Identification and characterization of genes and signaling pathways involved in proliferation and differentiation of mammary epithelial cells

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Marcin Jankiewicz
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ABSTRACT

The mammary gland is a perfect system to study the pathways regulating organogenesis during development of an individual. The proper development of the mammary gland requires a tight coordination of expression of many genes involved in proliferation and differentiation. The aim of this work was to identify novel genes and pathways involved in the development of the mammary gland and to find possible correlations between the signaling pathways and their downstream targets that are activated during proliferation and functional differentiation of mammary epithelial cells. In this study rapamycin has been used to inhibit the mTOR protein to analyze its role during mammary gland development. Further a genomic approach was used to identify genes differently expressed during this process.

The analysis of the effects caused by the inhibition of the mTOR signaling pathway by using rapamycin on mammary epithelial cells for the first time demonstrate that mTOR plays central role in the coordination of pathways governing the proliferation and differentiation of epithelial cells during mammary gland development. More detailed analysis led to the identification of Id1 and Id2 as two major downstream effectors of the mTOR signaling pathway regulating proliferation and differentiation, respectively.

The genomics analysis revealed several interesting genes involved in the regulation of a proliferative or secretory phenotype of normal epithelial cells *in vitro*. Various genes identified by microarray analysis are of high interest and to determine their role in mammary gland development. Among the identified genes some contribute to process of proliferation like Nol5 and Kpna2, whereas other genes are required for proper functional differentiation such as Nkd2 and Cited4. Importantly, the mentioned candidate genes are also interesting regarding cancer development, since deregulation of their expression migth contribute to tumor formation.

The findings described in this work clearly contribute to our better understanding of the mTOR signaling pathway regulating expression of the genes involved in the development of mammary gland. In additon, the presented results should allow broadening our view of the events that contribute to breast cancer development and help to design better anticancer therapies in the future.

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1. INTRODUCTION

The development of all life forms starts from a single cell. The formation of complicated tissue structures and organs in higher organized organism requires multiple cell divisions. This process also depends on coordinated signaling between single cells as well as between the thousands of cells forming a tissue structure or organ. The proliferation and functional differentiation of all cells needs to be tightly controlled, since deregulation of this process may lead to tumor formation. Investigating the genes, proteins and signaling pathways that control cell proliferation and differentiation will increase our understanding of how its deregulation leads to disease and cancer development. To study these genes, proteins and signaling pathways, which control organogenesis, the mouse mammary gland is a very attractive model. The development of the mammary gland occurs mainly after birth and unlike most other organs, the majority of the studies on developmental regulation can therefore be performed in juvenile or adult organisms (Medina, 1996).

1.1 Development of the mouse mammary gland

The development of the mouse mammary gland including the cycles of proliferation, functional differentiation and apoptosis of the secretory epithelium which occur with each pregnancy is tightly controlled by hormones (Hennighausen and Robinson, 2005). To understand the various signaling events occurring during proliferation and differentiation of the epithelial cells the anatomy and development of the mammary gland will be described in more detail.

1.1.1 Embryonal development of the mammary gland

In mice, embryonal development of the mammary glands starts at day 10-11 after fertilization of the egg. Five pairs of embryonic mammary buds are formed after invagination of embryonic epithelial cells at ten positions along the so called “milk line”. The milk line is a single layer of ectoderm with enlarged cells positioned bilaterally and extending from the anterior to the posterior limb. At day E12 the mammary epithelium consists of several cell layers and has a shape of a light bulb. At this point it is surrounded by a specialized fibroblast layer known as dense mammary mesenchyme derived from the subdermis. In male embryos, the fetal testes start to produce androgens on day E13 causing the destruction of the mammary buds.

