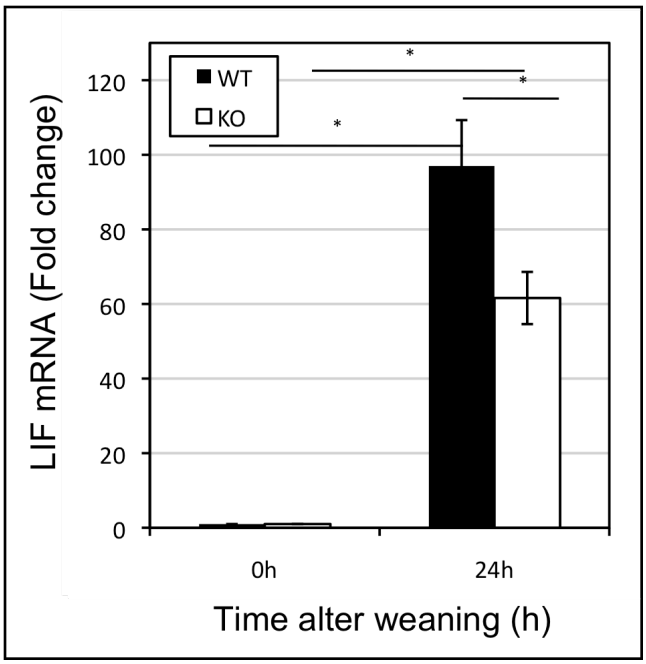

Figure 24. Reat-time qPCR to determine LIF expression in the mammary gland of control and 24h weaned mice. Each PCR was normalized against the housekeeping gene 18S. Results are means± S.E.M. relative amounts of the replicate tissue samples. *p<0.05 when comparing WT and NOS2-KO mice at the same time point also WT and KO 0h vs 24h.

Finally, in order to determine the physiological relevance of a lower STAT3 activity in NOS2-KO mice, the expression pattern of *bcl-3*, a known STAT3 transcriptional target (Clarkson et al, 2006), was analyzed. Figure 25 shows that *bcl-3* was induced after 24h weaning in both WT and NOS2-KO mice.

Nevertheless, *bcl-3* mRNA levels where increased significantly more in WT than in KO mice (6.5±0.4 fold increase in WT compared with 3.3±0.1 fold increase in NOS2-KO mice), reaching the same steady-state mRNA levels after 72h weaning.

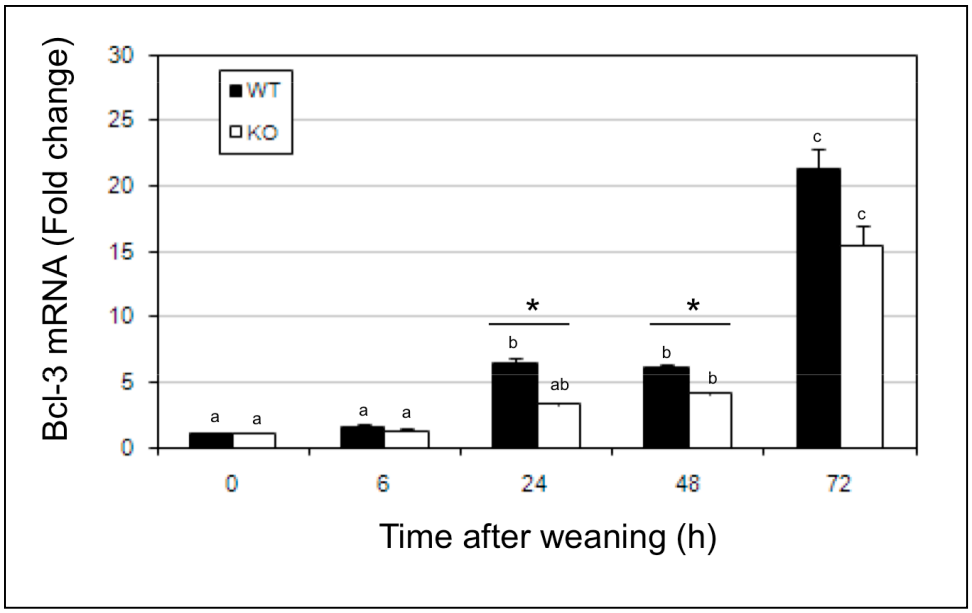

Figure 25. Real-time qPCR to determine *bcl-3* expression during lactation and at different timepoints after weaning. Each PCR was normalized against the housekeeping gene 18S. Results are presented as means ± S.E.M. relative amounts of the replicate tissue samples. To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. *$p<0.05$ when comparing WT and NOS2-KO mice at the same time point.

1.8 AKT REGULATION BY NO DURING MAMMARY GLAND INVOLUTION

The PI3K/AKT pathway is one of the main hubs that controls mammary gland involution. It has been described that during the apoptotic phase of the process AKT levels are clearly decreased, but most of all, its activation is abrogated. Since AKT is a pro-survival pathway, its inhibition is logical. Beyond the first 48 hours, in the context of tissue remodelling, AKT becomes activated again (Baxter et al, 2007). Having observed the delaying effects a lower

concentration of NO has on the involution process globally, a more specific analysis on its effects on the AKT pathway was performed.

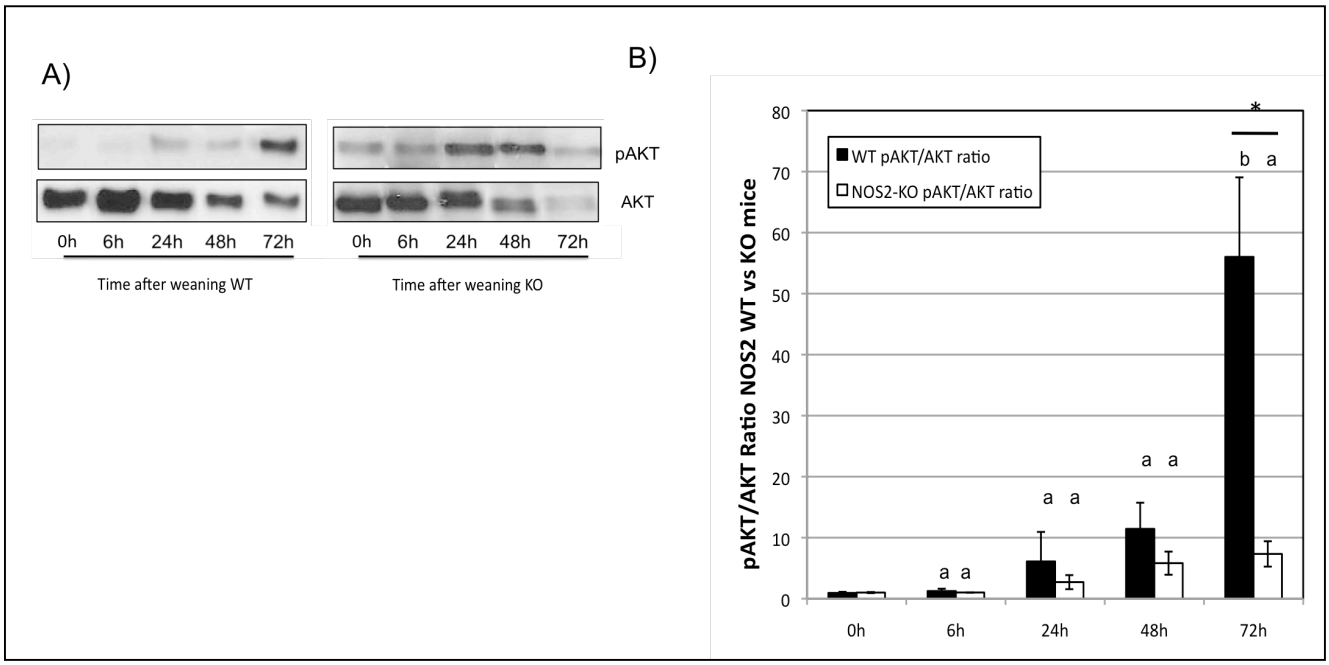

Figure 26. A) Western blot to determine phosphorylated AKT (pAKT) and total AKT (AKT) in WT and NOS2-KO mice during different time points: peak of lactation (0h) and 6, 24, 48 and 72h post-weaning (6h, 24h, 48h and 72h respectively). The figure is representative of at least 3 experiments for each time point. B) Analysis of the ratio between phosphorilated AKT (pAKT) and total AKT (AKT) in NOS2-WT and NOS2-KO mice (at different weaning time points (control 0h, 6h, 24h, 48h and 72h). To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. T-student test was performed to compare WT with KO mice within the same timepoint. *$p<0.05$.

Figure 26 shows a western blot to compare phosphorilated AKT (pAKT) and total AKT throughout different times of involution (panel A). Although total AKT levels were diminished, p-AKT increased during the second phase of involution in both WT and KO mice. To confirm AKT phosphorylation and activation, quantification was performed in order to compare phosphorylated AKT with respect to total AKT for both WT and NOS2-KO mice. Panel B is the

graphical representation of this quantification. It can be observed that the pAKT levels increase dramatically (50 fold with respect to control 0h) for the WT beyond the first 48h, coinciding with the start of the remodelling phase. The results are in concordance with the literature, since an increase in AKT activation has been described after the first 48h of weaning (Baxter et al, 2007). As for the KO mice, although a trend towards increase is seen, this is not statistically significant (5 fold increase when comparing the 72 hour time point with control lactating mice). This evidence further supports that NO has a role in the regulation of the weaning process, also in this specific pathway.

1.9 MODULATION OF NF-κB ACTIVITY BY NO DURING MAMMARY GLAND INVOLUTION

The other main regulator of mammary gland involution that has also been linked to pro-inflammatory processes is NF-κB. We investigated whether this factor could be modulated by NO and be responsible, at least in part, for the delayed involution observed in NOS2-KO mice. The differences found in NF-κB-binding activity during lactation and early involution are known to be caused by the modulation of its nuclear translocation (Geymayer & Doppler, 2000) and do not affect the p65/p50 protein levels. Thus we analysed by western blotting the decrease in the cytoplasmic levels of IκBα protein, indicative of IκBα degradation and, indirectly, of NF-κB activation. As shown in Figure 27(A), IκBα degradation, while maximal after 24 and 48h of weaning in WT mice, was delayed to 48 and 72h in NOS2-KO mice. As expected, IκBβ protein levels did not change during weaning (Zaragoza et al, 2005). These results strongly suggest that NF-κB is differentially activated in WT and NOS2-KO mice.

NF-κB activation was further assessed through analyzing its DNA binding activity at lactation and during weaning, again, using an ELISA-based assay. Panel B in figure 27 shows that NF-κB activity was increased at 24h after weaning, reaching a maximum after 72h in WT mice. This pattern of increased activity was also evident in the NOS2-KO mice, but delayed in time, in

agreement with the delay in involution seen in the histological changes, the Stat5 inactivation and the IκBα degradation seen before.

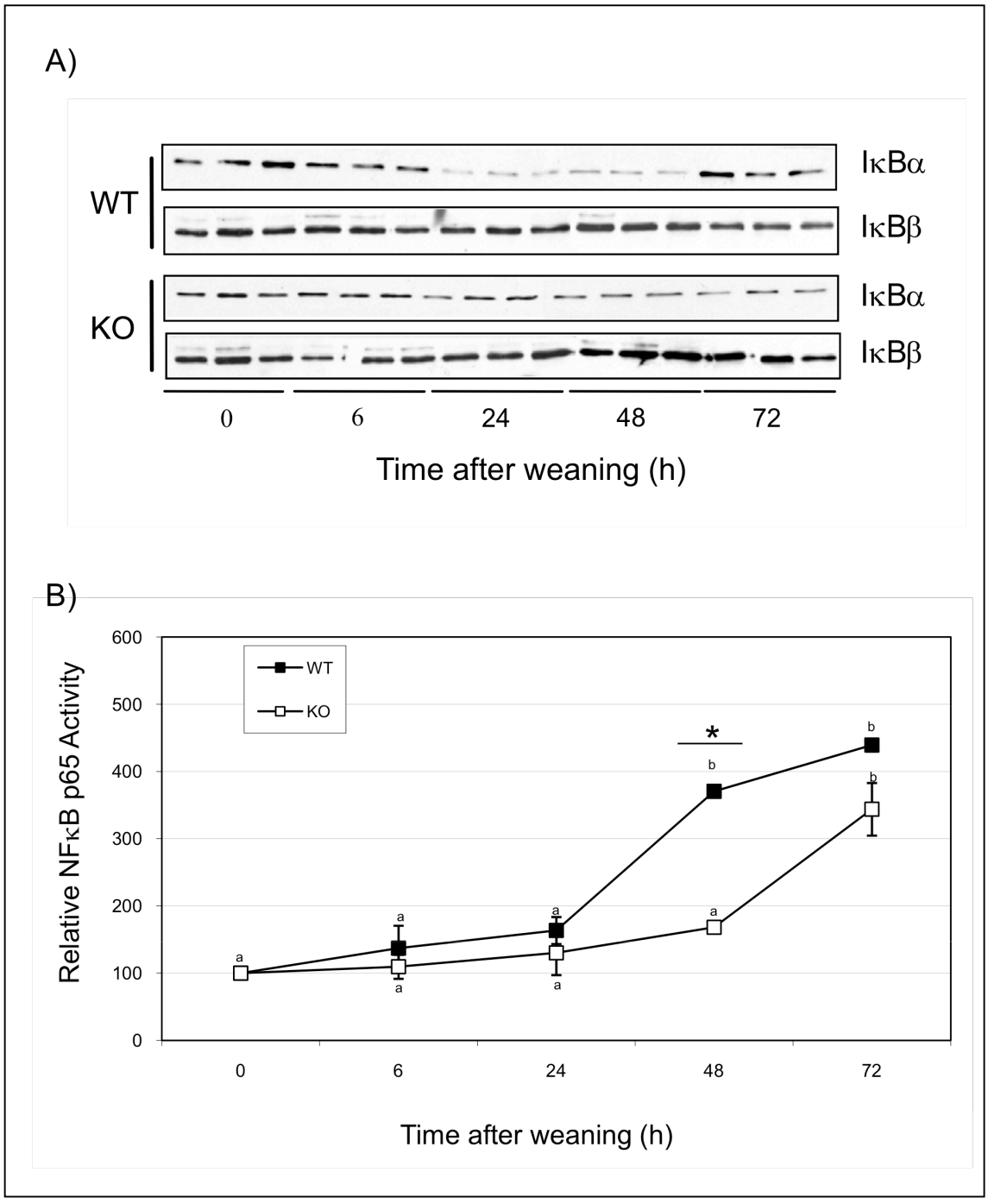

Figure 27. A) Western blot analysis for IκBα and IκBβ in mammary gland extracts obtained from WT and NOS2-KO mice at day 10 of lactation (0h), and 6, 24, 48 and 72 hours of involution. B) Graph to show assessment of NF-KB p65 activation. Nuclear extracts from WT and NOS2-KO mammary gland

extracts were assayed for NF-κB p65 activation (n=4 for each genotype and time point). The results are presented as means ± S.E.M. To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. A Student's t test with the Bonferroni correction was used for comparison between WT and KO at each experimental time point; *$p<0.05$.

1.10 ROLE OF NO IN THE MODULATION OF NF-κB ASSOCIATED GENES DURING WEANING

On the basis of the results of NF-κB activation, the expression of 84 genes, known to be associated with NF-κB pathway, was analyzed using super-array technology. The results showed that, for WT condition, when comparing 48h weaning with peak of lactation, 30 genes were up-regulated and 13 were down-regulated. Table 7 shows the genes that were differentially regulated in weaned mammary gland with respect to lactating controls in WT mice. The expression pattern found was similar to the values described previously by microarray studies (Clarkson et al, 2004; Stein et al, 2004). For the NOS2-KO mice (table 8), although the expression pattern was similar to that of the WT mice, only 21 genes were up-regulated at 48h involution with respect to peak of lactation.

Table 7. Summary of the NF-κB pathway related genes that are differentially expressed in WT mice at 48h weaning with respect to peak of lactation. The table shows the genes that were differentially regulated ($p<0.05$). Out of the 84 genes tested, 30 were up-regulated and 13 down-regulated.

GenBank® accession number	Gene Symbol	Description	Fold change
NM_009652	Akt1	Thymoma viral proto-oncogene 1	0,51
NM_007497	Atf1	Activating transcription factor 1	0,68
NM_009740	Bcl10	B-cell leukemia/lymphoma 10	0,26
NM_033601	Bcl3	B-cell leukemia/lymphoma 3	2,97

NM_009778	C3	Complement component 3	7,11
NM_130859	Card10	Caspase recruitment domain family, member 10	0,43
NM_009807	Casp1	Caspase 1	3,15
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	5,16
NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	0,13
NM_001025432	Crebbp	CREB binding protein	1,69
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	0,34
NM_010336	Edg2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	0,49
NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	2,32
NM_010493	Icam1	Intercellular adhesion molecule	3,97
NM_019777	IκBke	Inhibitor of kappaB kinase epsilon	4,29
NM_010547	IκBkg	Inhibitor of kappaB kinase gamma	2,28
NM_010548	Il10	Interleukin 10	2,23
NM_010554	Il1a	Interleukin 1 alpha	2,5
NM_008361	Il1b	Interleukin 1 beta	0,54
NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1	0,33
NM_008390	Irf1	Interferon regulatory factor 1	2,2
NM_010591	Jun	Jun oncogene	5,84
NM_010736	Ltbr	Lymphotoxin B receptor	1,76
NM_011952	Mapk3	Mitogen activated protein kinase 3	3,37
NM_010851	Myd88	Myeloid differentiation primary response gene 88	6,35
NM_008689	Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	2,14
NM_019408	Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	2,56
NM_020005	Pcaf	P300/CBP-associated factor	0,42
NM_009045	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	2,07
NM_009046	Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B	4,04
NM_138952	Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	2,62
NM_015747	Slc20a1	Solute carrier family 20, member 1	0,49
NM_030682	Tlr1	Toll-like receptor 1	4,4
NM_126166	Tlr3	Toll-like receptor 3	0,42
NM_133212	Tlr8	Toll-like receptor 8	5,58
NM_031178	Tlr9	Toll-like receptor 9	2,76
NM_013693	Tnf	Tumour necrosis factor	5,67
NM_020275	Tnfrsf10b	Tumour necrosis factor receptor superfamily, member 10b	2,22

NM_011610	Tnfrsf1b	Tumour necrosis factor receptor superfamily, member 1b	4,33
NM_011611	Cd40	CD40 antigen	2,78
NM_019418	Tnfsf14	Tumour necrosis factor (ligand) superfamily, member 14	2,51
NM_011632	Traf3	Tnf receptor-associated factor 3	0,42
NM_009539	Zap70	Zeta-chain (TCR) associated protein kinase	3,87

Table 8. Differentially regulated genes ($p<0.05$) when comparing control with 48h weaned NOS2-KO mice.

GenBank® accession number	Gene Symbol	Description	Fold change
NM_009652	Akt1	Thymoma viral proto-oncogene 1	0.32
NM_007497	Atf1	Activating transcription factor 1	0.53
NM_009740	Bcl10	B-cell leukemia/lymphoma 10	0.37
NM_033601	Bcl3	B-cell leukemia/lymphoma 3	2.53
NM_009778	C3	Complement component 3	2.68
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	4.92
NM_007700	Chuk	Conserved helix-loop-helix ubiquitous kinase	0.17
NM_009969	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	0.05
NM_009971	Csf3	Colony stimulating factor 3 (granulocyte)	0.25
NM_010336	Edg2	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	0.54
NM_007913	Egr1	Early growth response 1	3.65
NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain	0.51
NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	0.41
NM_008311	Htr2b	5-hydroxytryptamine (serotonin) receptor 2B	2.04
NM_010493	Icam1	Intercellular adhesion molecule	2.3
NM_008337	Ifng	Interferon gamma	0.27
NM_031168	Il6	Interleukin 6	4.82
NM_008363	Irak1	Interleukin-1 receptor-associated kinase 1	0.3
NM_172161	Irak2	Interleukin-1 receptor-associated kinase 2	0.5
NM_010591	Jun	Jun oncogene	4.93
NM_011952	Mapk3	Mitogen activated protein kinase 3	1.82
NM_010851	Myd88	Myeloid differentiation primary response gene 88	6.77
NM_010907	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	0.54
NM_020005	Pcaf	P300/CBP-associated factor	0.39

NM_029780	Raf1	V-raf-leukemia viral oncogene 1	1.71
NM_009045	Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	1.74
NM_009046	Relb	Avian reticuloendotheliosis viral (v-rel) oncogene related B	5.53
NM_138952	Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	2.05
NM_030682	Tlr1	Toll-like receptor 1	3.8
NM_133212	Tlr8	Toll-like receptor 8	4.56
NM_013693	Tnf	Tumour necrosis factor	3.39
NM_020275	Tnfrsf10b	Tumour necrosis factor receptor superfamily, member 10b	5.72
NM_011609	Tnfrsf1a	Tumour necrosis factor receptor superfamily, member 1a	2.72
NM_011610	Tnfrsf1b	Tumour necrosis factor receptor superfamily, member 1b	3.2
NM_011611	Cd40	CD40 antigen	4.02

A comparison between the WT and NOS2-KO mice during weaning revealed that 24 genes where significantly down-regulated in weaned NOS2-KO mice compared with WT and none was up-regulated (Table 9). The fact that all genes found to be differentially regulated in the KO animals compared with the WT were down-regulated further supports that the NF-κB signalling pathway during weaning is delayed in KO mice and thus NO could be an important signal involved in the mechanisms that trigger NF-κB response.

Table 9. Fold down-regulation in genes associated with the NF-KB signalling pathway during weaning in NOS2-KO compared with WT mice. The 23 genes that were differentially expressed ($p<0.05$) are listed above.

GenBank® accession number	Gene symbol	Description	Fold change
NM_033601	Bcl3	B-cell leukaemia/lymphoma 3	0.70
NM_009778	C3	Complement component 3	0.47
NM_130859	Card10	Caspase recruitment domain family, member 10	0.43
NM_009807	Casp1	Caspase 1	0.61
NM_001025432	Crebbp	CREB (cAMP-response-element-binding protein)-binding protein	0.64
NM_009969	Csf2	Colony-stimulating factor 2 (granulocyte-macrophage)	0.20

NM_009971	Csf3	Colony-stimulating factor 3 (granulocyte)	0.39
NM_007922	Elk1	ELK1, member of Ets oncogene family	0.53
NM_010169	F2r	Coagulation factor II (thrombin) receptor	0.56
NM_010177	Fasl	FasL (tumour necrosis factor superfamily, member 6)	0.21
NM_008311	Htr2b	5-Hydroxytryptamine (serotonin) receptor 2B	0.56
NM_010493	Icam1	Intercellular adhesion molecule	0.45
NM_008337	Ifng	Interferon γ	0.3
NM_019777	IκBke	IKKε	0.45
NM_008362	Il1r1	Interleukin 1 receptor, type I	0.42
NM_008390	Irf1	Interferon regulatory factor 1	0.61
NM_010735	Lta	Lymphotoxin A	0.47
NM_011952	Mapk3	Mitogen-activated protein kinase 3	0.59
NM_019408	Nfkb2	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2, p49/p100	0.52
NM_029780	Raf1	V-raf-leukaemia viral oncogene 1	0.62
NM_013693	Tnf	Tumour necrosis factor	0.65
NM_009397	Tnfaip3	Tumour necrosis factor α-induced protein 3	0.62
NM_011610	Tnfrsf1b	Tumour necrosis factor receptor superfamily, member 1b	0.67
NM_009539	Zap70	ζ -Chain (T-cell receptor)-associated protein kinase	0.39

1.11 CASPASE 3 ACTIVITY

In order to correlate the phases of mammary gland involution seen in the histology preparations with apoptosis and analyze the existence of differences between WT and NOS2-KO mice, the activity of caspase 3 was measured. After 24h of weaning, no difference in caspase activity was observed (figure 28) but after 48h of weaning, caspase 3 activity was clearly increased in the WT mice, but this increase was not observed in the KO mice until the 72h time point. Hence, apoptosis levels, in terms of caspase 3 activation, are also 'delayed' in *NOS2*-KO mice. From these results, we can hypothesize that NO is not only responsible for the changes observed at the early stages of involution, but that the effect of this molecule is extended far beyond the first phase of apoptotic cell death, somehow regulating the second phase of tissue remodelling.

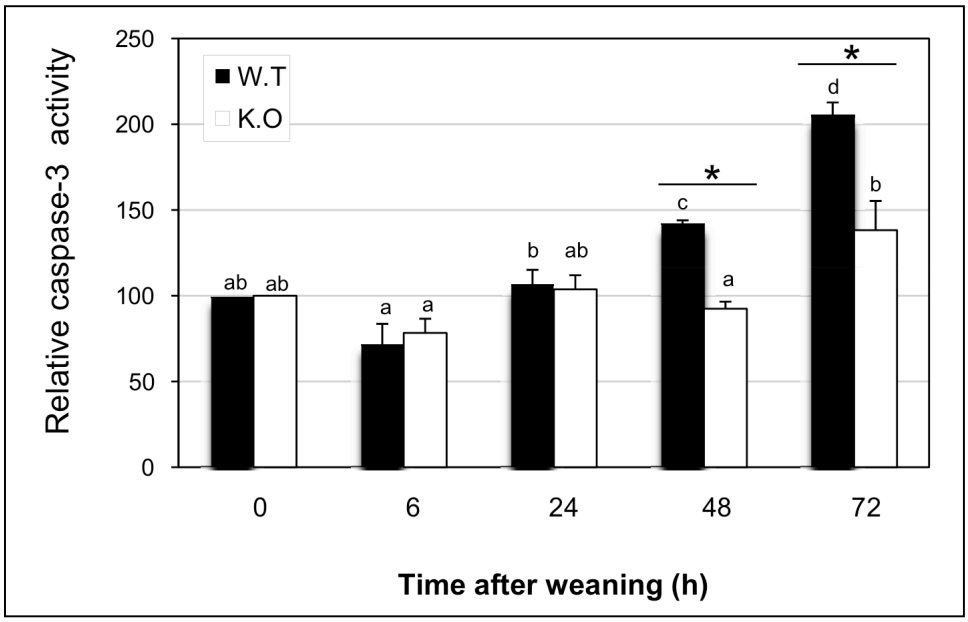

Figure 28. Caspase 3 activity in mammary tissue at different times of involution. Results are means ± S.E.M. relative amounts of the replicate tissue samples, for three different experiments performed in triplicate * $p<0.05$ when comparing WT and NOS2-KO mice at the same time point. . To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group.

1.12 PROTEASE ACTIVITY IN WT AND NOS2-KO MICE

Second phase involution is intimately related with the first, and some of its principal actors are also modulated by key regulators of the first phase. In fact, the extent of apoptosis has been reported to correlate with an increase in MMP activity during the second phase of involution (Clarkson & Watson, 1999; Lund et al, 1996). MMPs are essential in the remodelling of the ECM that takes place during the second phase mammary gland involution (Lund et al, 1996). MMP-9 is a key protease in the degradation of basement membrane collagen and different types of gelatine (Green & Lund, 2005) and accumulating evidence suggests that the activation of NF-κB is one of the most important signalling events required for the inducible expression of MMP-9 in response to different stimuli (Li et al, 2009).

We next wanted to assess whether the functional consequences of delayed first phase involution had also functional consequences during the second phase in NOS2-KO mice. This question was addressed through the analysis of MMPs expression and function.

Real-time PCR analysis was performed in WT and NOS2-KO mice at the peak of lactation and in weaned animals. Figure 29 shows that the mRNA levels of MMP-2 and MMP-9 were significantly higher after 48h of weaning than those mice at the peak of lactation. The maximal induction of both MMP-2 and MMP-9 was observed after 72h of weaning. While no difference in MMP-2 mRNA up-regulation was observed between WT and KO mice under all conditions studied (panel A); MMP-9 induction was more pronounced during weaning of WT mice vs NOS2-KO mice (panel B).

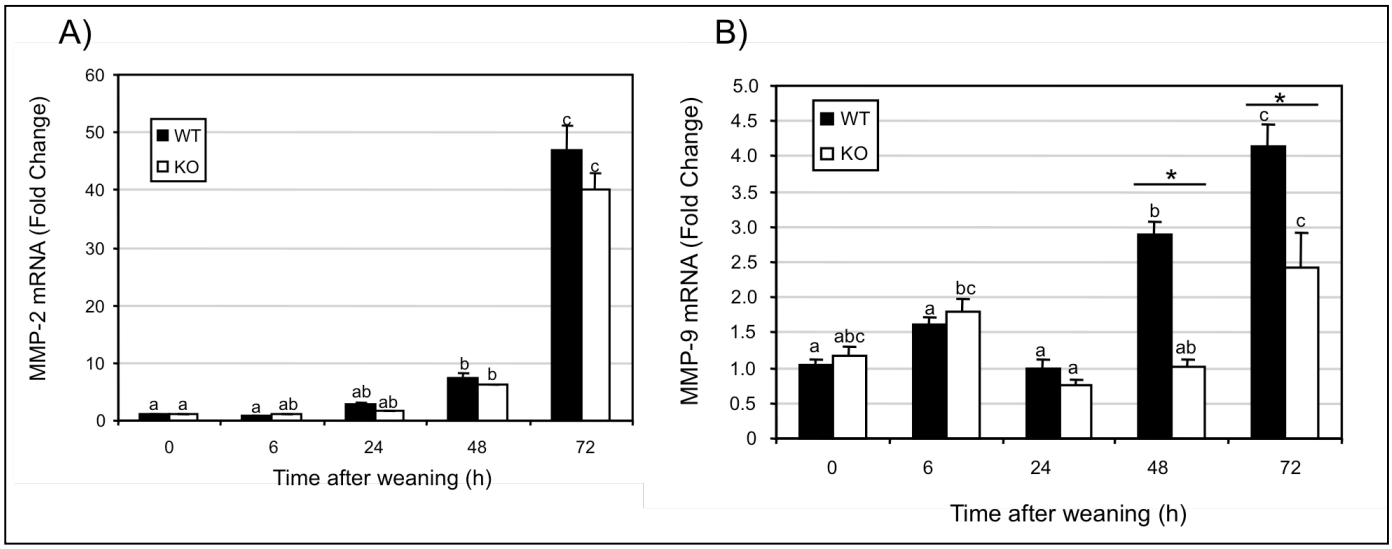

Figure 29. Real-time qPCR for both gelatinase genes, MMP-2 and MMP-9 (Panels A and B respectively). Expression levels were compared at each time after weaning (0, 6, 24, 48 and 72h) between WT and NOS2-KO mice. Each PCR was normalized against the housekeeping gene 18S. Results are means ± S.E.M. *p <0.05 when comparing WT and NOS2-KO mice at the same time point.

Taking into consideration the fact that NF-κB activation is delayed in the KO mice, the question rose whether NF-κB was the transcriptional factor

inducing MMP-9 translation during the involution process. In order to answer this question, a ChIP assay was performed. The ChIP assay consists on immunoprecipitating all the chromatin that is linked to a specific protein at a specific time. Beforehand, the DNA is cross-linked with the attached proteins by means of formaldehyde. After the cross-linking, the chromatin has to be sonicated into smaller fragments of around 500 bp (a size that will allow the rest of the experiment to run adequately). Following sonication, immunoprecipitation is carried out, this way, only the DNA attached to a specific protein such as a transcription factor will be recovered. Finally, the DNA will be purified and a PCR performed using primers of the promoter region of the specific gene sought. Should there be an amplification product, it will mean that the specific transcription factor immunoprecipitated was attached to the promoter region of the specific gene, which increases the chances of it being transcribed at the moment of the initial chromatin extraction.

In this experiment, chromatin was immunoprecipitated with anti-p65 and anti-p50 antibodies, the most frequent NF-κB dimer. Primers specific to the promoter region for MMP-9 gene were used to amplify the DNA isolated from the ChIP assay. Panel A in figure 30 shows the average size of the chromatin fragments obtained (~500 bp). Panel B shows that p65/p50 was bound to the MMP-9 promoter at 48h of involution in WT mice. On the other hand, the NOS2-KO mice showed no binding of the NF-κB on the MMP-9 promoter. This finding is in accordance with the delayed activation of this transcriptional factor that was observed in the previous ELISA experiment and with the lower levels of MMP-9 mRNA observed by RT-qPCR. For positive control, an aliquot of the input chromatin was used. For negative control, an unrelated IgG antibody was used.

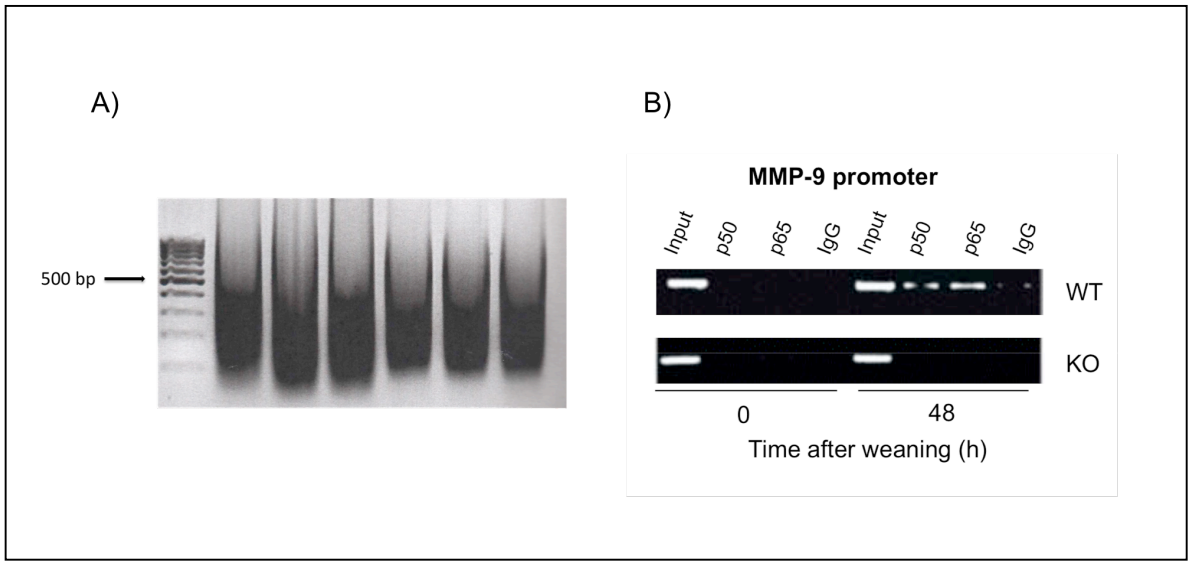

Figure 30. A) Size of the chromatin fragments obtained after isolation and sonication of chromatin from mammary gland samples in lactating controls and after 48h of weaning in both WT and NOS2-KO mice. B) *In vivo* association of p65/p50 with the MMP-9 promoter during weaning. Results of a representative ChIP experiment, performed in samples from control lactating mammary gland, and 48 h weaned WT and NOS2-KO mice are shown.

MMP activation was analyzed by zymography studies. The zymography assay is an electrophoretic technique that includes a substrate copolymerised with the polyacrylamide gel for the detection of enzymes and their activity. Gelatine is the most commonly used substrate, and is useful for demonstrating the activity of gelatine-degrading proteases, such as MMPs. Following electrophoresis, the gel is washed with a re-naturing buffer followed by incubation in an enzyme activation buffer. This allows the enzymes present in the sample to become active and digest the gelatine substrate copolymerised in the gel. The zymogram is subsequently stained and the areas of enzyme activity and digestion become visible.

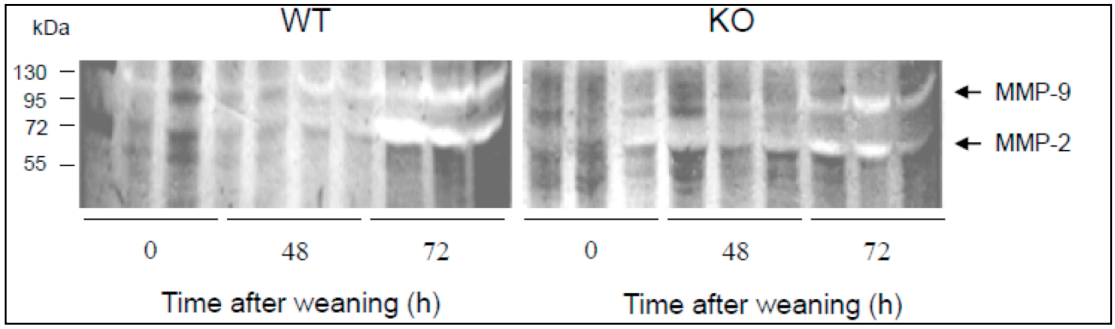

Figure 31. Gelatine zymography measuring MMP-2 and -9 activation. Protein extracts from lactating (0h) and weaned glands (48 and 72h) of both WT and NOS2-KO genotypes were used.

Figure 31 shows that in WT mice, MMP-9 proteinase activity (105KDa) appeared faintly after 48h of weaning, increasing after 72h of weaning. On the other hand, the activation of pro-MMP-2 (70KDa band) to an active form occurred after 72h of weaning. The increased enzymatic activity correlated with MMPs mRNA levels in the weaned mammary tissue. When comparing MMPs activity, the intensity of the bands for MMP-2 and -9 is lower after 72h of weaning in KO when compared with WT mammary glands. Also, no band was observed for MMP-9 at 48h of involution in NOS2-KO mice which would correlate with the absence of histological changes seen in the hematoxilin-eosin pathology sections studied in figures 15 and 16.

2. ROLE OF NO IN SPECIFIC POST-TRANSLATIONAL MODIFICATIONS DURING MAMMARY GLAND INVOUTION

Given the differences observed in mammary gland involution between NOS2 WT and KO mice, nitric oxide (NO) seems to play an important regulatory role in the involution of mammary gland after weaning. It is noteworthy that NO has been characterized as highly reactive molecule that can play contradictory signalling roles depending on concentration and temporal exposure. In general, lower NO concentrations promote cell survival and proliferation, while higher levels favour cell cycle arrest, apoptosis and senescence (Thomas et al, 2008). More specifically, it can induce a series of nitrosative post-translational

modifications on proteins and lipids that play a key signalling mechanism in cell physiology, with a clear role in pathophysiology (White et al, 2010). In proteins, these modifications occur in two main forms, either as S-nitrosylation of cystein thiols or as nitration of tyrosine residues. A set of experiments were performed in order to further explore the possible role protein nitration in the mammary gland involution setting. The modification sought was tyrosine nitration, but a possible role of protein s-nitrosylation in mammary gland involution is more than plausible, since s-nitrosylation is highly dependant on the redox-status of the cell and mammary gland involution entails the depletion of glutathione (GSH), hence altering the redox status in the weaned gland (Zaragoza et al, 2003). The chosen animal model for these experiments was rat, for technical reasons and in order to further confirm that the role of NO in weaning was a generalized phenomenon and not species-specific.

2.1 NITROPROTEOMIC ANALYSIS OF LACTATING AND WEANED MAMMARY GLAND FROM RATS

Previous work had already revealed that protein nitration is increased during weaning (Zaragoza et al, 2005). Our group had established that in the rat model used, this nitration was maximal 72h after weaning. Hence, to ensure identification of differentially nitrated specific proteins in the weaned mammary gland, proteomic studies were performed on whole tissue extracts from lactating and 72h weaned mammary glands. Nitrated proteins were identified using a combination of procedures that included 2D-PAGE immunoblotting with specific antibodies against nitro-tyrosine (NO-tyr) residues and MS of proteins recovered from two-dimensional gels. A total of 20 proteins in the molecular mass range of 15-75kDa with a pI between 5 and 8 were identified by 2D-IEF and matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF-MS). Since milk stasis plays an important role in the process of involution, milk-depleted mammary tissue was not used for proteomic analysis. This way, a complete nitrated protein profile could be obtained. The caveat of this was that, among the nitrated proteins identified, there were relative abundance of albumin, immunoglobulin chains and casein fragments. This higher proportion of albumin and other milk-secreted proteins probably impaired the recognition of

low abundance proteins. Nevertheless, it is possible that these fragments of nitrated albumin could play a more important role than one might think at first. Indeed, it has been described that the injection of small peptides derived from milk into the mammary gland accelerates the process of involution (Shamay et al, 2002).

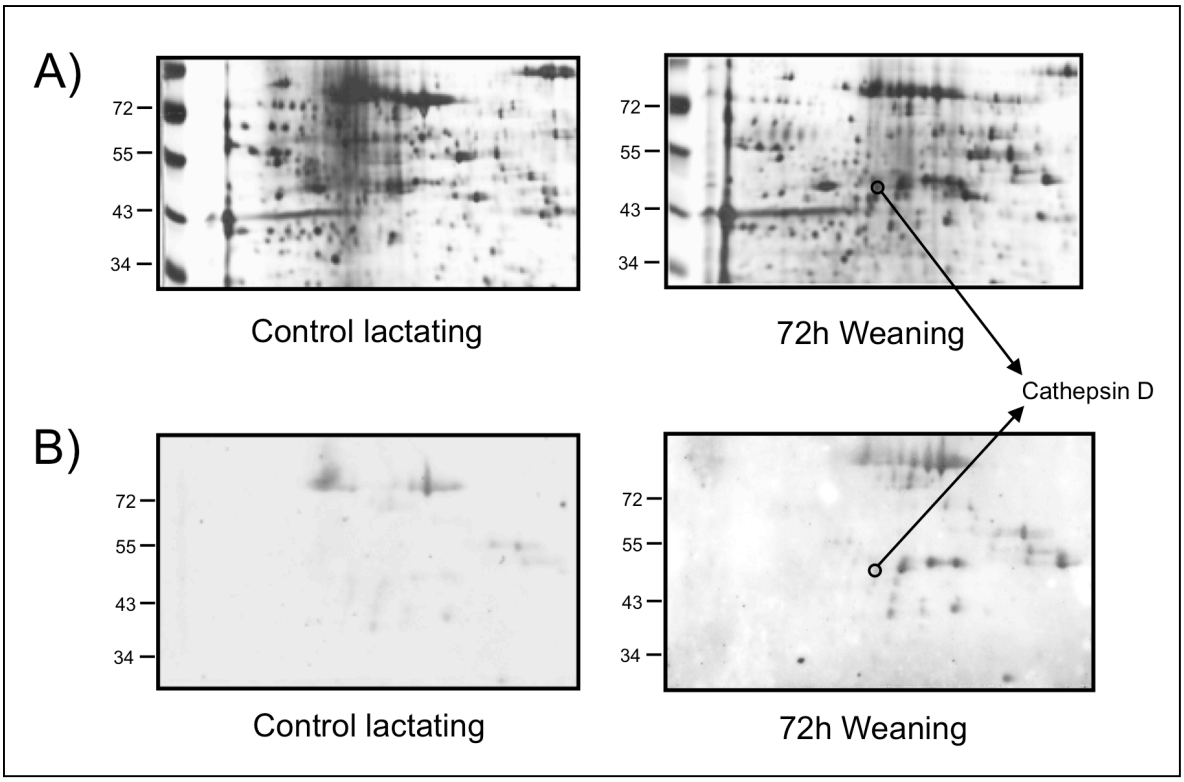

Figure 32. Protein expression profiles of control lactating and 72h weaned mammary glands separated by IEF 2D-PAGE and silver stained (A) or staining of nitrated proteins by immunoblotting with anti-nitro-tyrosine antibody (B). Figure B shows an increase of nitrated proteins (NO-tyr) in the weaned gland when compared to the lactating control. One of the proteins found differentially nitrated was cathepsin D (spotted on right hand panels fig A and B).

Still, within these other more abundant proteins, a 48kDa nitrated protein was identified, with coverage of 2.5%, as Cathepsin D. Differential nitration can be seen in figure 32 that shows representative silver stained gels and nitrated proteins (panels A and B respectively) in control and 72h weaned mammary glands.

2.2 CONFIRMATION OF CATHEPSIN D NITRATION IN WEANED MAMMARY GLAND

2.2.1 Immunoprecipitation of Cathepsin D with Specific Cathepsin D Antibody

To further confirm the nitration of this enzyme after 72h weaning, immunoprecipitation with anti-cathepsin D antibody, followed by immunoblotting against NO-tyr residues was performed. Two different forms of cathepsin D immunoprecipitated from the lactating mammary gland homogenates were found to be nitrated. One was the mature single-chain cathepsin D above the 43kDa molecular mass marker. The other corresponded to the heavy 34kDa chain from the mature double-chain form. These results of the immunoprecipitation and blotting can be seen in figure 33. Equal loading of both samples was assessed by loading on to the gel 1% of control or weaned mammary gland homogenate (input). Besides, to corroborate that cathepsin D was immunoprecipitated, Western blotting against cathepsin D was performed on the immunoprecipitate from 72h weaned mammary gland (Figure 33, right-hand panel).

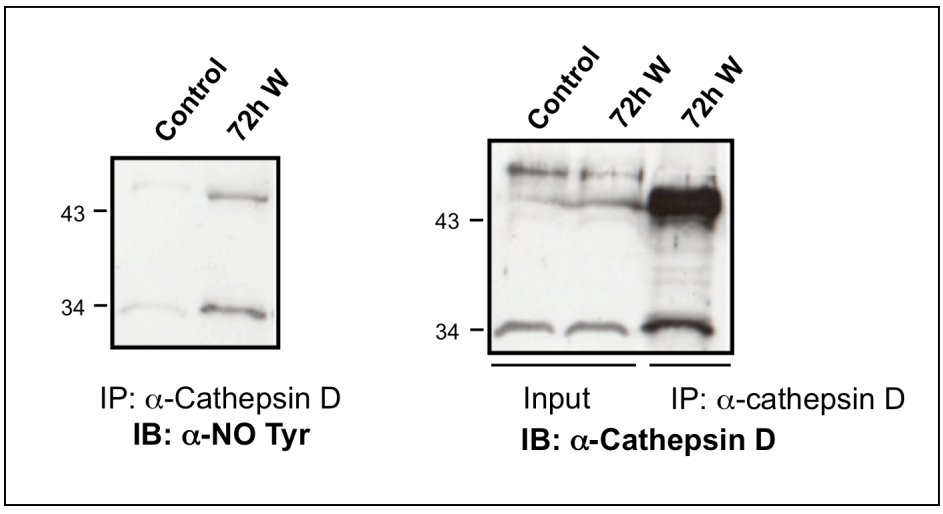

Figure 33. Immunoprecipitation of Cathepsin D. Tissue homogenates from control lactating or 72h weaned ('72h W') mammary glands were immunoprecipitated (IP) by anti-(cathepsin D) antibody, and the precipitates

were then immunoblotted (IB) with anti-nitro-tyrosine antibody or anti-(cathepsin D) antibody. Input represents an equal amount of starting homogenate.

2.2.2 Immunoprecipitation of Cathepsin D with Specific Pepstatin-Coated Beads

Furthermore, in order confirm the previous results, purification of cathepsin D from the mammary gland samples was performed, this time using an approach with pepstatin A. Pepstatin A is a cathepsin D inhibitor that binds specifically to this protease. Pepstatin A-coated agarose beads were used to specifically precipitate cathepsin D from weaned mammary gland homogenates to be thereafter blotted against cathepsin D and NO-tyr, as shown in figure 34. This experiment corroborated that cathepsin D was in fact nitrated during weaning. As a positive control, an equal amount of starting homogenate (Input) was also loaded onto the electrophoresis gel.

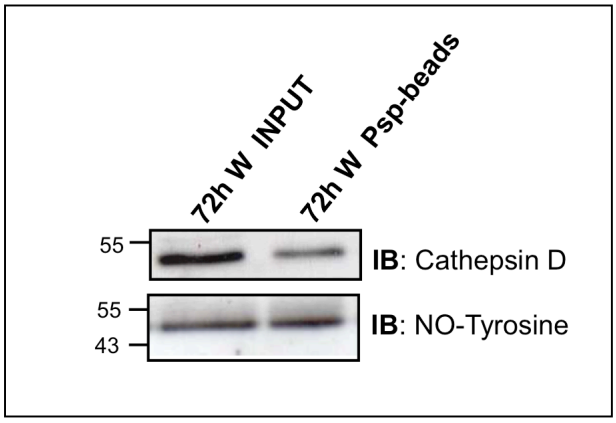

Figure 34. Immunoprecipitation of cathepsin D with pepstatin beads. Mammary gland homogenates from 72h weaned rats ('72h W') were incubated with pepstatin A-coated agarose beads. After elution, the bound fraction was analysed by Western blotting, including an equal amount of starting homogenate (named Input). The membrane was immunoblotted (IB) with anti-(cathepsin D) antibody and then stripped and reprobed with anti-nitrotyrosine to verify protein nitration.

2.3 EFFECT OF WEANING ON THE EXPRESSION AND ACTIVITY OF CATHEPSIN D IN RAT LACTATING MAMMARY GLAND

Having spotted cathepsin D as a protein that becomes nitrated during weaning, and knowing its role in the involution process, we sought to further explore the implications of this post-translational modification on its function. Nevertheless, before this, mRNA and protein levels of this protease were analyzed.