In female embryos the embryonic fat cells start to proliferate extensively at day E14 forming the mammary gland fat pad (Sakakura 1987). The fad pad is essential for the development of the mammary gland during puberty and the formation of the secretory compartment during pregnancy (Neville et al., 1998). The mammary buds remain quiescent until day E16. Then they start to elongate and penetrate into the surrounding mammary fat pad (Sakakura 1987). The adipocyte precursors in the embryonic fat pad start to accumulate the fat droplets and differentiate into monolocular adipocytes until three days after birth (Sakakura et al. 1987). Simultaneously, the mammary buds continue to elongate until birth (day E21) leading to the formation of a rudimentary ductal tree consisting of 15-20 branching epithelial ducts embedded within the mammary fat pad (Sakakura 1987). After the embryonic development is complete the mammary gland remains inactive until approximately 3 weeks of age when hormones are secreted by the ovary glands (Daniel 1987). Postnatal development of mouse mammary gland can be divided in two steps. Firstly, out-growth of a ductal tree initiated at puberty and secondly the formation of the alveolar compartment, which occurs during each pregnancy **(**Richert et al., 2000).

1.1.2 Development of the mammary gland during puberty

At birth the mammary gland consists of several ductal branches filling the stroma (fat pad). Each duct consists of a single layer of epithelial cells surrounding a central lumen. The cells building the lumen are called the luminal epithelial cells. The rudimental ductal tree structure (Figure 1.1A) remains quiescent until around 3 weeks after birth when the ovaries begin to secrete puberty hormones (oestrogen, progesterone).

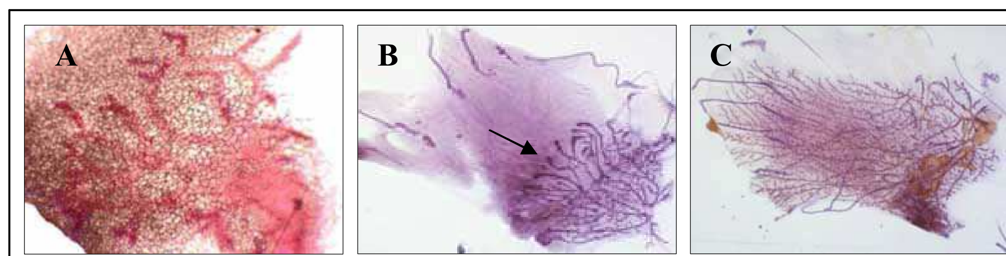

Figure 1.1 Development of the mammary gland in mice during puberty. (**A**) A small ductal tree in the mammary gland of a new born pup. (10 x magnification). (**B**) Growing ducts in the gland of a 4 weeks old virgin mouse with terminal end buds (TEB) present at their tips (arrow) (2 x magnification). (**C**) In a 12 weeks old virgin mouse the ducts reach the end of stroma (2 x magnification). (Figure B and C were adapted from Cardiff, R. D et al. “Tutorial for Comparative Pathology”).

Growing ducts in pubescent mice develop bulb-shaped structures at their tips, which are known as terminal end buds (TEBs) (Figure 1.1 B). Here, extensive cell proliferation occurs, causing extensive branching of the ductal tree into the surrounding stroma (Dunbar et al., 2001, Silberstein 2001). Two morphologically distinct cell types are found in the TEBs. The highly proliferative cap cells are organized in a single layer at the leading edges of the TEBs and give rise to basal cells. The body cells are organized in multicellular layers within the central cavity of the TEBs, and develop into luminal cells. Ductal elongation continues until the mice are approximately 12 weeks of age. At this point the branches have reached the borders of the fat pad (Figure 1.1 C). At the end of the ductal outgrowth the TEBs are not required any more and regress in an apoptotic way (Humphries et al 1996, Humphreys 1999).

1.1.3 Development of the secretory compartment during pregnancy

At the onset of pregnancy the mammary epithelial cells induced by pregnancy hormones (placental lactogens, progesterone) start to proliferate extensively and form terminal side branches and alveolar buds. During the second half of pregnancy the alveolar bud structures start to divide and differentiate into individual alveoli (compare Figure 1.2 A with 1.2 B).