Figure 35 shows the results of mRNA expression levels for cathepsin D in control and weaned (W) rat mammary gland at different time points (panel A). The mRNA levels peaked at 8h after litter removal and decreased thereafter. This finding correlated with the data obtained in the western blot analysis of lactating and weaned mammary gland lysates, which revealed two major cathepsin D-related bands that correspond to the enzymatically inactive precursor of ≈ 52KDa and the mature single-chain form of ≈ 48KDa. Figure 35 panel B shows that the amount of the latter (mature single chain) is increased after 8h of pup removal and remains elevated 3 days later, being barely detectable thereafter.

Since we had found that cathepsin D was differentially expressed during weaning when compared to lactation, differences in its activity were sought. Its proteolytic activity was measured by the hydrolysis of the fluorigenic substrate 7-methoxycoumarin as described in the materials and methods section. Panel C in figure 35 shows cathepsin D proteolytic activity at control and different weaning time points. Even thought the mRNA levels and protein levels elevated as early as 8h after weaning the protease activity is not increased until 48h after pup removal in the rat model. These results point to a possible post-translational modification needed for the protease to become active.

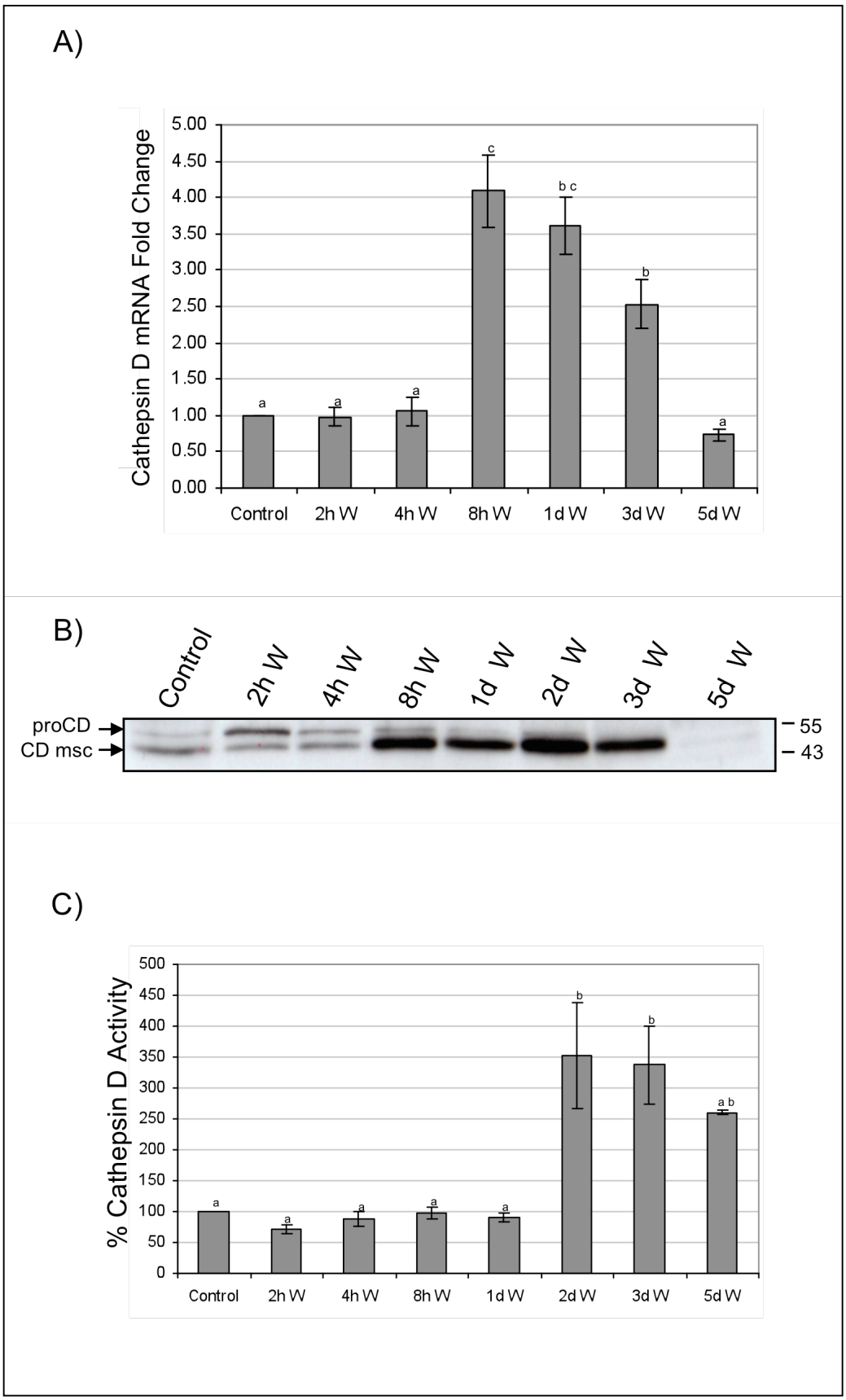

Figure 35. A) Cathepsin D mRNA expression in rat mammary tissue at the peak of lactation (control) and at different times of weaning, measured by real-time

RT–PCR. Each PCR was normalized against the housekeeping gene 18S. B) Western-blot for cathepsin D using control and weaned rat mammary gland homogenates. Two immunoreactive bands, corresponding to pro-cathepsin D (proCD) and mature single-chain cathepsin D (CD msc) forms of cathepsin D were visualized in control and weaned rats using anti-(cathepsin D) antibody. The position of the molecular mass markers (kDa) is shown on the right. W= weaning. C) Cathepsin D activity in mammary gland from control lactating and weaned rats by measurement of the hydrolysis of the fluorigenic substrate 7-methoxycoumarin-4-acetic acid. Results are means± S.E.M. relative amounts for three independent experiments. ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter “a” always represents the lowest value.

2.4 EFFECT OF *IN VITRO* $ONOO^-$ INDUCED NITRATION ON CATHEPSIN D ACTIVITY: STUDIES IN RAT MAMMARY GLAND HOMOGENATES AND IN RECOMBINANT CATHEPSIN D

So far, from the previous studies, it could be concluded that cathepsin D is induced during weaning as early as 8 hours after pup removal, but it does not become active until 48h after weaning. Also, cathepsin D was found to present tyrosine nitration as a post-translational modification at 72h weaning. Hence, it could be that this post-translational modification played a role in the activation of the protease.

To further explore this possibility, *in vitro* induced nitration experiments were performed using both mammary gland homogenates from lactating rats and human recombinant cathepsin D. After inducing protein nitration *in vitro*, cathepsin D activity was measured to directly confirm whether this post-translational modification was responsible, at least in part, for the protease activation.

Mammary gland homogenates from control lactating rats were incubated with different concentrations of $ONOO^-$ (PN) (ranging from 0 to 100μM). In figure 36 (panel A) it can be seen that at 20μM of $ONOO^-$ cathepsin D activity

was 50% higher when compared to the control. At concentrations higher than 50μM of $ONOO^-$, there was a linear decreased activity (probably due to the oxidative effects of $ONOO^-$ on a variety of amino acid residues in addition to nitration of aromatic acids). After corroborating that, in order to achieve the highest activity of Cathepsin D, the optimal dose of $ONOO^-$ was 20μM, it was also studied whether this activation was due to $ONOO^-$ by means of incubation of the mammary gland homogenates with $ONOO^-$. This increase was abolished in the presence of the flavonoid epicatechin that has been shown to interfere with protein tyrosine nitration by intercepting the tyrosyl radical (Schroeder et al, 2001) (Figure 36, panel B).

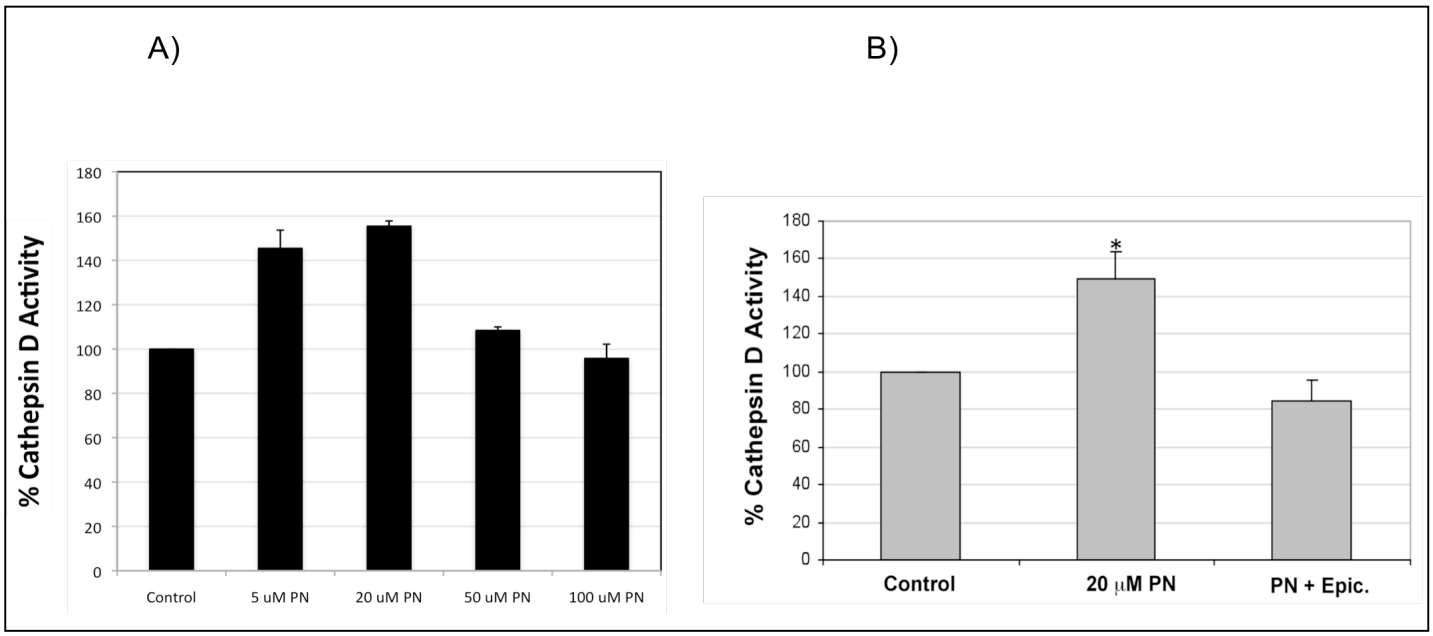

Figure 36. Cathepsin D activity in rat mammary gland homogenates. A) The graph shows the increase in cathepsin D activity in the nitrated samples of rat mammary gland homogenates ($ONOO^-$ concentrations ranging from 5 to 100μM to 100μM PN) when compared with control (treated with vehicle). Results are means ± S.E.M. of three different experiments. B) The graph shows cathepsin D activity in mammary gland homogenates treated with PN (20μM PN) or PN and epicatechin (PN+Epic) when compared with control (treated with vehicle). *p <0.05.

Western blotting with anti-NO-tyr antibodies showed that protein nitration was enhanced after the incubation with 20μM $ONOO^-$ and was undetectable in control lactating rats treated with vehicle or with $ONOO^-$ and epicatechin. The results of this experiment can be seen in figure 37A. It was also demonstrated that the different treatments (vehicle, $ONOO^-$ and $ONOO^-$ + epicatechin) had no effect on the amount cathepsin D protein levels. In order to assess whether cathepsin D was nitrated after the incubation with 20 μM $ONOO^-$, immunoprecipitation of control and nitrated samples with cathepsin D antibody was performed. Cathepsin D was immunoprecipitated from tissue lysates incubated with vehicle or 20μM $ONOO^-$, and analyzed for tyrosine nitration by western blotting. The results in figure 37 (panel C) show that nitrated cathepsin D was increased after $ONOO^-$ induced nitration.

The prior results pointed to two possibilities: the first, direct nitration of cathepsin D as a post-translational modification enhances its activity. The second is that NO activates cathepsin D through indirect mechanisms, such as nitration of other proteins that modulate cathepsin D activity. To gain further understanding of the mechanisms by which protein nitration might affect directly cathepsin D activity, as well as to test the first hypothesis, *in vitro* experiments with recombinant cathepsin D from human liver were performed. As seen in figure 38, commercial human cathepsin D reacted with 0 or 20μM $ONOO^-$ (PN) in the presence or absence of 30μM epicatechin (Epic.). This recombinant cathepsin D from human liver corresponds mainly to the heavy chain of the mature cathepsin D form (34kDa). Samples were analyzed for tyrosine nitration or cathepsin D activity. Panel A in figure 40 shows that when human cathepsin D is treated with 20μM $ONOO^-$, an increase in nitro-tyrosine residues can be clearly observed. This increase in tyrosine nitration was abolished when 30μM epicatechin was added. On the other hand, the direct effect of $ONOO^-$ on cathepsin D activity was measured (figure 38 panel B). A significant increase in the activity of this protease was observed when $ONOO^-$ (PN) was added, with a subsequent abolishment when treated also with epicatechin. Also, as expected, the activity of cathepsin D was completely abolished when incubating the recombinant human cathepsin D with its specific inhibitor pepstatin A (PspA).

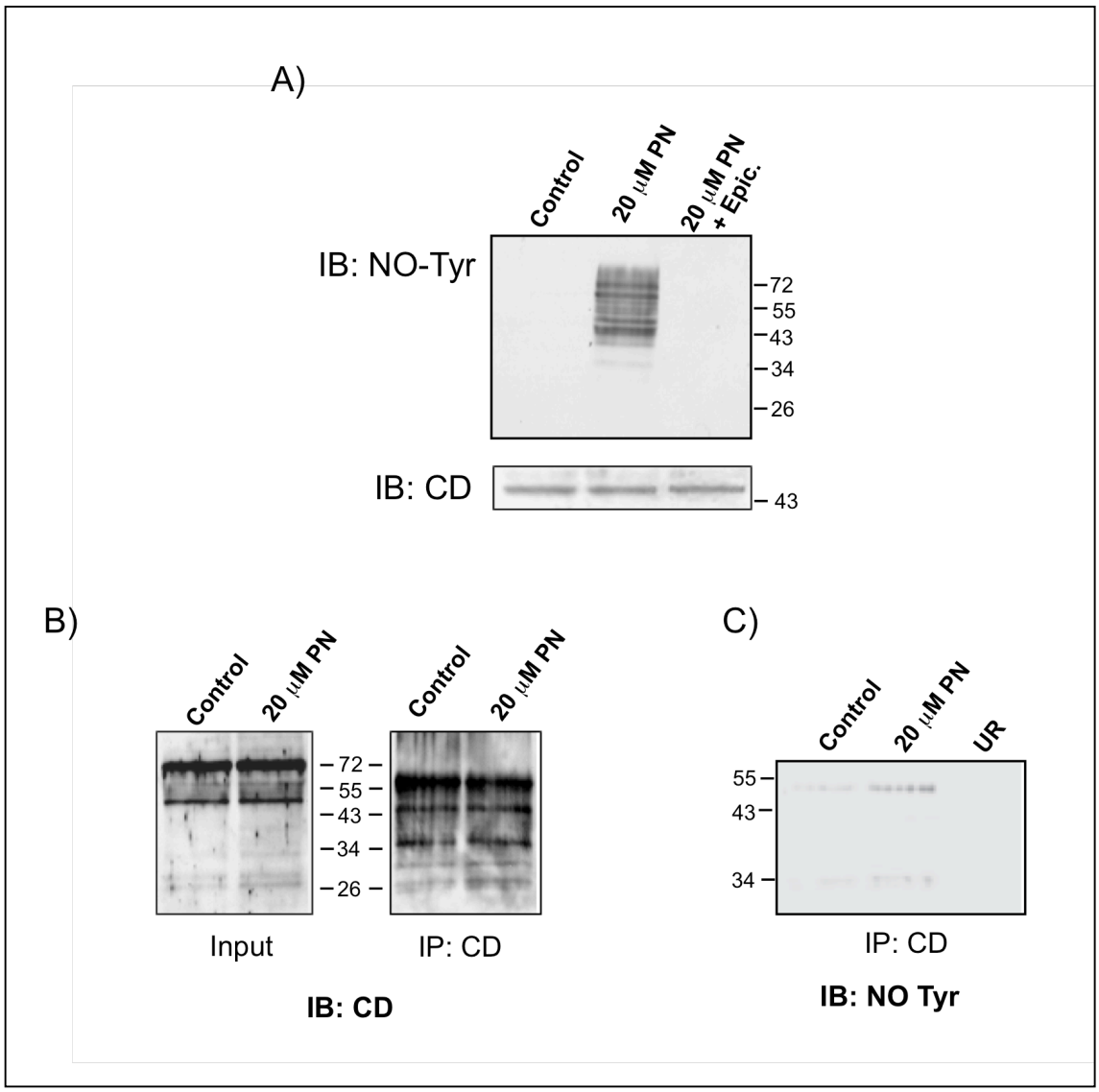

Figure 37. A) Western blot (IB) against tyrosine-nitrated residues (upper panel) or cathepsin D (lower panel) in samples from mammary tissue after treatment with $ONOO^-$ with or without epicatechin (20μM $ONOO^-$ and 20μM $ONOO^-$ + Epic respectively). B) Immunoprecipitation (IP) in tissue homogenates from control lactating mammary glands treated with vehicle (control) or with $ONOO^-$ (20μM PN) by anti-cathepsin D antibody and immunoblot(IB) with anti-cathepsin D antibody. C) Immunoprecipitation (IP) in tissue homogenates from control lactating mammary glands treated with vehicle (control) or with $ONOO^-$ (20μM PN) by anti-cathepsin D antibody and immunoblot (IB) with anti-NO-tyr antibody. Molecular mass markers (kDa) are indicated on each blot.

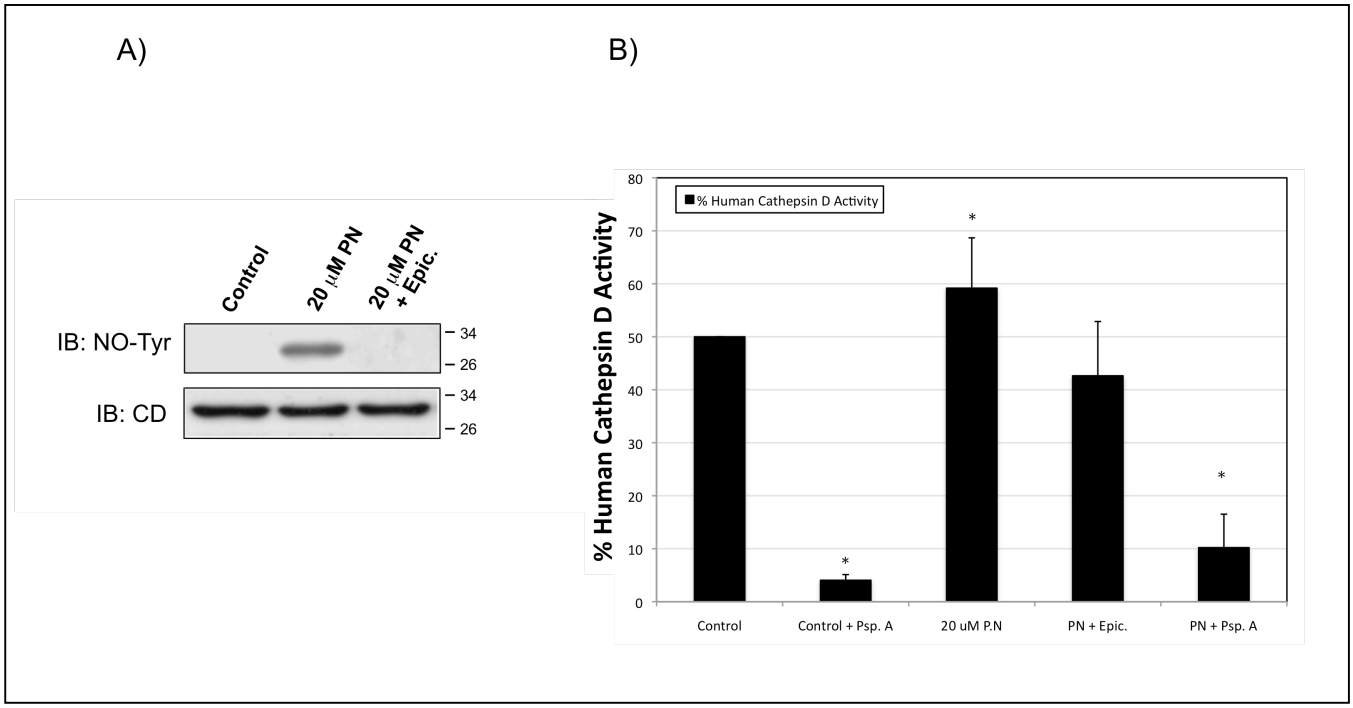

Figure 38. A) Western blot (IB) to analyze tyrosine nitration (NO-tyr) of human recombinant cathepsin D (CD) control, after treatment with 20μM $ONOO^-$ (20μM PN) and after treatment with both 20μM $ONOO^-$ and epicatechin (20μM PN+ Epic). The lower panel shows that equal amounts of cathepsin D (IB: CD) were loaded. Immunoblots are representative of three separate experiments. The position of molecular mass markers (KDa) is shown on the right. B) Effect of $ONOO^-$ on human cathepsin D activity. Treatment with 20μM $ONOO^-$ with or without 30μM epicatechin and with or without pepstatin A (20 μM PN, Psp, PN+Epic and PN+Psp A respectively). *$p<0.05$. Pepstatin A was used to verify the specificity of the assay.

Having found the same results for both rat cathepsin D (in mammary gland homogenates) and human recombinant, it proved that this specific action of $ONOO^-$ was an inter-species phenomenon. To further consolidate this concept, the effect of $ONOO^-$ was studied in bovine cathepsin D. Figure 39 shows that addition on $ONOO^-$ to bovine cathepsin D also results in a significant enhancement of this protease's activity; which is in turn abolished with the addition of epicatechin.

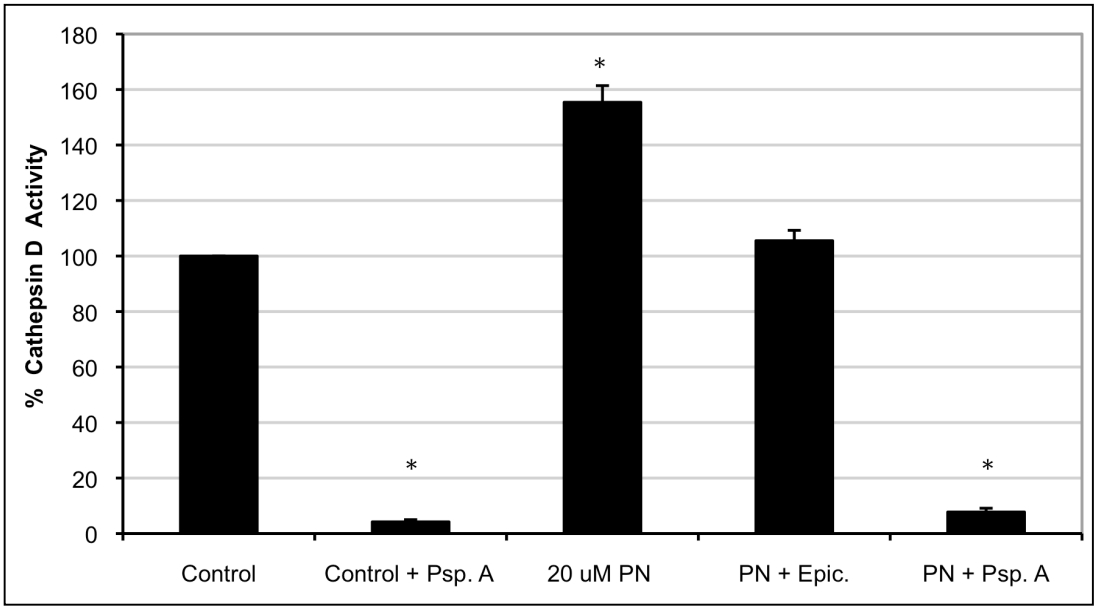

Figure 39. Effect of 20μM $ONOO^-$ with or without 30μM epicatechin and with or without pepstatin A on bovine cathepsin D (20 μM PN, Psp A, PN+Epic and PN+Psp A respectively). *p<0.05.

2.5 IDENTIFICATION OF THE CATHEPSIN D NITRATION SITE

From our previous results, it can be concluded that NO induces tyrosine residue nitration on cathepsin D. It can also be concluded that this post-translational modification has an effect on its activity, since we observed a 50% increase in activity when treating recombinant human cathepsin D with $ONOO^-$. Finally, In order to identify the tyrosine residue targeted by $ONOO^-$, tryptic digests of nitrated and non-nitrated human recombinant cathepsin D were characterized by MS. The doubly charged ion with m/z 823.92 was assigned as the nitrated form of peptide $L^{159}VDQNIFSFYLSR^{171}$. All y ions from y4 showed the extra 45Da, suggesting the nitration of Tyr^{168}. In contrast, no nitration was detected when tryptic digests from control cathepsin D were analysed. In particular, a doubly charged ion with m/z 801.42 was assigned as the non-nitrated $L^{159}VDQNIFSFYLSR^{171}$ peptide (figure 40). Nevertheless, although most of the tyrosine residues present in the protein (13 tyrosine residues out of 19), were sequenced, we were unable to sequence more than 40% of human cathepsin D, therefore it cannot be ruled out the possibility that other tyrosine

residues could also be nitrated. As shown in figure 41, tyrosine[168] is found in the heavy chain of cathepsin D and is highly conserved among species and other proteases, such as human renin, porcine pepsin and bovine chymosin. In fact, detailed protein sequence analysis reveals a possible nitration domain with the sequence F-S-F/V-**Y**-X-X-R/S.

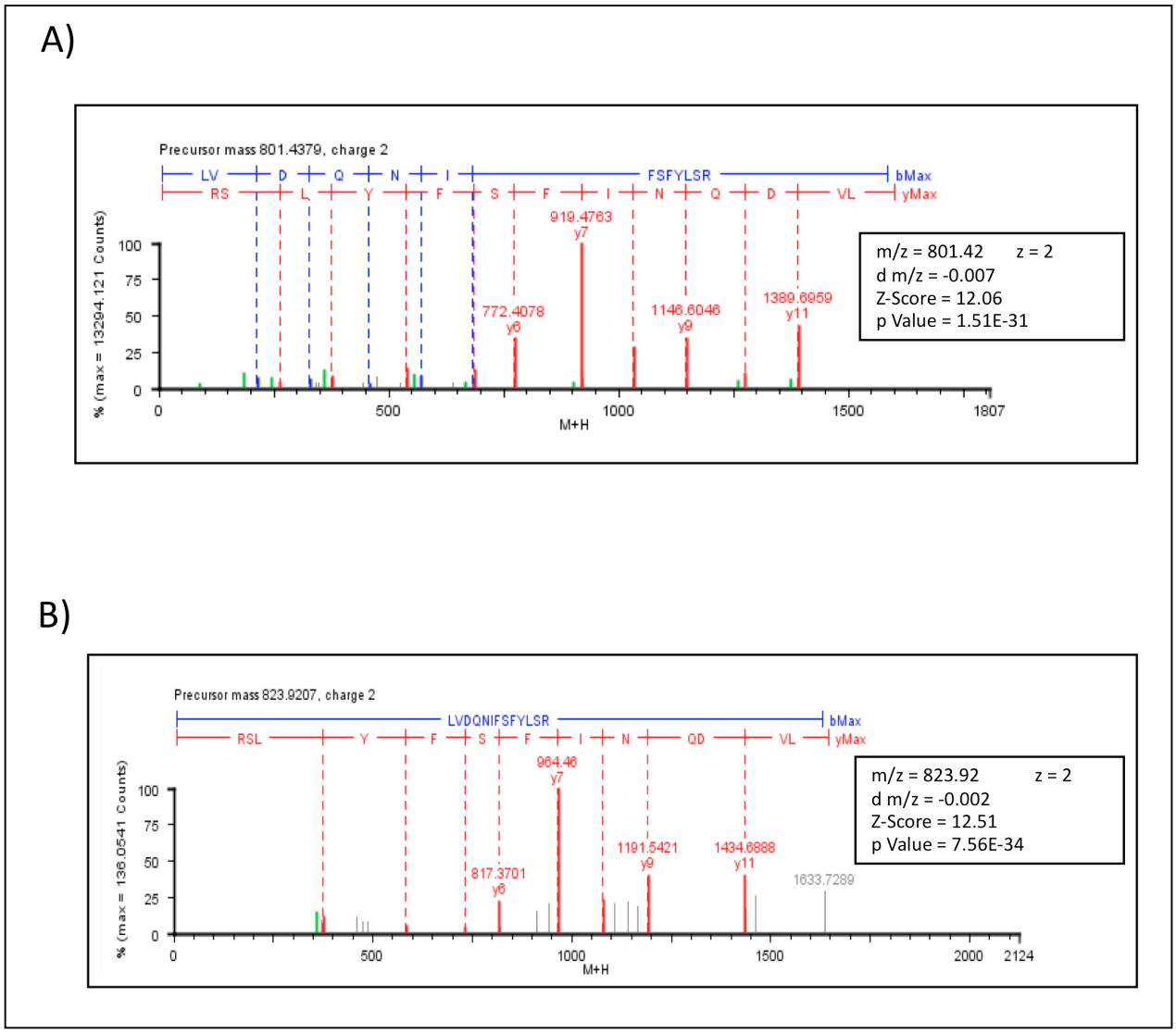

Figure 40. Representative MS/MS spectra of tryptic digests from human cathepsin D (A) vehicle or (B) $ONOO^-$-treated are shown. The mass/charge value of 823.92 for the peptide ^{159}LVDQNIFSFYLSR^{171} from CD treated with $ONOO^-$ contains a +45 Da adduct when compared to the vehicle-treated enzyme (m/z of 801.42), suggesting the nitration of Tyr[168].

Figure 41. Sequence alignments for the mammalian aspartic proteases: cathepsin D, cathepsin E, renin, pepsin, and chymosin. The possible nitration domain, which is highly conserved among different proteinases, is bolded in grey. Key: CD, cathepsin D; CE, cathepsin E; REN, renin; CHY, chymosin; PEP, pepsin. Sequence sources: Cathepsin D: Swissprot entries CATD_HUMAN, MOUSE and RAT. Cathepsin E: Swissprot entries CATE_HUMAN, MOUSE and RAT. Renin: Swissprot entries RENI_HUMAN. Chymosin: Swissprot entries CHYM_BOVIN. Pepsin: Swissprot entries PEPA_PIG.

2.6 EVALUATION OF THE EXPRESSION AND ACTIVITY OF CATHEPSIN D IN NOS2-KO AND WT MICE

Our results establish that NO plays a role in the involution process after weaning and that, although cathepsin D has increased expression early on in the process, its activity only increases after 48h of weaning (irreversible phase), and it undergoes tyrosine nitration in specific sites. Therefore, the data presented so far pointed to our initial hypothesis, tyrosine residue nitration as a post-translational modification does play a role in the activation of this protease. Of course, this does not mean that NO is solely responsible for the activation of cathepsin D, since this protease has to undergo many post-translational modifications in order to become active. Still, the increase in activity observed in

both mammary gland homogenates and human recombinant cathepsin D treated with $ONOO^-$ is evident.

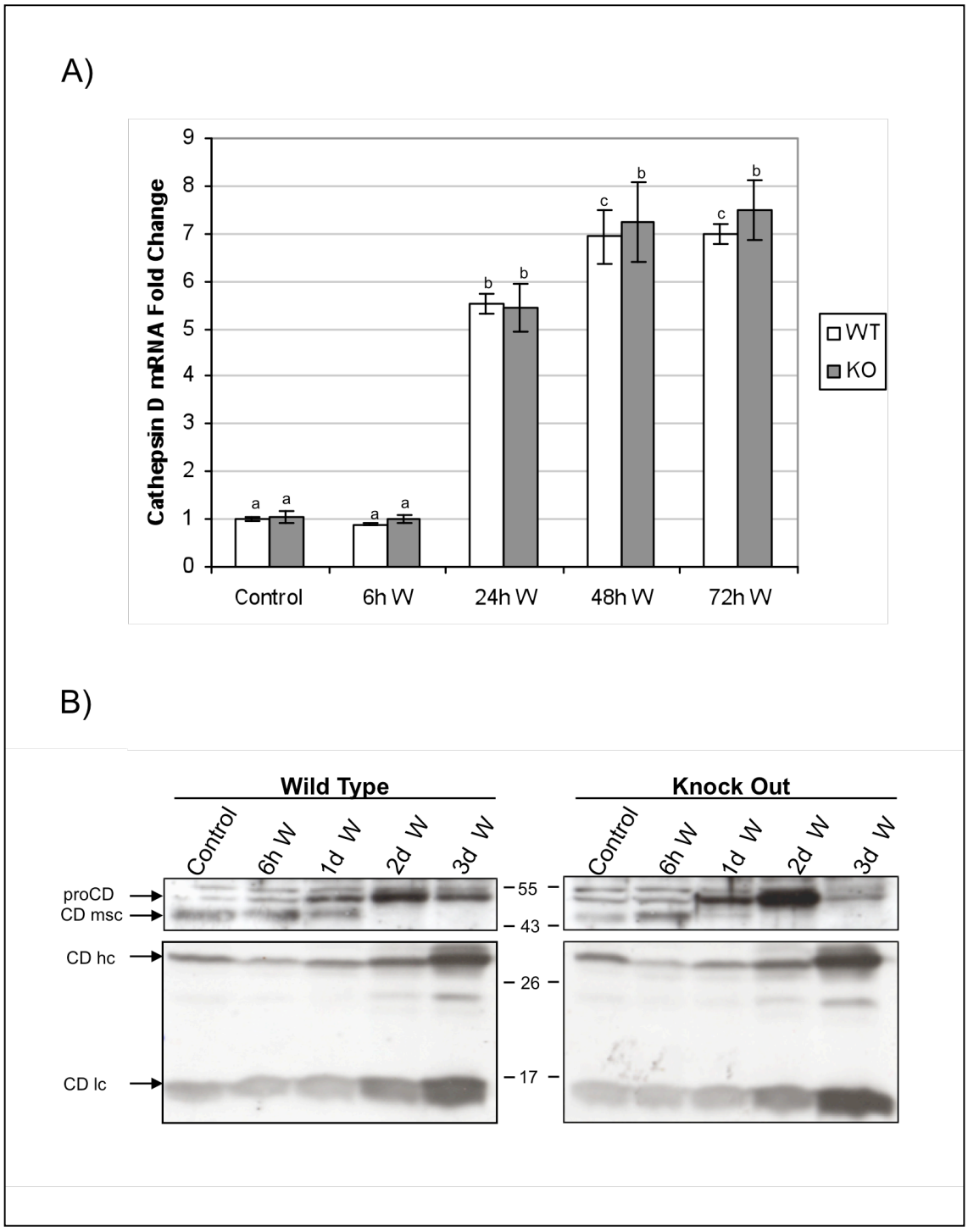

Figure 42. A) Gene expression of cathepsin from WT and NOS2-KO mice during lactation and weaning. To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p < 0.05$; the letter 'a' always

represents the lowest value within the group. A Student's t test with the Bonferroni correction was used for comparison between WT and KO at each experimental time point. *p<0.05. B) Western blot analysis of cathepsin D at the peak of lactation (control) and during mammary gland involution (weaning) in WT and NOS2-KO mice. The blot shows the presence of precursor (proCD) and mature single-chain (CD msc) forms of cathepsin D and the two degradation products of ~30 kDa (CD heavy chain; CD hc) and ~14 kDa (CD light chain; CD lc).

In the first objective of the present work, it has been established that lack of NOS2 implies a delay in mammary gland involution in the first phase of the process. In this second objective, it seemed plausible to demonstrate whether our *in vivo* model of NOS2-KO had also differences in cathepsin D activity, which would imply that NOS2 did play a role also in the second phase of mammary gland involution. First, a possible role in cathepsin D expression regulation was explored but no changes were found in mRNA and protein levels of cathepsin D between WT and KO mice, as seen in figure 42 (A). Protein levels were also checked and compared in WT vs NOS2-KO animals. In figure 42 (panel B) it can be observed that cathepsin D protein levels increased during weaning in a time-dependent manner for both WT and KO mice. This protein increase was maximal for the pro-cathepsin D 2 days after weaning.

After discarding a role of NO in cathepsin D expression regulation, its possible role in function modulation in this *in vivo* model was studied. For this, cathepsin D activity was measured in WT and NOS2-KO mice. Figure 43 shows that cathepsin D activity is significantly lower in NOS2-KO mice when compared with WT mice at 2 days and 3 days after weaning (2dW, 3dW) when cathepsin D is normally activated. NOS2-KO mice showed decreased protease activity throughout involution when compared to WT animals.

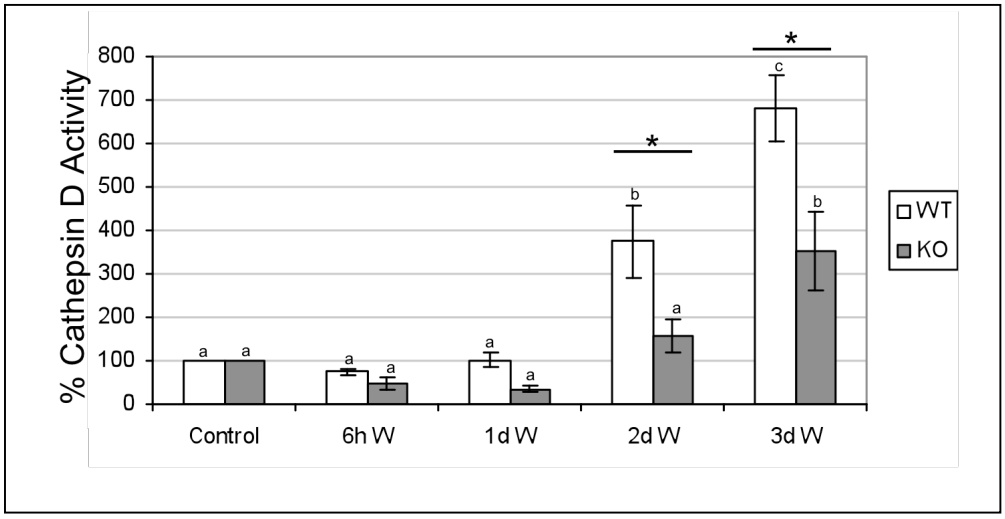

Figure 43. Cathepsin D activity measured in control and weaned mammary gland for both WT and NOS2-KO mice. To compare between the different conditions in WT or KO mice, ANOVA was performed for the statistical analysis where different superscript letters indicate significant differences, $p<0.05$; the letter 'a' always represents the lowest value within the group. A Student's t test with the Bonferroni correction was used for comparison between WT and KO at each experimental time point; *$p < 0.05$.

Recapitulating the *in vitro* and *in vivo* work in this second part of the project; the magnitude of the increase in cathepsin D activity seen in the *in vitro* work is of the order of 0.5 fold increase. This is much lower than the increase in activity seen between the WT and NOS2-KO mice (2.5 fold maximal difference). This difference was expected, since in the *in vitro* model only the direct effect of tyrosine-residue nitration on activity was measured. Of course, in the *in vivo* model many other factors will influence cathepsin D activation. These factors range from the delay in the first phase due to NO decreased levels, the many post-translational changes that cathepsin D undergoes, physiological inhibitors of cathepsins, etc, all of which could also be influenced by NO variation in concentration, that add to the direct effect of NO on the protease activity.

3. ROLE OF NITRIC OXIDE IN A LUMINAL BREAST CANCER MODEL. PRELIMINARY DATA AND FUTURE DIRECTIONS.

The third objective was to explore which of the studied physiological mechanisms did a transformed epithelial neoplastic cell recruit when exposed to high doses of NO. For this, we used a very well characterized breast cancer cell line of the luminal lineage, MCF-7, and we exposed it to varying concentrations of a NO donor, SIN-1, as described in the material and methods section.

3.1 NITRITE CONCENTRATIONS IN MCF-7 MEDIA AFTER SIN-1 TREATMENT

The first step in this part was to establish that indeed, higher concentrations of SIN-1 translated into higher nitrite concentrations. This was analysed by means of a colorimetric assay, as described in the materials and methods section. Figure 44 represents the quantitative results of this analysis and it can be observed that increasing concentrations of SIN-1 in the mM range (1 to 3mM) for 24h translate into a significant increase in nitrite concentrations. The time point of 24 hours was chosen because it is biologically plausible to think that NO concentrations are stable in a pro-inflammatory environment and we sought to study stable modifications, rather than fast signalling changes.

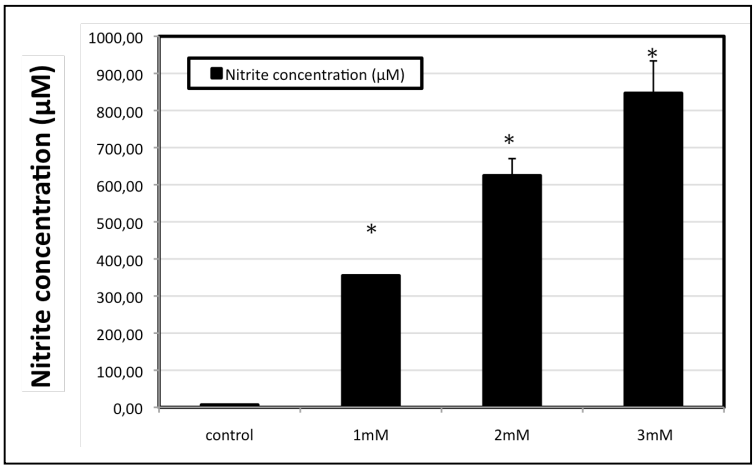

Figure 44. Nitrite concentration achieved in MCF-7 culture after treatment with different concentrations of the NO donor SIN-1 (Control, 1mM, 2mM, 3mM)

during 24h. T-test was performed to compare nitrite concentrations between control samples and treated with different SIN-1 concentrations. *p< 0.05.

3.2 MCF-7 VIABILITY AFTER EXPOSURE TO HIGH NO CONCENTRATIONS

It has been amply described that high concentrations of NO can induce apoptosis. To address this point, a MTT cell viability assay was performed. Figure 45 shows that increasing concentrations of SIN-1 resulted in a decrease of cell viability. In fact, when treating MCF-7 cells with 2 and 3mM concentrations of SIN-1, a significant decrease in cell viability was observed (20% and 34% decrease respectively).

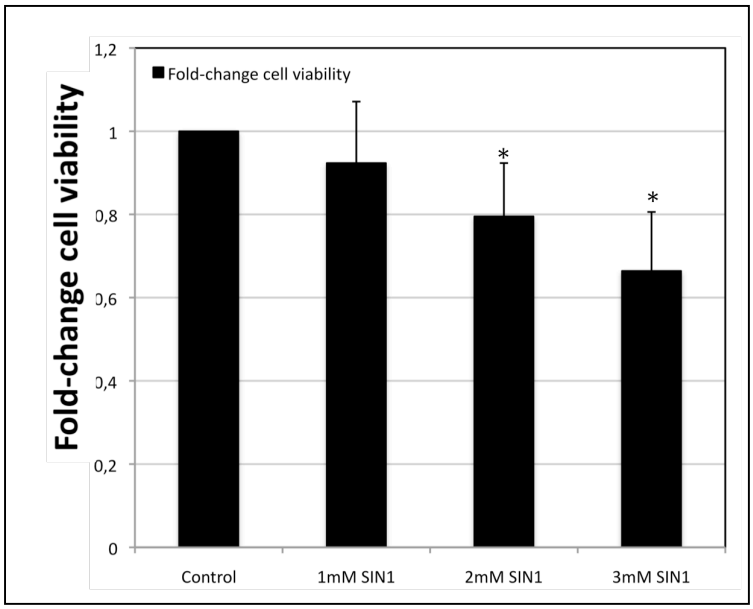

Figure 45. Cell viability in control and SIN1 treated MCF-7 cells. Treated cells were incubated with different concentrations of SIN1 for 24h and viability was measured using the MTT method as described in the materials and methods sections. T-test was performed to compare viability between control and treated samples. *p< 0.05.

3.3 EVALUATION OF AKT ACTIVATION IN MCF-7 CELLS AFTER NO EXPOSURE

AKT is a well described hub in both mammary gland involution and cancer signalling, the next exploratory step was to find if AKT was activated, hence phosphorylated, after MCF-7 exposure to high concentrations of NO. Figure 46 (panel A) shows a representative western blot were it can be seen that with increasing NO concentrations, there is an increase in pAKT, with stable concentrations of AKT. This is, to our knowledge, the first time that AKT activation has been described after long exposure to high concentration of NO. This increase in pAKT was consistent throughout repeated experiments and confirmed to be significant with a semi-quantitative method, as described in the materials and methods section (figure 46 panel B).

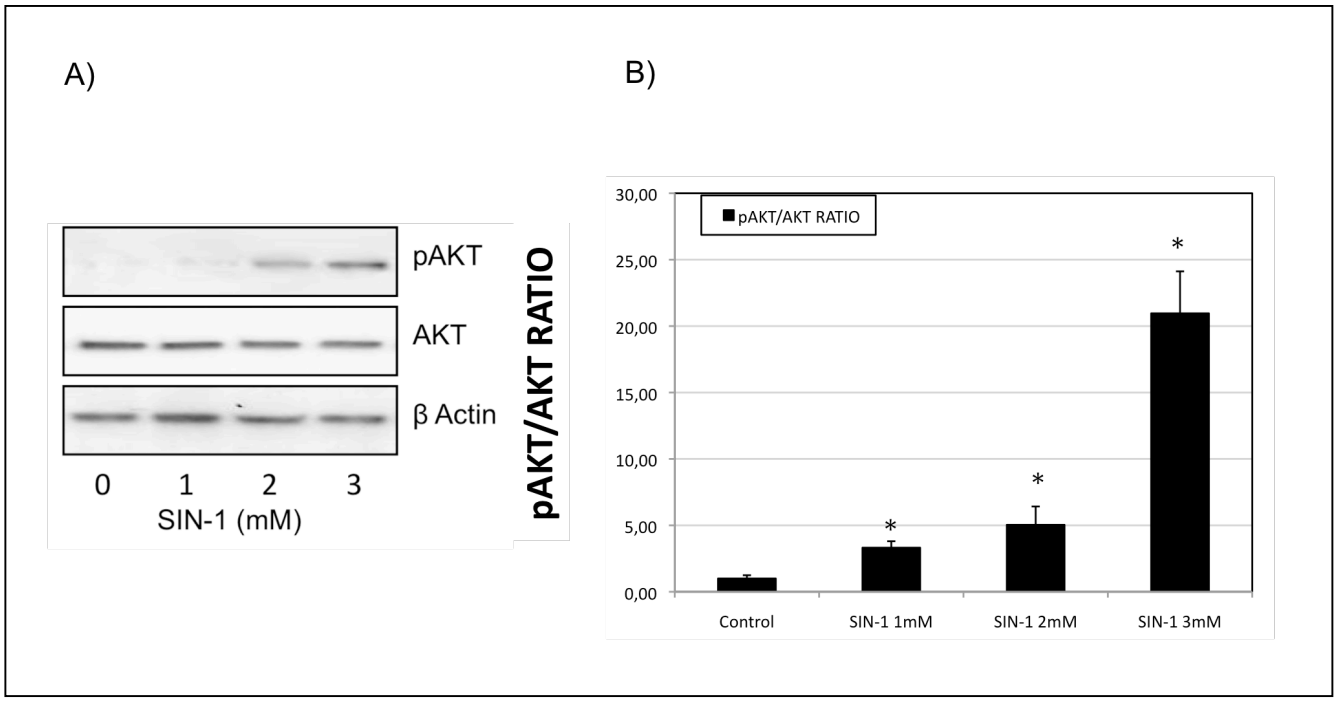

Figure 46. A) Western blot to determine AKT and phosphorilated AKT (pAKT). β-Actin was used as a loading control. The western blot is representative of at least three experiments. B) Analysis of pAKT/AKT ratio. T-student was performed to compare the pAKT/AKT ratio between the control experiment and after treatment with different concentrations of SIN-1. *$p<0.05$.

3.4 EVALUATION OF NF-κB ACTIVATION

After corroborating that NO had an effect on AKT phosphorylation in epithelial breast cancer cells, we also wanted to explore the possible effects on another of the major hubs controlling mammary gland involution. NF-κB, as well as being a major regulator of the involution after weaning process, plays an important role in tumour progression. Since it had been established that NO is a regulator of its activity during the physiological process of weaning it seemed logical to explore its possible effects on the regulation of this transcription factor in cancer cells. In this sense, levels of the NF-κB negative regulator, IκBα were evaluated by means of western blotting. Figure 47 (panel A) shows that the levels of IκBα clearly decrease after 24h exposure of MCF-7 cells to increasing concentrations of the NO donor SIN-1. When quantifying this decrease, a clear reduction pattern can be seen (figure 47 panel B).

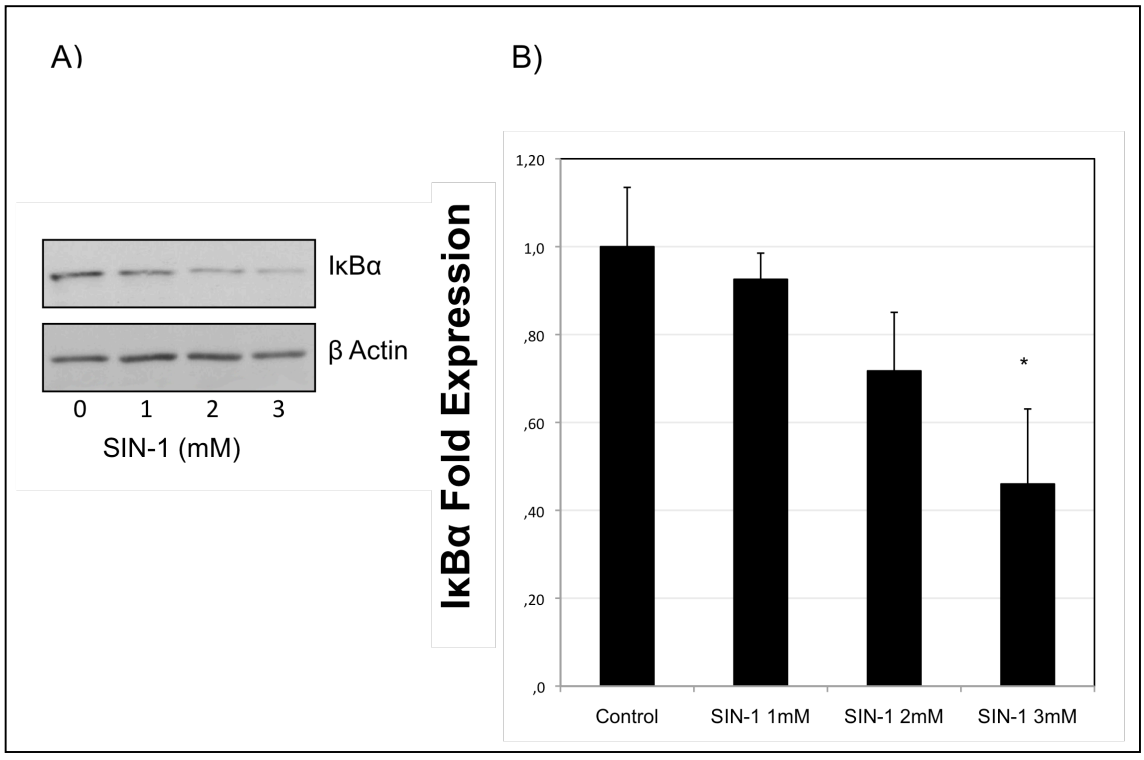

Figure 47. A) Western blot showing the decrease of IκBα in MCF-7 cells after 24h exposure to increasing concentrations of SIN-1 (0 to 3mM). β Actin was used as a loading control. The blot is representative of western blots with at least 3 samples per condition. B) Relative quantification of IκBα levels in MCF-7

normalized with β actin. The levels are relative to IκBα levels in control (non-exposure) conditions. A t-test was performed to compare IκBα levels after exposure to increasing concentrations with respect to MCF-7 non-exposure IκBα levels. *p<0.05

Another indirect approach used to further prove the possible activation of NF-κB was to evaluate the expression of MMP-9. Since we had established that MMP-9 is a transcriptional target of activated NF-κB in physiological weaning conditions in mice, we wanted to explore if MMP-9 expression was also up-regulated after treating MCF-7 cells with a high concentration of NO. The breast cancer cell line was treated with 2mM SIN-1 for 24h. Figure 48 shows up-regulation of MMP-9 gene expression after exposing MCF-7 to high concentrations of NO. This increase was statistically significant.

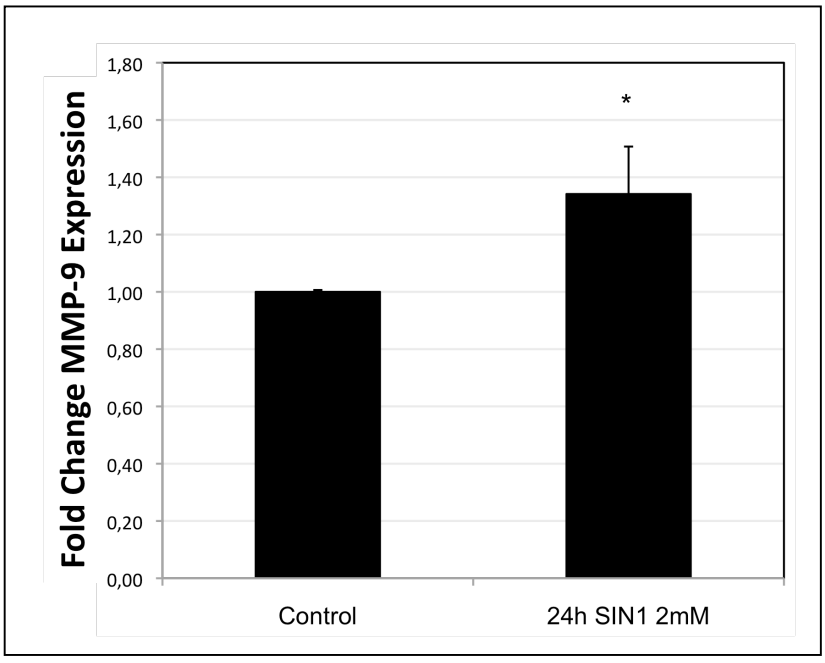

Figure 48. Real-time qPCR that analyzes de expression of MMP-9 in MCF-7 cells after treatment with high concentrations of a NO donor for different periods of time (SIN-1 2mM during 24h). The expression is related to baseline RNA levels of MMP-9 (control). *p<0.05

All in all, our preliminary data point clearly to the possibility that breast tumour cells, upon NO stimulation, activate the same mechanisms observed during weaning and that, as in this physiological process, also seem to depend on NO exposure.

DISCUSSION

DISCUSSION

Mammary gland is a highly dynamic and specialized tissue. Its development takes place mainly post-natally and its terminal functional differentiation is reached during pregnancy and lactation. This final differentiation process is regulated mainly by E, Pg, GH, glucocorticosteroids, insulin and PRL, being the most important Pg and PRL. During pregnancy, the branched ductal system subdivides into decreasingly smaller ductules, which terminate in lobules. These lobules, composed of various alveoli, consist of secretory epithelial cells that will undergo final differentiation upon parturition. When lactation ends, the excess of secretory epithelium has to be removed, in order to return the gland to a virgin-like state. This implies the need for massive apoptosis and tissue remodelling. Hence, it involves two distinct steps: a first step which is reversible and where apoptosis is the main event; and a second irreversible step that involves a change in structure, the remodelling of the ECM and an infiltration of adipocytes, so the mammary gland returns to a quiescent pre-pregnant state, ready for the next pregnancy. Selective teat sealing studies have concluded that the first phase is probably triggered by local factors such as milk accumulation, rather than by a fall of systemic lactogenic hormones (Li et al, 1997b; Marti et al, 1997). Apoptosis is thought to be initiated by anoikis (loss of cell adhesion), although this is still speculative, and the signal that initiates the extrusion of the epithelial cells to the alveolar lumen remains unknown (Monks & Henson, 2009). Involution is regulated by four main regulatory pathways, PI3K/AKT, NF-κB, STAT3 and TGFβ, which have also been implicated in cancer progression (Karin et al, 2002; Massague, 2008; Yu et al, 2009).

Gene expression studies have enabled researchers to define multiple phases of the controlled regulatory response to forced weaning on the basis of the transcriptional profile. In fact, they have allowed for a definition of three distinct phases within the first stage of involution (Clarkson & Watson, 2003; Stein et al, 2007); namely, a transitory change in gene expression within the first 24h, a prolonged change by 24h, and an increase in expression by 48h.

The analysis of the genes that make up these subsets identifies a correlation between involution-related genes and genes associated with an inflammatory response. It is of note that pro-inflammatory mediators are transiently activated as early as 12h post-weaning. This up-regulation is followed by an increase in APR genes including C/ebpδ and serum amyloids. Finally, by 48h, there is a significant increase in immune-related genes which indicates a macrophage and plasma cell infiltration. Beyond the 96h mark, apoptosis has passed its peak, the gland is being remodelled and apoptotic cells are being removed. The involution profile is characterized by expression of genes related with wound-healing and tissue metalloproteases as well as carboxypeptidases (Clarkson & Watson, 2003; Stein et al, 2007; Zaragoza et al, 2005). All in all, these published data demonstrate that gland involution utilizes some of the same tissue remodelling programs activated during wound healing. Specifically, immune cell infiltration and provisional wound healing matrix components. Hence, involuting mammary gland may present a window of opportunity for tumour cell progression. In fact, recent studies have shown that the involuting mammary gland stroma can promote tumour development and metastasis (Lyons et al, 2011; McDaniel et al, 2006).

Among the mechanisms activated in inflammatory processes, NO has been found to be generated by macrophages that take part in any such process. NO is a well-studied signal transduction agent that exerts a wide variety of functions, depending on the concentration and time of exposure. In a pro-inflammatory ambience NO is produced, mainly in macrophages, and it can reach steady concentrations of the μM range (Carcillo et al, 2003; Lee & Lee, 2005; Stadler et al, 1994). At these high concentrations, NO exerts its reactions mainly through indirect mechanisms, which require NO reacting with O_2 or superoxide, thus generating RNS. These RNS subsequently react with specific biological targets. In an inflammatory context, the vast majority of this NO is derived enzymatically form inducible NOS (NOS2). It has been previously described that NO is generated at a low rate during lactation, via NOS3 activity, but our group described a switch during involution, when NOS3 levels declined and NOS2 protein levels were increased at 24h after weaning

(Zaragoza et al, 2005). It was also established that NF-κB is bound to the promoter of this gene as early as 8 hours after weaning, and this results in the actual transcription of the gene. Taking into consideration this background, the main objective of this thesis was to further explore the role of NO in mammary gland involution, on a global level and on specific post-translational protein modifications. Our interest in further deepening on the role of NO was because NO rises in pro-inflammatory environments (such as our model of post-lactational involution), which in turn, facilitates tumour progression, hence, it is logical to think that a deregulation in the mechanisms where NO might play a role, could influence on tumour development.

1. GLOBAL ACTION OF NO IN THE INVOLUTING MAMMARY GLAND.

NO might be a signalling modulator not only during involution, but also during pregnancy and lactation where proliferation and differentiation of the mammary gland epithelial cells is maximal. Indeed, the three isoforms, NOS1, NOS2 and NOS3 are expressed in normal mammary epithelium (Khalkhali-Ellis & Hendrix, 2003); being up-regulated during involution (Islam et al, 2009; Zaragoza et al, 2005). In our model, nitrite levels as an end-product of NO did significantly increase in mammary tissue of both WT and NOS2-KO mice, but these were significantly higher in the WT animals (figure 13). The increment observed in the KO mice may be due to the activation of constitutive NOS forms, as we and others have demonstrated (figure 14) (Islam et al, 2009).

The difference in NO production between the WT and the NOS2-KO mice correlated with a different involuting phenotype, being delayed in the genetically modified animals. This is the first evidence that NO can have a role in the physiologic process of involution. Corroborating this hypothesis, mammary gland weight/total weight ratio was significantly higher in the KO mice (figure 17), the milk production was higher as was the mean pup weight (figures 18 and 19). These differences where also established in the pathology studies, where we saw that NOS2-KO mice displayed a 24h delay in the changes mammary gland goes through after weaning (figures 15 and 16). This difference could be due to alterations in the control of either lactation

or involution. Given these two possibilities, we performed further experiments to elucidate when NO was acting, taking into account that both physiological processes could be altered by the failure to increment NO levels.

The main regulatory hormone in lactation is PRL which acts in the epithelial cells of the mammary gland through binding to its receptor (PRLR) and this will activate the JAK-STAT5 pathway. Upon activation, STAT5 becomes phosphorylated in specific tyrosine residues. This phosphorylation results in the dimerization of STAT5 which translocates to the nucleus and binds to its recognition site, where it activates transcription of many genes, among which feature milk proteins such as β-casein (Miyoshi et al, 2001). It has been established in other works that NO has an inhibitory control on Ca^{2+} dependent PRL release in the adenohypophysis (Andric et al, 2003; Duvilanski et al, 1995), which could mean that a decrease in circulating NO levels would allow for further PRL release, thus prolonging lactation. This hypothesis was the basis to deepen on the STAT5 pathway, the downstream effector of PRL, in our experimental model. In our involution model we found that lack of functional NOS2 implied persistence of phosphorylated STAT5 up to 48h after weaning (figure 20). Of course, the increased PRL levels observed in NOS2-KO mice could be modulating the JAK/STAT signalling pathway avoiding p-STAT5 down-regulation during the first phase of involution. However, it is important to highlight that NO might also have a direct effect on STAT5 activity, that could account for the diminished p-STAT5 observed in WT mice after weaning. Indeed, NO has been described to inhibit Stat5 signalling pathway in macrophages either by decreasing STAT5a and STAT5b protein levels (Starzynski et al, 2006) or by reducing tyrosine phosphorylation (Bingisser et al, 1998). We also decided to check whether this lesser down-regulation was influencing STAT5 final function in mammary gland tissue. Since the levels of STAT5 expression and activation have been demonstrated to affect the extent of milk protein expression and secretion during lactation (Clarkson et al, 2006; Geymayer & Doppler, 2000; Watson & Brown, 2008) and the casein promoter is more sensitive to STAT5 transactivation than other milk proteins (Iavnilovitch et al, 2002); we studied

the pattern of β-casein expression at the peak of lactation and during the time course of involution. Increased mRNA levels of this gene were observed at the peak of lactation and after 6h of weaning (figure 21). This is not surprising since the β-casein mRNA half-life is approximately 9h (Kuraishi et al, 2002); and it has been described that levels of mRNA for α, β and γ-caseins remain high for at least 12h after weaning, decreasing thereafter (Wiens et al, 1992). However, accompanying the inactivation of STAT5, the β-casein gene expression was decreased after 24h of weaning in the WT mice, but this was not the case for NOS2-KO mice. Although decreased, we found that β-casein mRNA levels were significantly higher after 24 and 48h of weaning, in accordance with the sustained activation of STAT5 we had previously observed. The findings in this first set of experiments could explain, in part, the increased milk production in KO mice, the higher ratio in mammary gland weight/ total weight and the higher pup weight gain. Furthermore, these results support our initial hypothesis that the NOS2-KO mammary tissue is held in a functional stasis during early involution despite the loss of the suckling stimulus. Since teat-sealing experiments demonstrated that involution is triggered not only by decay of serum PRL levels, but rather by loss of the sucking stimulus, this would further support the idea that NO does have a direct effect in the first phase of involution, and not all of its effects are due to sustained PRL levels (Cross & Silver, 1956; Vina et al, 1981).

As it has been explained amply in the introduction, there are four main regulatory pathways in mammary gland involution: STAT3, NF-κB, PI3K/AKT, and TGFβ. Hence, the next step was to study the effect of NO on these. It has been reported that STAT5 and STAT3 are inversely activated during the course of a lactation/involution cycle (Clarkson et al, 2006; Watson & Brown, 2008). It has also been demonstrated that blocking STAT3 activation induces a strong delay in mammary gland involution (Chapman et al, 1999). Thus we analyzed STAT3 phosphorylation at the peak of lactation and during involution, finding that even if STAT3 activation was not delayed in NOS2-KO mice as compared to WT animals, the phosphorylation was clearly weaker (figure 22). The apparent lower phosphorylation observed by western blotting

was concordant with a significantly lower DNA-binding activity of STAT3 in the NOS2-KO mice (figure 23). Although we cannot rule out the possibility that NO could have a direct effect on STAT3 activation, it is predictable that activated STAT5 has a regulatory role in this pathway. This hypothesis is in agreement with a previous report demonstrating that over-expression or forced activation of STAT5 in transgenic mice promotes a lower level of STAT3 activation (Iavnilovitch et al, 2002).

We set out to determine the physiological relevance of a lower STAT3 activity in the NOS2-KO. We analysed *bcl-3* expression pattern since it is a known target of the STAT3 pathway (Clarkson et al, 2004). Although the functional role of this protein is still unclear, it is known to be strongly induced in mice mammary glands after 24h of weaning (Clarkson et al, 2006). Indeed, *bcl-3* expression was increased at 24h weaning, in accordance to literature, but this increase was significantly higher in the WT mice (figure 25). It is worth emphasizing that the magnitude of *bcl-3* up-regulation after 24h of weaning is the same as that found for STAT3 activity in both animal types at that time point which further supports that STAT3 is involved in the modulation of *bcl-3* expression at this time point. However, the pattern of *bcl-3* expression after 48 and 72h of weaning does not correlate with the STAT3 activation we found. Therefore, from our results we cannot discern the particular contribution of STAT3 to *bcl-3* expression during the time course of the experiment. Our data clearly show that, at least after 24h of weaning, STAT3 is phosphorylated, its DNA binding activity is increased and *bcl-3*, one of its target genes, is up-regulated to a higher level in WT compared with NOS2-KO mice. Hence, we can conclude that NO seems to play a role in the modulation of those signals leading to STAT3 molecular and functional activation in mammary tissue after 24h of involution.

Another of the main regulatory pathways involved in involution regulation is the NF-κB pathway. NF-κB is a key transcription factor in the modulation of different stages of mammary gland development and has been suggested to act as a checkpoint modulator of apoptosis (Clarkson & Watson,

1999). NF-κB is activated during pregnancy, almost completely suppressed during lactation and reactivated in early involution (Clarkson et al, 2000; Zaragoza et al, 2005). In different experimental models, NO has been shown to indirectly up-regulate NF-κB binding activity, via IκBα degradation through its phosphorylation (Fernandez-Martinez et al, 2004) or nitration (Yakovlev et al, 2007). We investigated if in our model, this factor could be regulated by NO and be responsible, at least in part, for the delayed involution seen in NOS2-KO mice.

Since the differences found in NF-κB binding activity during lactation and early involution are known to be caused by the modulation of its nuclear translocation (Geymayer & Doppler, 2000) and do not affect the p65/p50 protein levels, we analyzed by western blotting the decrease in cytoplasmic IκBα levels, which is an indirect form of measuring NF-κB activation. We found that IκBα degradation was maximal after 24 and 48h of weaning in WT mice and delayed to 48 and 72h in the KO mice. These results strongly suggest that NF-κB is differentially activated in WT and NOS2-KO mice (figure 27 A). This observation was further confirmed by the analysis of NF-κB DNA-binding activity at lactation and during weaning. Indeed, NF-κB binding activity started to increase at 24h after litter removal in the WT mice, reaching a maximum after 72h of involution, whereas, in the NOS2-KO mice, NF-κB activity was also increased but to a significantly lesser extent (figure 27 B) in agreement with the 24h delay in IκBα degradation. Of note, in the WT animals, at 72h after weaning, IκBα was re-synthesized, whereas the p65 subunit of NF-κB was still active. This is in line with the fact that NF-κB modulates the expression of its own negative regulator, this way, the negative feedback loop controls the extent of the NF-κB-dependent response. Once IκBα has accumulated again, it would interact with p65 within the nucleus and facilitate its export back to the cytoplasm. In our experimental model, our results after 72h of weaning most probably reflect the final step of this NF-κB signalling pathway, where NF-κB is involved in IκBα expression and therefore is still active. However, it is interesting to remark that this process is known to be more complicated than a simple stoichiometric-dependent regulation. For

example, the binding of p65 to IκBα and its subsequent nuclear export to the cytoplasm is modulated by deacetylases/acetyltransferases activated by different stimuli. In this way, acetylation of p65 would block its interaction with IκBα and therefore would be still active within the nuclear compartment (Chen et al, 2002; Neumann & Naumann, 2007).

On the basis of the result that NF-κB activation during weaning was delayed in NOS2-KO mice, we investigated the extent of this effect on those pathways known to be associated with the NF-κB response. We therefore analyzed the expression of 84 genes associated with NF-κB using super-array technology. In WT mice, the expression pattern found after 48h of pup removal was similar to that described in previous microarray expression studies (Clarkson et al, 2004; Stein et al, 2004). Out of the 84 genes included in the study, 30 where found to be up-regulated when compared with the lactation point. In the KO mice, even if the expression pattern was also similar to the WT at 48h of weaning, we only found 21 genes to be up-regulated. Furthermore, when comparing WT and NOS2-KO mice at 48h of weaning, 23 genes were significantly down-regulated in weaned NOS2-KO mice compared with the WT mice, and none was up-regulated. The detailed study of the data shown in table 9 provides additional and important information on the role of NO in the regulation of mammary gland involution. That is, (i) the fact that all genes found to be differentially regulated in KO animals compared with WT were down-regulated, suggests that the NF-κB signalling pathway during weaning is delayed in KO mice, and thus, NO could be an important signal involved in the mechanisms that trigger the NF-κB response. Since none of these genes was up-regulated, we conclude that NO does not participate in the silencing of any NF-κB associated genes. Also (ii) some of the down-regulated genes, such as caspases, *bcl-3*, colony-stimulating factors, *elk-1* or *tnf*, are largely considered to be pro-apoptotic (Gouon-Evans et al, 2000; Varela & Ip, 1996). For instance, an 80% decrease in *fasl* expression in NOS2-KO mice would suggest a protection from apoptosis in these mice. In fact, no apoptotic cells were found during the first 3 days of involution in *fasl* deficient mice (Song et al, 2000). Moreover, the authors of that study suggest

a defect during the first phase of involution that does not affect the second phase. And (iii), some other genes are know to be anti-apoptotic, and one would expect them to be up-regulated in a process with less severe apoptosis as in the NOS2-KO mice. Strikingly, those genes where also down-regulated. This is the case of intercellular adhesion molecule 1 (ICAM-1), which is reduced 55% in NOS2-KO compared with WT mice. It has been suggested that the response of adjacent cells to localized apoptosis is to induce the expression of cell adhesion molecules such as ICAM-1 to maintain survival. Nevertheless, a correlation between the extent of apoptosis and the expression of ICAM-1 has been demonstrated (Clarkson & Watson, 1999). Therefore, it would be reasonable to think that a less severe apoptosis during the first phase of involution would induce a poorer response in terms of ICAM-1 expression. This could also apply to the poorer activation seen of the pro-survival signal exerted by AKT (figure 26). The definition of the first stage of involution can explain this apparent functional contradiction. On the whole, involution is the addition of both pro- and anti-apoptotic signals that, upon persistence of forced weaning, will unbalance the response towards the irreversible phase of apoptosis and remodelling of the gland; but the remaining surviving cells will recruit survival pathways in a controlled manner in order to ensure the adequate remodelling. Finally, the data suggest that, although NF-κB is in some way involved in the modulation of all of the genes listed, there is not a solely specific pathway that can be affected by the absence of NOS2 expression after 48h of weaning.

Given the amount of pathways intervening and all the events that take place as well as the strict order they take place in, it can be inferred that, like in any physiological process, mammary gland involution has to be extremely well orchestrated. This way, nothing is left to chance. The characteristics of the process and the early differences seen between WT and NOS2-KO mice suggest that, although NO targets are at the very onset of involution, including the PRL receptor/JAK/STAT pathway, the nature of the mammary gland allows the amplification of the signal through the modulation of several pathways. This means that NO does not seem to only affect one pathway, but many.

As explained, mammary gland involution has two distinct phases, the first phase, characterized by apoptosis, and the second phase, characterized by gland remodelling. This second phase is intimately related with the first, because, although the second can happen without the first (Chapman et al, 2000) some of its principal actors are also modulated by key regulators of the first phase. Such is the case of metalloprotases (MMPs). Activation of NF-κB can induce apoptosis, with concomitant loss of extracellular matrix contacts (Gukovskaya et al, 1997). The rise in NF-κB binding activity in our model coincided with an increased rate of apoptosis in the mammary gland. On the other hand, the extent of apoptosis has been reported to correlate with an increase in MMP activity at the onset of major remodelling of the gland during the second phase of involution (Clarkson & Watson, 1999; Lund et al, 1996). In fact, MMPs are synthesized as latent zymogens, which have to be biologically activated by cleavage of their proenzyme peptide. Accumulating evidence suggests that the activation of NF-κB is one of the most important signalling events required for the inducible expression of MMP-9 in response to different stimuli (Li et al, 2009). Consequently, we explored the functional consequences of delayed NF-κB activation in NOS2-KO mice during the second phase of involution. Since we found that MMP-9 induction was significantly more pronounced during weaning in WT than in NOS2-KO mice (figure 29), concordant with the delay found in NF-κB binding activity in the latter, we hypothesized that MMP-9 transcription could be directly dependent on NF-κB. Our hypothesis was confirmed by ChIP assay (figure 30), were we found that, in WT mice, at 48h after weaning, NF-κB was in fact bound to the MMP-9 promoter, and this was not the case for NOS2-KO mice.

MMPs are essential in the remodelling of the ECM that takes place during mammary gland involution (Lund et al, 1996). In fact, MMP-9 is a key protease governing degradation of basement membrane collagen as well as different types of gelatine (Green & Lund, 2005). Our results suggest that the effect of NO in the regulation of mammary gland involution is not limited to the first phase of involution. Therefore, it seems that by the modulation of either PRL signalling and transcriptional factors activities and/or their interactions,

the signal generated by NO during the first phase of involution is amplified throughout the cascade of events triggered during the second phase.

2. SPECIFIC ROLE OF NO IN REGULATION OF MAMMARY INVOLUTION AFTER WEANING. NITRATION AS A POST-TRANSLATIONAL MODIFICATION.

As said before, NO at high concentrations exerts its functions through the reaction with O_2 or superoxide to generate reactive nitrogen oxide species (RNS) that subsequently react with biological targets, inducing nitrosative modifications. Nitrosative modifications are selective processes that target precise molecular sites in proteins or lipids for gain or loss of function, in a manner somewhat analogous to the better-known phosphorylation or acetylation signal transduction mechanisms (White et al, 2010).

Tyrosine nitration is a post-translational modification of protein tyrosine residues that is mediated by $ONOO^-$. Nitration of tyrosine or tyrosyl groups of a protein modulates protein function and its involved in signal transduction, which leads to an alteration of cellular metabolism and function. In particular, $ONOO^-$ -induced protein oxidation often leads to a loss of protein activity (Gorg et al, 2007; MacMillan-Crow et al, 1998), seldom to activation (Ji & Bennett, 2003; Ji et al, 2006; Rebrin et al, 2007). Because of its recognized significance, there is an increasing interest in identifying nitrated proteins in order to gain a better understanding of their involvement in different biological processes, as is the case for mammary gland remodelling.

So far, in our work, we have established that NO concentration rises with weaning, and this is due, at least in part, to an up-regulation of NOS2. We have also established that NO targets are at the very onset of the process, but given the dynamic nature of the mammary gland tissue and the multiple interconnected pathways that partake in involution, the specific changes/signals induced by NO are amplified throughout the whole process. We have established that a decrease in its concentration translates into a global delay in involution; still, we also wanted to assess if NO induced specific post-translational modifications.

In order to approach this question, proteomic studies were performed using mammary gland homogenates from lactating rat and after 72h weaning. From the 2D gel studies (figure 32), it was confirmed that protein nitration is increased during weaning. The differentially nitrated proteins were recovered and from the gel and identified using MALDI-TOF-MS. As explained before, mammary glands were not milk-depleted because milk stasis plays an important role in involution and we were interested in the complete protein profile. As a consequence, low abundance proteins could not be detected, hence it cannot be ruled out that more proteins could be nitrated after weaning. The analysis of the protein map showed a relative abundance of albumin, immunoglobulin chains and casein which is expected, due to milk stasis. Nevertheless, it is possible that these fragments of nitrated albumin could play a more important role since it has been described that the injection of small peptides derived from milk into the mammary gland accelerates the process of involution (Shamay et al, 2002). However, a 48KDa nitrated protein was identified, with coverage of 2.5% as cathepsin D in mammary gland 72h after weaning.

Cathepsin D is a major aspartic protease of endosomes and lysosomes and member of the pepsin family of proteases (Tang & Wong, 1987). Cathepsin D is increased during weaning, playing a key role in protein degradation during mammary gland apoptosis; indeed, cathepsin D is known to be involved in the mitochondrial caspase-dependent intrinsic pathway of cell death (Castino et al, 2007). Despite the relevance of this protease in physiological and pathological events, little data exists on the factors that modulate its secretion, activity and extracellular function in lactating mammary tissues. It is known that cathepsin D undergoes a complex series of post-translational modifications from its pre-pro form to its fully activated double-chain mature form, and recently it has been proved that these changes may vary depending on the physiological process it participates in (Margaryan et al, 2010). Finding cathepsin D among the nitrated proteins oriented to the possibility of tyrosine nitration as an added post-translational modification.

Indeed, the stagger between the rise of mRNA and protein levels and the increase in the protease activity (figure 35), pointed to a possible post-translational modification. Immunoprecipitation studies (figures 33 and 34) confirmed cathepsin D nitration in mammary gland homogenates after 72h weaning; hence rose the hypothesis that this post-translational modification was, at least in part, responsible for its activation. As presented in the results section, cathepsin D of rat, human and bovine origin does become more active after exposure to $ONOO^-$. This increase in activation was of the order of 0.5 fold in the *in vitro* studies (figures 37, 38 and 39). With these results we can conclude that tyrosine nitration does play a role in cathepsin D activity enhancement and that this post-translational modification is conserved among different species. Of course, even though we have described in this work cathepsin D nitration for the first time, and that post-translational modification seems to play a direct role in its increased catalytic activity, other proteins, also affected by NO, could be modulating cathepsin D activity indirectly. Also, this protease needs of other modifications that could also be modulated by NO, in order to become fully activated, this could explain the 2 fold difference observed between the WT and the NOS2-KO mice experiments (figure 42 A).

The next question raised by the results obtained was how nitration of a tyrosine residue can affect cathepsin D activity. Immature cathepsin D can be partially auto-activated, leading to the mature form of the enzyme; therefore a specific nitration could be somehow accelerating or interfering with the autoprocessing of the immature form of cathepsin D. However, our experiments with WT and KO animals show the same pattern of proteolytic cathepsin D cleavage (figure 42B). It has been shown that pro-cathepsin D requires lysosomal cysteine proteases for the final processing of its pro part and we have demonstrated that recombinant human cathepsin D undergoes *in vitro* nitration and activation in a cysteine protease-free buffer (figure 38). Therefore it can be excluded that auto-processing is the mechanism by which nitration increases activity in cathepsin D. Intriguingly, in the rat mammary gland homogenates, we found that both the 48kDa mature single chain and the 34kDa heavy chain of the mature two-chain enzyme had tyrosine nitration (figures 33, 34 and 37 panel C), which points to the possible cellular

compartment this nitration could occur in, and this also suggests that cathepsin D becomes nitrated at an earlier step of the maturation process along the endosomal-lysosomal pathway.

Our results strongly suggested the existence of nitration domain conserved among species, since both rat, human and bovine cathepsin D can be nitrated by $ONOO^-$ (figures 37, 38 and 39). Moreover, the fact that this nitration induced a slight increase in enzyme activity compared with non-nitrated cathepsin D suggests that NO can modulate cathepsin D activity in a direct manner. One could argue that the protein context surrounding cathepsin D or the conformation of cathepsin D *in vivo* could be other than that favoring the nitration. Nevertheless, we have shown that cathepsin D from extracts of control lactating mammary gland can also be nitrated *in vitro*, and that this nitration induces an increase in the enzymatic activity (figure 36).

On the whole, our experiments show that low degree of nitration increases cathepsin D catalytic activity, not only in recombinant human cathepsin D, but also in tissue homogenates, suggesting that direct nitration of this enzyme is responsible for the enhanced activity observed. Also, the specific tyrosine nitration site was sought. Figure 40 shows MS characterization of human recombinant cathepsin D before and after $ONOO^-$ treatment. After being exposed to $ONOO^-$, the tyrosine residue in the 168 position appeared nitrated. Although most of the tyrosine residues present in cathepsin D structure were sequenced (13 residues out of 19), we were unable to sequence more than 40% of human cathepsin D, hence, it cannot be ruled out that other tyrosine residues could be nitrated. Tyr^{168} is found in the heavy chain of cathepsin D and it is highly conserved among species (figure 41). In fact, a detailed protein sequence reveals a possible nitration site within the sequence F-S-F/V-**Y**-X-X-R/S. Further studies would be needed in order to determine if other proteases with the same conserved domain can also undergo *in vitro* and *in vivo* nitration.

It has been amply described by other groups that tyrosine nitration can induce profound changes in protein structure and function due to a shift in the

pKa of the tyrosine hydroxyl group (Sokolovsky et al, 1967). Also, the importance of pH in the modulation of cathepsin D activity has been extensively documented (Beaujouin & Liaudet-Coopman, 2008; Khalkhali-Ellis et al, 2008; Matsuyama et al, 2000). Indeed, while an acidic pH leads to a fully active enzyme, at pH 7.5 a molecular bond between specific tyrosine residues and the catalytic aspartate residues stabilizes the protein structure in an inactive conformation (Masa et al, 2006). Although Tyr^{168} is 17.76 Å (1 Å=0.1 nm) away from the active site and such a distance is not compatible with a direct hydrogen bond with the catalytic residues, it would be conceivable that the nitration of Tyr^{168} could favor an irreversible active conformation of the enzyme by blocking a molecular bond crucial for cathepsin D folding. Moreover, an inhibitory domain has been localized at the N-terminus of cathepsin D (Masa et al, 2006); at pH 7.5 the mature N-terminus is repositioned into the active site cleft, interacting with the catalytic residues. Since we cannot exclude the possibility of nitration in other non-identified tyrosine residues, the *in vivo* nitration of a specific tyrosine at this site could block the binding of the inhibitory pro-peptide and allow free access of the substrate.

In our work we find that levels of cathepsin D mRNA are highly up-regulated in the first day of involution, while its catalytic activity is delayed and it increases in days 2-3 of involution, this further supports the importance of post-translational modifications in protein function (figures 35, 42 and 43). In line with our finding, another published paper studied mammary cathepsin D protein levels and proteolytic activity in a mouse model from late pregnancy and throughout lactation and involution (Margaryan et al, 2010). The authors also find cathepsin D protein levels to be elevated from the initial stages of involution, but in the first three days they find primarily a mixture of procathepsin D and active single-chain in the tissues. On day 4 of involution, a large quantity of mature, double chain cathepsin D appears. So it seems that cathepsin D does not have a single function or state, but rather, it holds a highly plastic activity. In the same paper, another observation is the potential role of the glycosilation pattern of this protease as a regulatory tool. Indeed, changes in glycosilation are unlikely to impact catalytic activity or substrate

specificity of cathepsin D (Beyer & Dunn, 1996), but alterations in glycosilation may contribute to altered trafficking and compartmental localization of pro-cathepsin D and cathepsin D, thus dictating changes in biological functions (Erickson et al, 1981; Fortenberry et al, 1995; Varela & Ip, 1996).

Finally, we present a schematic figure on the global role NO exerts during post-lactational mammary gland involution. As discussed, we have found that NO in this pro-inflammatory setting can modulate certain key pathways (STAT5, STAT3, NF-κB and AKT) as well as induce specific protein modifications that will add to the perfect orchestration of this highly dynamic process.

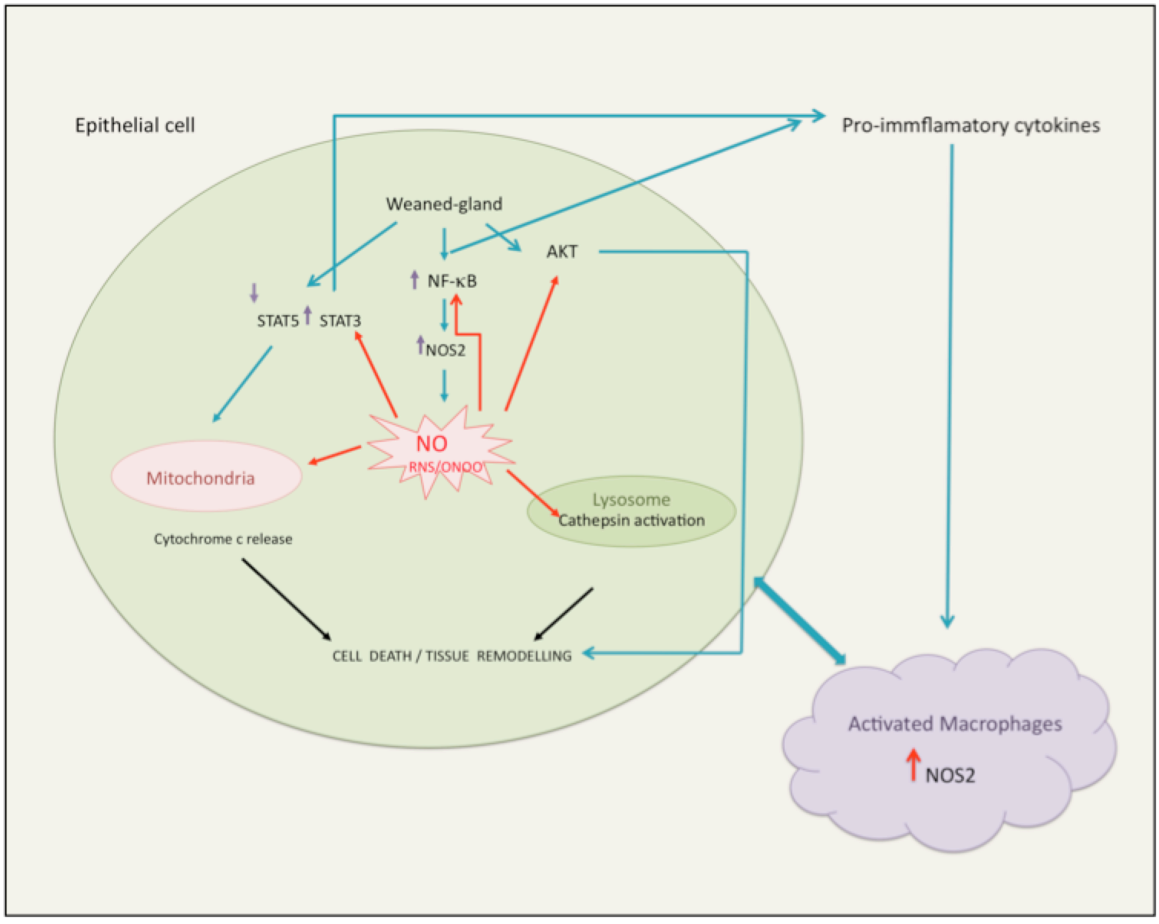

Figure 49. Global effect of NO during weaning. Upon weaning, involution starts and initial activation of Stat3 and NF-κB occurs. NF-κB induces NOS2 which will activate NO formation. NO, by means of formatting RNS will modulate the involution process, having an impact on Stat, NF-κB and AKT

function. Also, it will induce specific nitrosative modifications, eg, tyrosine nitration of cathepsinD, which will enhance its activity, having an impact on the tissue remodelling process. The NO source has also been proved to be activated macrophages that invade the mammary tissue upon pro-inflammatory signalling, exerted by STAT3 and NF-κB activation. Macrophages phagocyte apoptotic cells and suppress inflammation, allowing for adequate tissue re-structuring.

3. ROLE OF NO IN THE REGULATION OF PATHWAYS INVOLVED IN BOTH INVOLUTION AND CANCER PROGRESSION: PRELIMINARY DATA

Understanding the relationship between the physiological functions of different molecules during development and their pro-tumorigenic functions in carcinogenesis is critical for developing appropriately targeted therapeutics. The relevance of our findings is granted by the fact that the main regulatory pathways in mammary gland involution: STAT3, NF-κB and PI3K/AKT pathways are related with pro-survival mechanisms in cancer. This means that physiological processes have to be very well orchestrated, the signalling pathways that are recruited have to be switched on and off at the appropriate times and that depending on the context, transcription factors can have pro-survival or pro-apoptotic functions. An error in the regulation may facilitate an oncogenic event that, in the appropriate microenvironment, can progress and give rise to a neoplasm.

Breast cancer incidence is rising among young women, and this is due to many factors, but one is delay in age of the first conception (Lord et al, 2008). This might seem a paradox, since it is a well established epidemiological fact that pregnancy is associated with a reduction in a woman's lifetime risk of developing breast cancer (Albrektsen et al, 1994; MacMahon et al, 1973; Rosner et al, 1994). However, it is also recognized that the protective effect of pregnancy is neither immediate nor constant, and, while parity is associated with a lifetime reduction in breast cancer risk,

studies in breast cancer incidence have demonstrated a transient increase in breast cancer risk in the years immediately following pregnancy, independently of age (Albrektsen et al, 2005; Chie et al, 2000; Lambe et al, 1994). This increase has been shown to persist for at least ten years for a woman under the age of 25 at delivery (Lambe et al, 1994). Also, delaying pregnancy further increases this transient risk, and a maternal age greater than 30 at first birth results in both an increase in incidence and a longer increased risk that persists for up to 30-50 years post-partum (Albrektsen et al, 2005; Chie et al, 2000; Dupont & Page, 1987; Lambe et al, 1994; Liu et al, 2002). Hence, pregnancy might both suppress and promote breast cancer, since, the initial effect of any completed pregnancy is an increased risk, but a cross-over to long term protection is seen in women of younger maternal age, and high parity (Lord et al, 2008). This protective cross-over effect is lost when the age of first birth is above 35 (Lyons et al, 2011; Lyons et al, 2009; Rosner et al, 1994; Schedin, 2006).

There are two main hypotheses to explain this transient increased risk in breast cancer associated with pregnancy. The first, exposure to pregnancy related hormones such as E, Pg, GH and IGF-1 are associated with promotion of breast cancer cell proliferation (Schedin, 2006). The second hypothesis postulates that mammary gland involution, which goes hand-in-hand with a terminated pregnancy implies the activation of certain mechanisms that resemble pro-tumorigenic wound-healing environment. In fact, the latter theory seems to better explain the worse prognosis of patients when breast cancer is diagnosed after birth in comparison to those who are diagnosed during pregnancy (Guinee et al, 1994; Schedin, 2006).

To further support the hypothesis that the involuting mammary gland drives breast cancer progression, a recent work published by the Schedin group (Lyons et al, 2011) proved that tumour cell exposure to the involuting mammary microenvironment promotes breast cancer metastasis and this is via ECM proteins, such as fibrillar collagen, and COX-2. The authors demonstrate that COX-2 activity in the normal involuting gland is required for the deposition of fibrillar collagen and that this collagen up-regulates COX-2

expression in tumour cells which promotes invasion. Notably, COX-2 inhibition by NSAIDs during normal post-partum mammary gland regression blocked the production of fibrillar collagen so it is hypothesized that NSAIDs taken during mammary gland involution could be an effective chemoprevention strategy for women who are at higher risk of developing breast cancer (Lyons et al, 2011). In agreement with this, epidemiological studies have correlated COX-2 inhibition with NSAIDs with decrease breast cancer recurrence and related deaths (Holmes et al, 2010; Kwan et al, 2007).

Having reviewed the argument for the use of NSAIDs in this context, we postulate that the use of anti-inflammatory drugs could also have an impact on the NO regulated mechanisms we have described in the present work. Indeed, NO activates COX-2 (Cahlin et al, 2000; Kim et al, 2005) and aspirin-like drugs have been described to inhibit both NOS2 expression and catalytic activity (Amin et al, 1995). However, other influencing factors cannot be ruled out.

In the final part of the present work, we raised the question of whether high concentrations of NO had a specific impact on the pathways that are regulated by this molecule during mammary gland involution, but in an epithelial breast cancer model. The effect we have found in our model supports that high doses of NO could be having a dual effect: on one side, the induction of apoptosis, which is in concordance with other studies (Borutaite & Brown, 2006; Cleeter et al, 1994; Thomas et al, 2004). MCF-7 cells where less viable when exposed to increasing concentrations of NO (figure 45); on the other, high concentrations of NO seem to be activating pathways clearly related with survival such as AKT and NF-κB (figures 46 and 47) and interestingly, there is a parallelism with the physiological process of involution.

4. FUTURE WORK

All in all, our data point to the possibility that breast tumour cells upon NO stimulation activate the same mechanisms observed during weaning; and that, like in the physiological process, the activation of these pathways also

seem to depend, at least in part, on NO exposure. Of course, these are only very preliminary data. Indeed, the direction to take this would be to further corroborate activation of the PI3K/AKT/mTOR pathway through the analysis of activation of downstream effectors and the quantification of expression of targets genes of this pathway. Another interesting set of experiments would be to specifically address at which point of the signalling pathway does NO interfere, does it activate AKT directly, or through the activation/inhibition of up-stream regulators? Or on the contrary, does it become phosphorylated through the loss of the negative feed-back loop exerted by mTOR should mTOR be inhibited by direct or indirect effects of NO?

Although other works have amply proved that NF-κB can be activated by NO through mechanisms that implicate direct IκBα tyrosine nitration (Yakovlev et al, 2007) or its phosphorylation (Gochman et al, 2011), it would be interesting to know if at these high concentrations, NF-κB is activated and which target genes does it have. In this line of thought, it would be important to corroborate if the up-regulation in MMP-9 expression we have observed in our MCF-7 model (figure 45) is due to NF-κB transactivation.

It would also be pertinent to study these pathways in more physiological conditions, since the nitrite concentrations achieved with the amount of SIN-1 used in our work model are above physiological (figure 41), even when the microenvironment is that of an inflammatory status. But all in all, we believe that the data provided so far, does support the hypothesis that the same regulatory activity exerted by NO in different pathways implicated in mammary gland involution after weaning is also seen in malignant breast epithelial cells. Of course, many studies have already been published on the role of NO in cancer, and most of these works support the hypothesis that a concentration of NO in the μM range, such as the concentrations achieved in pro-inflammatory environments, promote tumour regression and cell death (Singh & Gupta, 2011). This has been the rational for the development of drugs such as NO donating NSAIDs (NO-NSAIDs) (Williams, 2011). Still, many questions are still pending on the real anti-tumour effects of these

drugs, their safety and whether if their anti-tumour effects are really dependant on the NO-donating part or, just on the anti-inflammatory effects of these agents (Rigas & Williams, 2008).

Understanding the relationship between physiological functions of a particular process during normal development and homeostasis is crucial, since all physiological processes are a finely balanced mix of signalling pathways that can be contradictory in their functions. Indeed, in our NOS2-KO mouse model there is a de-regulation in this balance during mammary gland involution. This is due to an alteration of NO concentration which gives rise to a delayed apoptosis, but also to a down-regulation of pro-survival-related genes. This phenomenon could be due to the fact that losing NO pro-apoptotic stimulus also alters the pro-survival counter-back. Hence, in a pro-inflammatory microenvironment such as post-lactational regression, NO will induce apoptosis, but could also facilitate proliferation in the remaining cells via the activation of pathways such as the PI3K/AKT pathway.

We know that a normal cell, on its way to neoplastic transformation will recruit already existing mechanisms in order to survive, only that these mechanisms will be de-regulated and adapted in order to meet the cell's new needs. Indeed, in our *in vitro* preliminary data in a breast cancer luminal model, we found that, after exposure to high concentrations of NO, transformed cells recruited some of the specific mechanisms used during the physiological process of involution. Of note, these mechanisms have been amply described as pro-survival in a neoplasic setting, which could mean that, although high concentrations of NO imply cell death, the surviving cells could be ensuring their endurance activating pro-survival mechanisms that use the apoptosis-inducing noxa in their own benefit, and this concept has deep therapeutical implications.

CONCLUSIONS

CONCLUSIONS

The present study emphasizes the importance of NOS2 up-regulation and of NO concentration increase in the fine-tuning of the coordinated changes that occur during mammary gland involution. We conclude that maintaining the correct levels of NO, together with other regulatory factors in the mammary gland, is essential for the modulation of transcription factor activities, post-translational modifications and interactions that lead to the involution and remodelling of the mammary tissue once lactation is terminated.

1. In the present work we have studied the effects of the absence of NOS2 in mammary gland involution after lactation. This absence results in lower NO levels in NOS2-KO mice, which in turn delays involution. We have evaluated this delay by means of immunohistochemistry studies, milk production and caspase 3 activation.

2. In order to gain a deeper knowledge on the differences observed at a macroscopic and histological level, molecular studies were carried out. The main signalling pathways that control post-lactational regression are clearly delayed in NOS2-KO mice. The most remarkable differences have been observed after 48 hours of weaning, specifically in the activation of the Stat5/Stat3 switch and the activation of NF-κB. This results in a de-regulation of their direct transcriptional targets (e. g. β-casein, Bcl-3, MMP-9); affecting the first phase of the process, where apoptosis is mainly involved, and also the ECM remodelling that takes place during the second phase of involution.

3. NO not only plays a role on the fine tuning of the global process of involution, but also induces specific post-translational modifications that impact on protein function. In our model we found a specific tyrosine-nitration in cathepsin D, a lysosomal aspartic endopeptidase, crucial for tissue remodelling. Our *in vitro* and *in vivo* experiments demonstrate that this tyrosine nitration results in cathepsin D activation.

4. Concerning breast cancer, *in vitro* exposure to high concentrations of NO reduces viability of epithelial breast cancer cells; but the remaining cells recruit some of the specific mechanisms activated during the physiological process of involution. In our work we found that high concentrations of NO induced cell death but also AKT phosphorylation and NF-κB activation, which are pro-survival signals.

RESUMEN

INTRODUCCIÓN

La glándula mamaria es un tejido complejo y muy especializado que, a lo largo de la evolución se ha ido modificando para proveer al recién nacido de leche, alimento básico en la primera etapa del desarrollo de los mamíferos, que aporta nutrientes y protección inmunológica (Vorbach et al, 2006). El tejido mamario sufre una extensa remodelación en cada ciclo de embarazo/lactancia. Durante el embarazo, se produce una proliferación y diferenciación del epitelio ductal y alveolar para adquirir un fenotipo secretor (Benaud et al, 1998). Cuando cesa la succión, al final de la lactancia, se produce la muerte de las células epiteliales secretoras por apoptosis y se induce la diferenciación de los adipocitos circundantes de modo que la glándula mamaria queda preparada para un nuevo ciclo embarazo/lactancia. Este fenómeno fisiológico, conocido como involución, está estrechamente regulado y se produce en dos fases; la primera fase tiene lugar hasta las 48h del destete, es reversible y se caracteriza por la apoptosis del epitelio secretor alveolar, sin que se produzcan grandes cambios en la estructura de la glándula. Conforme avanza la involución, entramos en la segunda fase, de carácter irreversible, en la que se produce la activación de proteasas que degradarán la membrana basal y la matriz extracelular (Reinhardt & Lippolis, 2009). A su vez, en esta segunda fase, se produce la adipogénesis, o rediferenciación de los adipocitos, de modo que la glándula se remodela hasta llegar a un estado similar al que tenía antes del embarazo (Baxter et al, 2007; Green & Streuli, 2004).

Este proceso fisiológico y secuencial está sometido a una estrecha regulación para que se produzca la perfecta coordinación entre cada una de las fases. Diversos estudios han demostrado que muchos de los genes sometidos a importantes cambios de expresión en la glándula mamaria durante la involución participan también en el proceso y desarrollo de la transformación neoplásica del tejido mamario (Watson, 2006). Por lo tanto, la glándula mamaria en el ciclo de lactancia/destete se puede considerar un excelente modelo fisiológico para establecer las bases moleculares de la

regulación de expresión de genes que, en condiciones patológicas como el cáncer de mama, se encuentra alterada.

En cuanto a las vías de señalización que regulan las diferentes fases de involución, dos estudios de microarray de expresión génica han revelado que existen hasta cuatro perfiles transcripcionales que se suceden y se solapan parcialmente en las 96 primeras horas tras el destete. Un primer grupo presenta un perfil de expresión que alcanza su pico máximo a las 12 horas (grupo 1) y contiene genes relacionados con la vía de los death receptors (DR) y genes de respuesta de fase aguda (APR) de clase II; en el que se incluyen genes activados por NF-κB. Un segundo grupo que presenta un pico de expresión a las 12h de destete pero que se mantiene al menos hasta las 96h. Este segundo grupo de expresión se caracteriza por estar compuesto por genes regulados por el factor de transcripción STAT3. Un tercer grupo cuya expresión máxima se da a las 24h de destete con un perfil similar al del grupo 2. Finalmente, el cuarto grupo, cuyo máximo pico de expresión se da a las 96h del destete. Este grupo también se caracteriza por genes de APR clase II, pero sobretodo se caracteriza por la expresión de genes relacionados con la inmunidad innata, inflamación y la regulación de la fagocitosis (Clarkson et al, 2004; Stein et al, 2004). Lo sorprendente de estos estudios es que han ligado íntimamente la involución post-lactancia con respuestas inflamatorias y de fase aguda. Las principales vías de señalización que se activan durante el destete son la vía de STAT3, NF-κB, TGFβ y PI3K/AKT.

En modelos murinos, tras la retirada de las crías se inicia el proceso de involución con la inducción de apoptosis en las primeras 12h (Lund et al, 1996). El inicio de la involución se caracteriza a nivel molecular por la activación de varias vías de señalización que deben estar bien orquestradas para que el proceso se dé de manera regulada. Es de señalar el cambio en actividad que se da en dos de los componentes de la familia STAT de factores de transcripción. Si durante la lactancia, una de las principales vías de señalización activadas era la de STAT5, que actuaba como efector de la

prolactina (PRL) regulando la transcripción de genes esenciales para la proliferación alveolar, diferenciación y síntesis de leche; durante la involución el estado de fosforilación (activación) de STAT5 cae mientras que aumenta la fosforilación de STAT3 activando la transcripción de genes pro-apoptóticos y pro-inflamatorios (ver tabla 2; página 26) (Clarkson et al, 2006; Li et al, 1997b; Liu et al, 1996).

Otra de las vías de señalización que regula la involución post-lactancia es la vía de NF-κB, grupo de factores de transcripción diméricos que regulan procesos de inmunidad, inflamación, proliferación y apoptosis (Sen & Baltimore, 1986). NF-κB se encuentra activo en varias fases de la evolución de la glándula mamaria. Su actividad es elevada durante el embarazo, momento en el que regula la proliferación epitelial (Baxter et al, 2007; Cao et al, 2001), pero se encuentra inactivo durante la lactancia (Clarkson et al, 2000; Geymayer & Doppler, 2000). Durante la involución, NF-κB se activa 1h tras el destete y su actividad aumenta considerablemente a las 24h, siendo su pico máximo a las 72h post-destete (Clarkson et al, 2000). Su activación se da a través de la vía canónica y el perfil de expresión génica que activa está relacionado con respuesta de fase aguda e inmune (ver tabla 3 página 31) (Clarkson & Watson, 2003).

La vía de PI3K/AKT es una vía pro-proliferativa y de supervivencia. Se encuentra inactiva en las primeras 48h post-destete (Schwertfeger et al, 2003), pero más allá de estas 48h, la glándula mamaria inicia la fase de remodelado y la vía PI3K/AKT se activa nuevamente. Además, AKT es un nodo no sólo de PI3K, sino que también lo es de la vía NF-κB y STAT3, lo que sugiere que AKT tiene una función de regulador central del proceso de involución. También es interesante señalar que si bien las células del epitelio alveolar entran en apoptosis durante la involución, algunas de ellas sobreviven, siendo biológicamente plausible que activen esta vía de supervivencia para evitar la apoptosis (Baxter et al, 2007).

Como se ha mencionado anteriormente, la involución de la glándula mamaria post-lactancia es un proceso dinámico en el que no sólo se da una fase de apoptosis para retirar el exceso de epitelio, sino que en último lugar se da un proceso de profunda remodelación que dará lugar a una estructura similar a la de antes de la concepción, de forma que la mama quedará lista para un nuevo ciclo embarazo/lactancia. Esta remodelación es llevada a cabo por una serie de proteasas como las metaloproteasas (MMPs) y las catepsinas, que contribuirán a la degradación de la matriz extracelular y la membrana basal y con ello, a una mayor apoptosis de las células luminales, favoreciendo así a la involución y remodelación glandular (Green & Lund, 2005). A medida que la involución progresa, aumenta la actividad de las MMPs, a la par que se facilita la invasión del estroma por adipocitos y se remodela el árbol ductal. Las principales MMPs que intervienen en este proceso son MMP-2, MMP-3, MMP-9 y uPA (Benaud et al, 1998; Lund et al, 2000; Talhouk et al, 1992).

Por otra parte, otra familia de proteasas relacionadas en la segunda fase de la involución es la familia de las catepsinas, que son hidrolasas lisosomales que degradan proteínas en un medio ácido, actuando como endopeptidasas en el compartimento endolisosomal (Liaudet-Coopman et al, 2006). Se piensa que las catepsinas modulan la involución a través de tres mecanismos: i) muerte celular mediada por lisosomas (necroptosis); ii) autofagia; y iii) remodelación tisular. En la actualidad no existen estudios concluyentes acerca de los roles específicos que cada miembro de esta familia ejerce durante este proceso fisiológico específico, pero estudios de expresión génica han demostrado un aumento en la expresión de las catepsinas B, K, S, L y D durante el destete. Es importante anotar que aunque su expresión aumenta a las 12h de destete, su actividad no aumenta hasta cierto tiempo después, dado que estas proteasas deben sufrir una serie de cambios post-traduccionales antes de estar activas (Clarkson et al, 2004; Watson & Kreuzaler, 2009).

La catepsina D presenta un proceso de maduración desde su síntesis en la forma de pre-pro-catepsina D (412 amoniácidos) hasta su forma madura

final compuesta por dos cadenas, una pesada de 196 aminoácidos y otra de 141 aminoácidos. A este proceso hay que añadir otras modificaciones como la formación de puentes disulfuro, la fosforilación y la glicosilación en diferentes residuos. El procesamiento de esta peptidasa tienen lugar en el compartimento endo-lisosomal (Minarowska et al, 2008). Durante el ciclo lactancia/involución, la catepsina D se ha visto que ejerce múltiples funciones: Es secretada como pro-catepsina D en la leche, se cree que para facilitar la digestión en el tracto digestivo del recién nacido. También es secretada por las células luminales hacia el lado basal, donde podría participar en el procesado de la PRL (Benes et al, 2008; Castino et al, 2008). Durante la involución, tal y como se ha mencionado, su función principal es como una endopeptidasa con un papel importante en la remodelación tisular y la necroptosis.

Como se ha descrito, el proceso de involución de la glándula mamaria integra una compleja serie de mecanismos de señalización que deben estar reguladas para asegurar un resultado final que permita a la glándula estar preparada para un nuevo embarazo y lactancia. Es evidente que los actores que intervienen en este proceso son múltiples. Nuestro grupo, en un trabajo previo, estableció que la transcripción de Óxido Nítrico Sintasa Inducible (NOS2) era regulada por NF-κB (Zaragoza et al, 2005). NOS2 está implicada en muchos procesos pro-inflamatorios a través de la formación de óxido nítrico (NO) a concentraciones más elevadas y estables que las obtenidas con las formas constitutivas NOS1 y NOS3. NO es una molécula altamente reactiva que, dependiente de la concentración y del tiempo de exposición, tiene propiedades tóxicas o protectoras en las células. De hecho, se ha demostrado *in vivo* que en microambientes pro-inflamatorios las concentraciones de NO pueden alcanzar el rango μM; siendo la mayor fuente de NO los macrófagos, que juegan un papel fundamental en las respuestas inmunes e inflamatorias. El NO a concentraciones altas induce parada de ciclo celular e inhibición de la respiración mitocondrial. El NO a concentraciones elevadas ejerce sus acciones a través de la generación de radicales libres como las especies reactivas de nitrógeno (RNS) (ver figura

10; página 46) que reaccionan con otras moléculas, induciendo modificaciones nitrosativas como la s-nitrosilación de tioles de cisteína y la nitración de residuos de tirosina (Hess et al, 2005; Ischiropoulos, 2003). Si bien estas modificaciones proteicas no son dependientes de una reacción enzimática, sí son procesos altamente selectivos pudiendo ejercer un papel importante en la regulación de diferentes vías de señalización. Por ejemplo, se ha descrito que las modificaciones nitrosativas regulan la activación de las vías de NF-κB y AKT (Nomiyama et al, 2004; Yakovlev et al, 2007). Tomando en consideración todos estos datos, se concluye que el NO es un mediador de la señalización celular que puede influir en múltiples vías. Dado que su concentración aumenta en ambientes pro-inflamatorios como la involución de la glándula mamaria post-destete, su rol específico en este proceso debería ser caracterizado en mayor profundidad.

El estudio de los mecanismos moleculares que intervienen en la regulación de la involución de la glándula mamaria revela diferentes procesos biológicos que se suceden en el tiempo, y a su vez, se superponen. La proliferación celular se da, no sólo durante el desarrollo puberal y el embarazo, sino que también sucede durante la involución post-lactancia (Wiesen & Werb, 2000). La inducción concomitante de señales de apoptosis, inflamación y proliferación podrían suponer un conflicto para la célula. En condiciones fisiológicas, un balance adecuado de todas estas vías de señalización condicionan la homeostasis tisular. Es importante recalcar, que la mayor parte de las proteínas y de las vías de señalización que regulan este proceso también han sido identificadas como oncogenes y factores de transcripción que se activan durante la transformación neoplásica. De hecho, STAT3 y NF-κB no sólo modulan la proliferación y la muerte celular, también han sido implicados en el desarrollo neoplásico a través de la inducción de ambientes pro-inflamatorios que en parte simulan el microambiente que se da durante la involución tras el destete (Chariot, 2009; Torres et al, 2011). De hecho, trabajos ya publicados sugieren que la des-regulación del proceso de involución post-lactancia puede facilitar la progresión tumoral (Lyons et al, 2011; Radisky & Hartmann, 2009; Torres et al, 2011).

OBJETIVOS

1. Nuestro grupo ha establecido que NOS2 es inducida por NF-κB a 8h del destete (Zaragoza et al, 2005). NOS2 está implicada en muchos procesos pro-inflamatorios, induciendo altas concentraciones de NO de manera más estable que las alcanzadas con NOS1 y NOS3. Estos datos, junto con los trabajos que definen la regresión mamaria post-lactancia como un modelo pro-inflamatorio, suscitaron la pregunta acerca del papel exacto de NO en el proceso de involución. Nuestro primer objetivo es establecer el papel de NO como molécula de señalización en el proceso de involución, específicamente en la regulación de dos de las principales vías implicadas en la involución, STAT3 y NF-κB.

2. NO es una molécula altamente reactiva que produce especies reactivas nitrosativas. NO puede ejercer roles contradictorios dependiendo de la concentración y la exposición temporal (Thomas et al, 2008). NO puede inducir una serie de modificaciones nitrosativas post-traduccionales en proteínas y lípidos que juegan un papel clave en la fisiología celular y patogénesis (White et al, 2010). En proteínas, estas modificaciones se manifiestan de dos formas, como s-nitrosilación de tioles de cisteína o como nitración de residuos de tirosina. El segundo objetivo del presente trabajo es explorar el posible papel de modificaciones nitrosativas específicas en la involución de la glándula mamaria y cómo estas modificaciones post-traduccionales afectan la función proteica.

3. El embarazo se asocia con una reducción del riesgo de desarrollar cáncer de mama a lo largo de la vida de una mujer (Albrektsen et al, 1994; MacMahon et al, 1973; Rosner et al, 1994). Sin embargo, estudios sobre la incidencia de cáncer de mama han demostrado que existe un aumento transitorio en el riesgo de cáncer de mama en los años que siguen al embarazo (Albrektsen et al, 2005; Chie et al, 2000; Lambe et al, 1994). Así pues, el embarazo puede suprimir o promover la carcinogénesis mamaria, dado que el efecto inicial de cualquier embarazo a término es de aumento de

riesgo, pero existe un efecto protector a largo plazo en mujeres de edad joven en el primer embarazo y un mayor número de gestaciones (Lord et al, 2008). Una de las hipótesis que explica este aumento de riesgo transitorio postula que la involución de la glándula mamaria que se da necesariamente tras una gestación a término, implica la activación de una serie de mecanismos que remedan un microambiente pro-inflamatorio y pro-carcinogénico (Schedin, 2006). NO es una de las moléculas inducidas en ambinentes pro-inflamatorios, y se ha demostrado que tiene efectos pro-apoptóticos o pro-supervivencia celular en función de la dosis y el tiempo de exposición (Thomas et al, 2008). El tercer objetivo es explorar en un modelo celular de cáncer de mama luminal las vías de señalización específicas que se activan tras la exposición a NO a concentraciones elevadas. El objetivo principal en este apartado es determinar qué mecanismos fisiológicos estudiados en los dos objetivos previos son reclutados por las células epiteliales transformadas cuando son expuestas a altas concentraciones de NO.

RESUMEN DE LAS PRINCIPALES TÉCNICAS UTILIZADAS EN MATERIALES Y MÉTODOS

1. OBTENCIÓN DE TEJIDO MAMARIO DE RATAS Y RATONES

Para la consecución de este trabajo se emplearon ratas wistar embarazadas (Harlan) y ratones hembra de la cepa C57BL/6 de Taconic, “wild type” (WT) y knock out para el gen NOS2 (NOS2-KO). En todo momento los animales fueron tratados siguiendo estrictamente la legislación vigente. Los protocolos experimentales fueron aprobados por la Comisión de Ética en la Investigación Experimental de la Universidad de Valencia.

Para la obtención de muestras tisulares los animales fueron anestesiados con tiopental sódico por inyección intraperitoneal (60 $mg.Kg^{-1}$ de peso corporal, en ClNa 0.9 %). Después las glándulas mamarias inguinales se extrajeron rápidamente, se lavaron con suero fisiológico y e inmediatamente se congelaron por la técnica del *freeze-clamped*.

Posteriormente los órganos enteros y congelados se almacenaron a -80 ºC hasta su procesado.
Una parte de la glándula mamaria de ratones de cada condición se fijó en paraformaldehído 4%(w/v) y se conservó en parafina para realizar cortes histológicos, tinción con hematoxilia-eosina y estudio microscópico.

2. CULTIVO CELULAR

La línea celular utilizada para los estudios *in vitro* fue MCF-7, obtenida de la American Type Culture Collection (ATCC). Los diferentes experimentos fueron realizados cuando las placas de cultivo alcanzaban una confluencia del 70%. Los experimentos con donantes de NO se realizaron utilizando hidrocloruro de 3-Morfolinosidnonimina SIN-1 (sigma-aldrich) a diferentes concentraciones y con tiempos de exposición crecientes. Los estudios de viabilidad celular se realizaron con MTT (Bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol), un tetrazol amarillo que se reduce a formazán (color lila) en células viables.

3. CUANTIFICACIÓN DE LOS NIVELES PLASMÁTICOS DE PRL

En el día 10 de lactancia se recogió sangre de ratones WT y NOS2-KO (n=3 para cada condición). Tras centrifugar, se recogió el sobrenadante (plasma) y se utilizó para medir niveles de PRL utilizando un kit comercial (SPIbio) basado en un inmunoensayo enzimático.

4. DETERMINACIÓN POR COLORIMETRÍA Y FLUOROMETRÍA DE LAS CONCENTRACIONES DE NITRITOS

En los estudios con líneas celulares, los niveles de nitritos se midieron con un kit de colorimetría (Cayman Chemical Company). Dado que este kit no era lo suficientemente sensible para medir la concentración de nitritos *in vivo* en tejido mamario, para este caso se utilizó un método de fluorimetría basado en la reacción del 2,3-diaminonaftaleno (Sigma-Aldrich) con nitrito en condiciones de pH ácido para formar 1-(H)-naftotriazol, un producto

fluorescente que cuantificamos utilizando el SPECTRAmax GEMINI XS Microplate Sectrofluorometer (Molecular Devices, Inc. Sunnyvale, CA. USA).

4. GENOTIPADO DE RATONES

Para determinar el genotio de los ratones WT y NOS2-KO se cortaron las colas que fueron incubadas en buffer TESNA (100mM Tris/HCl pH7,5; 10mM EDTA; 2% SDS y 10mM NaCl) con proteinasa K. El producto se mezcló con 1 volumen de Fenol:Cloroformo:Isoamil Alcohol (25:24:1) y se centrifugó. Se recuperó la fase acuosa a la que se añadió 500μL de para realizar una segunda centrifugación. Finalmente el pellet se lavó con etanol 80% y resuspenddido en 65μL de H_20. Posteriormente se realizó una PCR utilizando los primers mencionados en la tabla 4 de la página 68. El uso de tres oligonucleótidos se debió a que los ratones NOS2-KO sí presentan el gen, pero en una forma truncada, por lo que el primer forward para WT era diferente del primer para KO.
Condiciones de la PCR: 95°C (1min); [95°C (30s); 63°C (30s); 72°C (30s)] repeat for 40 cycles; 72°C (10min); 4°C(∝).

El producto de amplificación se cargó en un gel de agarosa al 1.5% agarosa y la lectura se realizó utilizando un sistema de análisis de imagen UVIdoc (UVITEC Limited, Cambridge, UK).

6. EXTRACCIÓN DE RNA

RNA total de tejido de glándula mamaria y de pellets celulares fue extraído utilizando el reactivo TRIzol (Invitrogen Life Technologies), cloroformo y alcohol isopropílico. Tras el aislamiento, se realizó una purificación por columnas adicional (RNeasy, Qiagen) siguiendo las instrucciones del fabricante. La concentración y pureza del RNA fueron medidas con NanoDrop ND-2000 (NanoDrop Technologies); y su integridad mediante la determinación del ratio RNA 28S/18S utilizando *RNA 6000 Nano Labchips* en un Agilent 2100 Bioanalyzer (Agilent Technologies).

7. RT-PCR EN TIEMPO REAL

El RNA fue retro-transcrito a cDNA utilizando el Real-Time RT-PCR *high capacity cDNA reverse transcription kit* (Applied Biosystems). Los productos de cDNA fueron amplificados por qPCR utilizando la *GeneAmp Fast PCR Master Mix* (Applied Biosystems). Se realizó una PCR cuantitativa en 7900HT Fast Real-Time PCR System. Los primers pre-diseñados fueron adquiridos Applied Biosystems (ver tabla 5 página 69).

8. ENSAYO DE INMUNOPRECIPITACIÓN DE CROMATINA (ChIP ASSAY)

El ensayo de inmunoprecipitación de la cromatina (ChIP assay) permite identificar la unión de una determinada proteína o factor de trascripción a un promotor específico. Esta técnica se realizó mediante los siguientes pasos: se trató con formaldehído las muestras de glándula mamaria para establecer enlaces carbamida entre la cromatina y cualquier proteína que estuviese unida a ésta en un momento específico. La cromatina fijada fue sometida a sonicación (15 pulsos de 10s a una amplitud del 38% con paradas de 50s) para obtener fragmentos de ≈500pb; y posteriormente centrifugada. Se determinó la concentración de la misma y su tamaño final. Esta cromatina se pre-lavó con las bolas de agarosa A/G durante 4h a 4°C en rotación. Tras centrifugar las muestras se cogió una alícuota de los sobrenadantes y se incubaron con 2µg de anticuerpos específicos frente las subunidades NF-κB p65 y p50 durante toda la noche a 4°C en rotación. Posteriormente se incubaron co las bolas de agarosa previamente bloqueadas y los inmunocomplejos se recuperaron con una serie de lavados con tampones de baja y alta salinidad, LiCl y finalmente con TE (Sandoval et al, 2004). La cromatina inmunoseleccionada fue eluída con tampón de elución y el DNA fue purificado con el kit QIAquick® DNA (QIAGEN, Hilden, Germany). También se trató una alícuota de cromatina en presencia de anticuerpo no relacionado (IgG) y se guardó parte de la muestra no incubada con las bolas de agarosa como input. Tras la purificación del DNA, se realizó una PCR con

los pares de primers para amplificar la región promotora de MMP-9 (Primer forward: 5'- GTGAACACGGTGGCTGAAA-3'. Primer reverse: 5'-CAGGCTCTTTGAAGCAGGATT-3').

La PCR se realizó con NETZYME® DNA Polymerase mediante el sistema Gene Amp PCR system 2700 (Applied Biosystems) y las condiciones de PCR fueron las siguientes:

93°C (2min); [93°C (30s); 58°C (30s); 72°C (30s)] repetir 37 ciclos; 72°C (10min); 4°C(∝).

El producto de la amplificación fue cargado en un gel de 1.5% de agarosa/TAE con tampón de carga de DNA y bromuro de etidio para su posterior lectura.

9. OBTENCIÓN DE PROTEÍNAS

El tejido mamario (de ratón o rata) o los cultivos celulares se homogenizaron en tampón RIPA con inhibidores de proteasas y, tras centrifugación para eliminar restos celulares, se guardó el sobrenadante en el que cuantificaron las proteínas por el método Bradford.

10. WESTERN BLOT E INMUNOPRECIPITACIÓN

Los niveles de proteínas se analizaron mediante procedimientos convencionales de inmunoblotting. 15μg proteínas se desnaturalizaron con tampón Laemli y calentando a 95ºC. Posteriormente se cargaron en un gel de acrilamida a concentraciones variables en función del peso molecular de la proteína que se deseaba determinar (entre 8% y 12%) para proceder a su separación por tamaño. Tras la electroforesis, se transfirieron las proteínas a una membrana de nitrocelulosa donde se detectaron los niveles de las diferentes proteínas con un anticuerpo específico (ver tabla 6 página 76). Los inmunocomplejos formados se revelaron usando un anticuerpo secundario conjugado con peroxidasa y una reacción quimioluminiscente inducida por la peroxidasa.

11. INMUNOPRECIPITACIÓN DE CATEPSINA D

Se utilizaron bolas de sefarosa de Proteina A y Protein G beads(GE Healthcare UK Limited) (50:50 v/v) bloqueadas con albúmina sérica bovina. 1mg de proteínas obtenidos de los tejidos de glándula mamaria lisados fue pre-lavado con las bolas previamente bloqueadas. El sobrenadante se incubó con anticuerpo anti-catepsina D o con un anticuerpo no relacionado y posteriormente con las bolas de sefarosa. Tras la centrifugación se utilizó el sobrenadante de estas muestras como input y los pellets formados por los inmunocomplejos unidos a las bolas se lavaron y eluyeron para obtener las proteínas inmunoprecipitadas y posteriormente evaluarlas por inmunobloting.

También se realizaron inmunoprecipitaciones de catepsina D utilizando un método alternativo con bolas de agarosa cubiertas con Pepstatina A (Sigma). Los homogenados de glándula mamaria fueron incubados con estas bolas durante 15h, a 4°C. Posteriormente las muestras se centrifugaron a 20000*g* y el pellet se lavó con tampón 20mM citrato-fosfato. Finalmente el pellet se resuspendió y desnaturalizó en tampón Laemly previo al análisis por western-blot.

12. DETERMINACIÓN DE LA ACTIVIDAD DE CATEPSINA D

La actividad proteasa de la catepsina D fue medida utilizando un kit comercial (Sigma-Aldrich) que se basa en el método de Yasuda (Yasuda et al, 1999). Brevemente, se añadió tampón del kit (pH3,5) a 5μg de proteínas obtenidas de tejido mamario de ratas y ratones lactantes o en diferentes momentos del destete. Posteriormente se añadió el sustrato fluorométrico 7-metoxicumarina-4-ácido acético 20μM. El enzima activo actuó sobre el sustrato que tras ser procesado libera fluorescencia que fue medida con el fluorómetro SPECTRAmaz GEMINI XS Microplate (Molecular Devices, Inc. Sunnyvale, CA. USA) durante 60min, en una reacción cinética a 37°C. Para comprobar que el sustrato sólo era hidrolizado por la catepsina D, se añadió

el inhibidor específico pepstatina A (Sigma) en muestras paralelas. El test también se realizó con catepsina D humana recombinante (Sigma-Aldrich) tratada con $ONOO^-$ para determinar si la nitración afectaba la actividad proteasa.

13. ANÁLISIS ESTADÍSTICO

Los resultados se muestran como medias ± S.E.M. Los datos obtenidos en los experimentos con ratas control y en destete así como con ratones WT y NOS2-KO dentro del mismo genotipo fueron analizados con ANOVA de una vía. La homogeneidad de las variancias se analizó con el test de Levene test. En el caso de que las variancias fueran desiguales, los datos se transformaron de forma adecuada antes de realizar la ANOVA. Las diferencias significativas fueron determinadas por el test de Tukey-Kramer. La letra “a” siempre representa el valor inferior dentro del grupo. Se consideraron estadísticamente significativas las diferencias con $p<0.05$. Un test de T-Student con la corrección de Bonferroni se utilizó para la comparación de dos grupos entre sí. Las diferencias se consideraron estadísticamente significativas si $p<0.05$. Estudios independientes se realizaron con un mínimo de tres replicas por cada condición para permitir la comparación estadística.

RESULTADOS Y DISCUSIÓN

1. ACCIÓN GLOBAL DEL NO EN LA INVOLUCIÓN DE LA GLÁNDULA MAMARIA

El NO puede ser un modulador de la señalización, no sólo durante la involución mamaria, si no también durante el embarazo y la lactancia, momento en el cual la proliferación y diferenciación de las células epiteliales es máxima. De hecho, las tres isoformas de óxido nítrico sintasa (NOS1, NOS2, NOS3) se expresan en el epitelio mamario normal (Khalkhali-Ellis & Hendrix, 2003) aumentando dicha expresión durante la involución post-lactancia (Islam et al, 2009; Zaragoza et al, 2005). En nuestro modelo, los niveles de nitritos aumentaron significativamente en el tejido mamario tanto

en ratones WT como en NOS2-KO (figura 13 página 88), pero estos era significativamente más elevados en los animales WT. El incremento observado en los ratones KO puede deberse a la activación de formas constitutivas de NOS, como se demuestra en la figura 14 de la página 89.

La diferencia en la producción de NO entre ratones WT y NOS2-KO se correlaciona con un fenotipo involutivo diferente, dado que en los ratones KO la involución se ve retrasada 24h (figuras 15 y 16). A estas diferencias en la histología se suman diferencias significativas en el peso de la glándula mamaria, la producción de leche y el peso de las crías (figuras 17, 18 y 19 respectivamente) que era mayor en los ratones genéticamente modificados. Estas diferencias podían deberes a alteraciones en el control de la lactancia o de la involución, o de ambos procesos, por lo que realizamos una serie de experimentos para dilucidar el mecanismo de acción de NO en este contexto.

La principal hormona reguladora de la lactancia es la PRL, que actúa en las células epiteliales a través de la unión con su receptor (PRLR). Esta unión activará la vía JAK/STAT activándose STAT5. STAT5 activo (fosforilado p-STAT5) se trasloca al núcleo donde activa la trascripción de múltiples genes, entre los cuales figuran genes de proteínas de la leche como la β-caseína (Miyoshi et al, 2001). Otros trabajos han establecido que NO actúa inhibiendo de la liberación de PRL dependiente de Ca^{2+} en la adenohipófisis (Andric et al, 2003; Duvilanski et al, 1995), lo cual podría significar que una disminución en los niveles circulantes de NO aumentaría la liberación de PRL, prolongando la lactancia. Esta hipótesis fue la base de partida para profundizar en la vía se señalización de STAT5, el efector de PRL, en nuestro modelo experimental. En efecto, encontramos que la pérdida de NOS2 implicaba la persistencia de p-STAT5 hasta 48h tras el destete (figura 20, página 97). Esto podría deberse en parte al aumento en los niveles plasmáticos de PRL que objetivamos en los ratones KO frente a los WT (213±16ng/mL vs 165±27ng/mL respectivamente), pero no podemos descartar que NO puede tener una acción directa en la actividad de STAT5. De hecho, otros trabajos han descrito que NO inhibe la señalización de

STAT5 en macrófagos a través de la disminución directa de los niveles de STAT5a y b (Starzynski et al, 2006) o de su fosforilación (Bingisser et al, 1998). También quisimos comprobar si este aumento en la actividad de STAT5 influía en su función en el tejido mamario, y para ello estudiamos el patrón de expresión de β-caseína en lactancia e involución; observando que en los ratones NOS2-KO presentaban mayores niveles de mRNA de dicho gen en los ratones genéticamente modificados (figura 21, página 98). Todo ello concordante con la activación sostenida de STAT5, el aumento de la producción de leche, el mayor peso de las crías y el mayor peso de la glándula mamaria en estos ratones con respecto a los WT. Más aún, estos resultados apoyan nuestra hipótesis inicial de que la glándula mamaria en los ratones NOS2-KO se mantiene en un estasis funcional a pesar de la pérdida del estímulo de succión. Dado que los experimentos de sellado de tetilla han demostrado que la involución se inicia, no sólo por la caída de hormonas lactogénicas, sino más bién por la pérdida del estímulo de succión, esto apoyaría también la idea de que el NO tiene un efecto directo en la primera fase de la involución, y no todos sus efectos se deben a una concentraciones plasmáticas de PRL sostenidas (Cross & Silver, 1956; Vina et al, 1981).

Ha sido descrito que STAT5 y STAT3 están inversamente activados durante el ciclo lactancia/involución (Clarkson et al, 2006; Watson & Brown, 2008). También ha sido publicado que el bloqueo de la activación de STAT3 induce un retraso en la involución de STAT3 (Chapman et al, 1999). Por todo ello analizamos la fosforilación de STAT3 en el pico de lactancia y durante la involución, encontrando que si bien la activación de STAT3 no estaba retrasada en ratones NOS2-KO, los niveles de la misma eran claramente inferiores cuando se comparaban con los niveles de ratones WT. Lo mismo sucedía cuando determinamos su capacidad de unión al DNA (figuras 22 y 23, páginas 99 y 100 respectivamente). Esta diferencia en la activación de STAT3 puede deberse al retraso en la inactivación de STAT5 visto previamente en los ratones KO o a un efecto directo de NO sobre STAT3, o ambos. La menor activación de STAT3 se vio traducida en una menor expresión de *bcl-3,* un gen diana de este factor de transcripción (figura 25, página 102).

Otra de las vías de señalización implicadas en la regulación del proceso de regresión post-lactancia es la vía de NF-κB, que se encuentra activada durante el embarazo, casi totalmente suprimida durante la lactancia y se activa nuevamente durante la involución tras el destete (Clarkson et al, 2000; Zaragoza et al, 2005). En diferentes modelos experimentales, se ha demostrado que NO puede regular su actividad de forma directa o indirecta (Fernandez-Martinez et al, 2004; Yakovlev et al, 2007). En nuestro modelo estudiamos si este factor de transcripción podría estar regulado por NO y ser responsable, al menos en parte, del retraso en la involución observado en los ratones NOS2-KO. Para ello medimos los niveles citoplásmicos de IκBα, su inhibidor fisiológico, confirmando un retraso de 24h en su degradación en los ratones KO (figura 27 A, página 105), lo que significa de una manera indirecta un retraso de 24h en la activación de factor de transcripción en los ratones NOS2-KO. Esta observación se confirmó con el estudio de unión a DNA de NF-κB (figura 27 B, página 105) en el que se objetivó que la actividad de unión a DNA de NF-κB en los ratones KO era significativamente inferior con respecto a los WT. En base a estos resultados, investigamos el alcance de este retraso en la activación de NF-κB en los ratones transgénicos evaluando por RT-qPCR la expresión diferencial de genes involucrados en la vía de este factor de transcripción. La tabla 9 (página 109) muestra una relación de los genes diferencialmente expresados a las 48h del destete en ratones WT y NOS2-KO, objetivándose que 23 genes estaban regulados a la baja en los ratones KO con respecto de los WT, y ninguno estaba sobreexpresado. Estos datos se interpretan de varias maneras: i) el hecho de que todos los genes diferencialmente regulados estén infraexpresados en los ratones NOS2-KO sugiere que la vía de señalización de NF-κB está retrasada en estos ratones durante el destete. ii) algunos de los genes diferencialmente expresados son pro-apoptóticos, como las caspasas, *bcl-3*, *elk-1* o *tnf* (Gouon-Evans et al, 2000; Varela & Ip, 1996) lo que sugiere que un déficit de NOS2 implica una protección de apoptosis. Por último iii) otros de estos genes son anti-apoptóticos, y sorprendentemente estos también están infraexpresados en los ratones KO, lo que nos plantea que una apoptosis menos severa se

traduce en una activación más pobre de las señales pro-supervivencia. Este planteamiento concuerda con la menor activación de AKT observada en nuestro modelo NOS2-KO (figura 26, página 103).

Estos datos sobre señales pro y anti-apoptóticas dándose conjuntamente pueden parecer contradictorios si uno atiende a la definición de la primera fase de involución (apoptosis del tejido epitelial). Sin embargo, globalmente la involución post-lactancia es la adición de señales pro y anti-apoptóticas que, si persiste el destete, inclinan la balanza hacia la apoptosis y la remodelación, pero las células restantes que sobreviven activarán mecanismos de supervivencia de manera controlada para asegurar una remodelación adecuada. Finalmente, los datos obtenidos sugieren que, si bien NF-κB está involucrado en la modulación de todos los genes listados, no existe una única vía que se encuentre afectada por la ausencia de NOS2, sino múltiples.

Finalmente, en este primer objetivo del presente trabajo, se profundizó en el posible papel del NO en la regulación de la segunda fase de la involución post-lactancia, caracterizada por la existencia de una remodelación tisular que depende, principalmente, de proteasas efectoras. Otros trabajos han descrito que la extensión de la apoptosis se correlaciona con la actividad de las metaloproteasas durante la segunda fase de la involución (Clarkson & Watson, 1999; Lund et al, 1996) y que la activación de NF-κB es una de las señales más importantes requeridas para inducir la expresión de MMP-9 (Li et al, 2009). Por todo ello se exploraron las consecuencias del retraso en la activación de NF-κB en ratones NOS2-KO en la segunda fase de la involución. Encontramos que la inducción de MMP-9 era más pronunciada en el destete en ratones WT que en los KO (figura 29, página 112), y dado que esto concordaba con el retraso de activación de NF-κB, comprobamos mediante ChIP assay que la expresión de MMP-9 dependía de este factor de transcripción (figura 30, página 114). Todos estos hallazgos apoyan la hipótesis de que el efecto de NO en la regulación de la involución de la glándula mamaria no se limita a la primera fase. A través de la modulación de la señalización de PRL, los diferentes factores de transcripción y sus

interacciones, la señal generada por NO en la primera fase se amplifica a través de las cascada de eventos que se dan en la segunda fase.

2. PAPEL ESPECÍFICO DEL NO EN LA REGULACIÓN DE LA INVOLUCIÓN POST-LACTANCIA. MODIFICACIONES POST-TRADUCCIONALES

El segundo objetivo del proyecto fue establecer si NO inducía modificaciones post-traduccionales concretas a nivel proteico que indujeran cambios funcionales a nivel molecular, y así explicar el impacto de NO en el proceso involutivo de forma más específica. Se realizó un estudio de nitro-proteómica utilizando homogenados de glándula mamaria de rata lactante o tras 72h de destete. En los geles 2D (figura 32, página 117) se confirmó que la nitración proteica aumenta con el destete. Se recuperaron las proteínas diferencialmente nitradas que fueron analizadas mediante MALDI-TOF-MS. Una de estas proteínas, de 48KDa fue identificada, como catepsina D.

La catepsina D es una proteasa aspártica endolisosomal que pertenece a la familia de las pepsinas (Tang & Wong, 1987) que aumenta durante el destete, donde juega un papel importante en la degradación proteica y la apoptosis celular. Se sabe que hasta alcanzar su forma totalmente activa debe sufrir una serie de modificaciones post-traduccionales. Esto podría explicar el en parte retraso que encontramos entre la activación de la proteasa (48h post-destete) y el aumento de mRNA y de niveles de proteínas (8h post-destete) (figura 35, página 121). Los estudios de inmunoprecipitación confirmaron la nitración de la catepsina D en homogenados de glándula mamaria tras un destete de 72h; de estos resultados se dio la hipótesis que esta modificación post-traduccional fuese, al menos en parte, responsable de su activación (figuras 33 y 34, páginas 118 e 119 respectivamente) .

Como se presenta en las figuras 37, 38 y 39 (páginas 125, 126 y 127 respectivamente) tanto la catepsina D de rata como la de origen humano y bovino presentan un aumento de orden de 0,5 en actividad en los estudios de

nitración in vivo. Con estos resultados podemos concluir que la nitración de tirosinas tiene un papel en la actividad de la catepsina D y que esta modificación post-traduccional parece jugar un papel directo en el aumento de su actividad catalítica y que esta modificación está conservada entre diferentes especies.

Sin embargo, no se puede descartar que el NO active otros mecanismos que modulen la actividad de catepsina D de forma directa o indirecta, esto podría explicar por qué en los estudios *in vitro* el NO inducía un aumento del orden de 0,5 en la actividad proteasa pero este aumento era del orden de 2 cuando comparamos los ratones WT con los NOS2-KO (figura 42 A página X). En los estudios *in vivo* se descartó que la ausencia de NOS2 y por ende, la menor concentración de NO provocara diferencias en el procesamiento proteolítico de la catepsina D; lo que apunta que la nitración del residuo tirosina no aumenta la actividad enzimática a través de un mecanismo de autoprocesamiento (figura 42 B, página 130) sino que tenga un efecto directo en la actividad catalítica. La figura 40 (página 128) ilustra la caracterización del punto concreto de nitración en la catepsina D humana. Se identificó la tirosina de la posición 168 como el residuo modificado tras la exposición a $ONOO^-$, sin embargo, dado que no pudimos secuenciar más del 40% de la proteasa, no podemos descartar la presencia de otro residuo que esté nitrado. La Tyr^{168} se encuentra en la cadena pesada de la catepsina D y es un residuo muy conservado entre las especies (figura 41 página X). Otros grupos han descrito ampliamente la importancia del pH en la modulación de la actividad de la proteasa (Beaujouin & Liaudet-Coopman, 2008); y que la nitración de tirosinas puede inducir cambios profundos en la estructura y la función proteica dado el cambio inducido en el pKa del grupo hidroxilo (Sokolovsky et al, 1967). De hecho, el pH ácido conlleva una activación de la proteasa, pero en un pH7,5 los enlaces moleculares entre residuos tirosina específicos y los residuos aspartato del dominio catalítico estabilizan la estructura en una conformación inactiva (Masa et al, 2006). Por todo ello, sería plausible que la nitración en Tyr^{168} favoreciera una conformación activa e irreversible del enzima a través del bloqueo de la formación de un enlace crucial en el plegamiento del la proteasa.

La conclusión de esta segunda parte de nuestro trabajo es que NO no solo juega un papel en la regulación global del proceso de involución, también induce modificaciones post-traduccionales específicas que tienen un impacto en la función proteica. En nuestro modelo encontramos una nitración de tirosina específica en catepsina D, una endopeptidasa aspáritca lisosomal crucial para la remodelación tisular. Nuestros trabajos *in vivo* e *in vitro* demuestran que esta nitración de tirosina resulta en la activación de catepsina D.

3. PAPEL DEL NO EN UN MODELO DE CARCINOMA DE MAMA LUMINAL. DATOS PRELIMINARES

Entender la relación entre las funciones fisiológicas de diferentes moléculas durante el desarrollo y sus funciones pro-carcinogénicas es crítico para desarrollar apropiadamente terapias diana-específicas. Nuestros hallazgos son relevantes en la medida que algunas de las principales vías involucradas en la involución post-lactancia: STAT3, NF-κB y PI3K/AKT están a su vez, íntimamente relacionadas con mecanismos de supervivencia y proliferación celular en cáncer. Esto significa que los procesos fisiológicos tiene que estar muy bien orquestrados y las vías de señalización reclutadas tienen que activarse e inhibirse en los momentos adecuados para una adecuada progresión del proceso. Significa también que, dependiendo del contexto, los factores de transcripción pueden tener funciones pro- o anti-apotóticas. Un error en la regulación de estos mecanismos podría facilitar un evento oncogénico que, en un microambiente adecuado, puede progresar y dar lugar a una neoplasia.

La incidencia de cáncer de mama está aumentando en mujeres jóvenes, y esto se debe entre otros muchos factores al retraso en la edad de la primera concepción (Lord et al, 2008). Esto podría resultar una paradoja, ya que se sabe que el embarazo se asocia epidemiológicamente a una reducción del riesgo de desarrollar cáncer de mama a lo largo de la vida (Albrektsen et al, 1994; MacMahon et al, 1973; Rosner et al, 1994). Sin

embargo se sabe que el efecto protector del embarazo no es ni inmediato ni constante, y, mientras que la paridad se asocia a una reducción global del riesgo de cáncer de mama, estudios sobre la incidencia de este tipo de tumor han demostrado un aumento transitorio del riesgo en los años siguientes a una gestación a término, independientemente de la edad (Albrektsen et al, 2005; Chie et al, 2000; Lambe et al, 1994). Así pues, la gestación puede aumentar y disminuir el riesgo de cáncer de mama a la vez, dado que el efecto inicial es de aumento de riesgo y el global es de protección, si bien este efecto protector sólo se produce cuando la edad del primer embarazo es inferior a los 35 años (Lyons et al, 2011; Lyons et al, 2009; Rosner et al, 1994; Schedin, 2006). Una de las hipótesis que intenta explicar este aumento transitorio del riesgo postula que la involución mamaria post-lactancia implica la activación de una serie de mecanismos que remedan el microambiente típico de la cicatrización, que es altamente pro-carcinogénico.

En la parte final de el presente trabajo se planteó la pregunta de si la exposición a altas concentraciones de NO tenía un impacto específico en las vías de señalización que son reguladas por esta molécula durante la involución tras el destete, pero en un modelo de carcinoma de mama perfil luminal. El efecto que encontramos en nuestro modelo apoya la idea que altas concentraciones de NO podría tener un efecto dual. Por un lado, y en concordancia con otros estudios, induciría apoptosis (Borutaite & Brown, 2006; Cleeter et al, 1994; Thomas et al, 2004). De hecho, las células MCF-7 presentaban menor viabilidad cuando eran expuestas a concentraciones crecientes de NO (figura 45, página 134). Por el otro lado, concentraciones elevadas de NO parecen estar activando vías de señalización claramente relacionadas con la supervivencia, como AKT y NF-κB (figuras 46 y 47, páginas 135 y 136 respectivamente) y lo más interesante es que parece existir un paralelismo con el proceso de involución tras la lactancia. En conjunto, nuestros datos señalan la posibilidad de que las células de cáncer de mama, tras ser estimuladas con NO activen los mismos mecanismos observados durante ela involución post-lactancia, y que, como en el proceso fisiológico, la activación de estas vías parece depender, al menos en parte, de la exposición al NO. Por supuesto, son datos muy preliminares; de hecho,

sería interesante corroborar la activación de la vía PI3K/AKT/mTOR mediante el análisis de la expresión de genes diana de la vía. También sería interesante profundizar en punto en el que la vía de AKT se activa tras exposición a NO.

Entender la relación entre las funciones fisiológicas de un proceso particular del desarrollo normal y la homeostasis tisular es crucial, ya que todos los procesos fisiológicos son un balance finamente regulado de una series de vías de señalización que pueden tener funciones contradictorias. En nuestro modelo NOS2-KO hemos encontrado una desregulación de este balance durante la involución glandular post-lactancia. Esta desregulación se debe a una alteración en las concentraciones tisulares de NO, lo que da lugar a un retraso en la apoptosis, pero también una Infra-expresión de genes pro-supervivencia. Este fenómeno podría deberse a que la pérdida del estímulo pro-apoptótico del NO también altera la contra-partida pro-supervivencia. Así pues, en un microambiente por-inflamatorio como la regresión post-lactancia, el NO induce apoptosis, pero también puede facilitar la proliferación de las células que quedan a través de la activación de vías como AKT.

Sabemos que una célula, en su camino hacia la transformación neoplásica reclutará mecanismos ya existentes para poder sobrevivir, sólo que estos mecanismos serán des-regulados y adaptados para suplir las nuevas necesidades de la célula. De hecho, en los datos preliminares obtenidos *in vitro* hemos visto que las células tumorales reclutan mecanismos específicos del proceso fisiológico de involución post-destete. Es de notar que estos mecanismos han sido ampliamente descritos como pro-supervivencia en el contexto de transformación neoplásica, lo que podría significar que, si bien altas concentraciones de NO implican muerte celular, las células viables podrían estar asegurando su supervivencia activando mecanismos que la faciliten, y así utilizar la noxa inicialmente inductora de apoptosis en su propio beneficio, lo cual podría tener implicaciones terapéuticas importantes.

CONCLUSIONES

Nuestro trabajo demuestra la importancia de NOS2 así como el incremento en la concentración de NO en la regulación de los cambios que se dan durante la involución mamaria post-lactancia. Concluimos que mantener los niveles adecuados de NO, junto con otros factores de regulación, es esencial para la correcta modulación de la actividad de factores de transcripción, de modificaciones post-traduccionales y la adecuada regulación de las interacciones que conducen a la involución y remodelación del tejido mamario cuando la lactancia finaliza.

5. En este trabajo hemos estudiado los efectos de la ausencia de NOS2 en la involución mammaria tras el destete. Esta ausencia resulta en una menor concentración de los niveles de NO en los ratones NOS2-KO, que conlleva un retraso en el proceso involutivo. Hemos evaluado este retraso por medio de estudios de inmunohistoquímica producción de leche y activación de caspasa 3.

6. Para comprender mejor las diferencias observadas a nivel macroscópico e histológico, realizamos estudios moleculares. Las principales vías de señalización que controlan la involución post-destete se ven claramente retrasadas en los ratones NOS2-KO. Las diferencias más importantes se han visto a las 48h post-destete, específicamente en la activación STAT3 y de NF-κB. Esto conlleva una alteración en la regulación de sus dianas transcripcionales (por ejemplo β-casein, Bcl-3, MMP-9); lo cual afecta la primera fase del proceso, donde la apoptosis es el principal evento, pero también la segunda fase en la que se da la remodelación de la matriz extracellular.

7. El NO no solo juega un papel en la regulación del proceso global, sino que también induce modificaciones post-traduccionales específicas que interfieren en la función proteica. En nuestro modelo objetivamos

que la nitración de tirosinas en la catepsina D tiene un impacto en su actividad proteasa.

8. Con respecto al cancer de mama, la exposición *in vitro* a altas concentraciones de NO reduce la viabilidad cellular; sin embargo, las células que sobreviven activan mecanismos específicos del proceso de involución post-lactancia. En nuestro trabajo objetivamos qie altas concentraciones de NO inducen muerte cellular pero también fosforilación de AKT y activación de and NF-κB, que son señales pro-supervivencia.

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EPILOGUE

Ooooooooh oooh oooh oooh oooh oooh oooh ooh ooh ooh ooh ooh ooh
Don’t worry.
Ooh ooh ooh ooh ooh ooh ooh.
Be happy.
Ooh ooh ooh ooh ooh
Don’t worry, be happy.

Bobby McFerrin