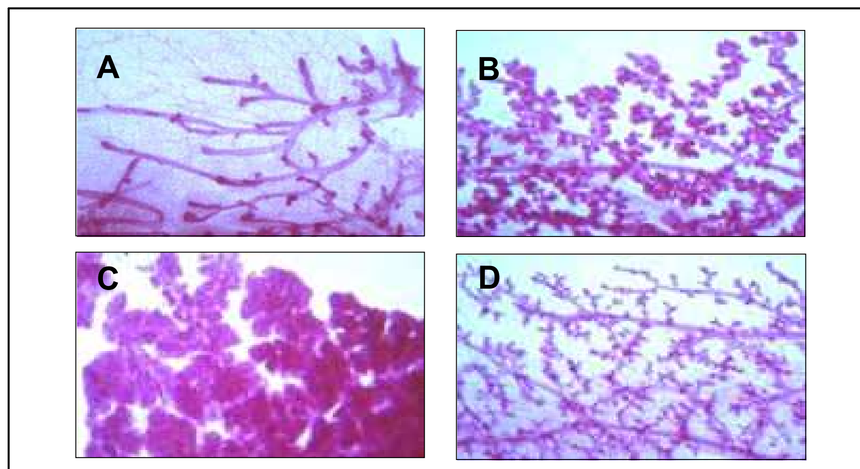

Figure 1.2 Development of the mammary gland in mice during pregnancy. Hematoxylin staining of whole mounts of the mammary gland during different stages of development. (**A**) Mammary gland of a virgin mouse, ducts with branches and terminal end buds are clearly visible. (**B**) During pregnancy, mammary epithelial cells start to form alveoli shown by the development of the terminal end buds. (**C**) During lactation, the secretory alveolar compartment is enlarged, filled with milk and covers almost the whole gland. (**D**) During involution, the secretory compartment is reduced via an apoptotis process (Adapted from Hennighausen, L., www mammary.nih.gov).

At day 18 of pregnancy the alveoli composed of secretory epithelial cells reach their terminal differentiation and are able to express milk proteins and produce lipids. The mammary gland development cumulates finally with the formation of a functional differentiated alveolar compartment secreting milk at parturition. (Nandi et al., 1958 Daniel et al., 1987). Before lactation starts there are still areas within the gland that are filled with adipocytes. At this point around 30% of fat pad is accessible for growth of the secretory compartment. When lactation is established the remaining fad pad is metabolised and the secretory epithelial cells expand to fill the entire mammary gland (Richter et al., 2000).

1.1.4 Lactation in the mammary gland

After 21 days of pregnancy the pubs are fully grown and birth is induced. After parturition the mammary gland is fully functional and produces milk required for proper growth and development of the newborn pups (Figure 1.2 B). The alveoli units are filled with milk proteins and lipid droplets. During this lactation period the secretory epithelial cells surrounding the alveolar lumen changed their shape from a cuboidal form, which was observed during pregnancy, to a more flattened phenotype (Figure 1.3 B). A discontinuous layer of myoepithelial cells, which helps to transport the secreted milk into the ducts, surrounds the alveoli. The adipocytes lost most of their fat content and are present as very long projections in the interstitial space between the alveoli (Elias et al., 1973). The process of lactation lasts for approximately 3 weeks after parturition, until the pubs are able to take solid food (Richter et al., 2000).

1.1.5 Involution of the mammary gland

When pups do not require feeding by their mother anymore the milk stasis in the lumen of alveolar units initiates a process of massive apoptosis of the secretory epithelial cells. This massive death of secretory epithelial cells causes remodelling of the whole gland structure called involution (Quarrie et al. 1996).

Initially involution is reversible and lactation can be reinitiated within 1-2 days by suckling. During this reversible stage the epithelium becomes flat due to the enlargement of the alveoli caused by the stacking milk (Furth A. 1999).

Two days after induction of involution the remodeling process is irreversible. The secretory epithelial cells of the alveolar compartment massively die due to apoptosis as measured by nick-end DNA labeling. Most cells die at day 4 of involution (Quarrie et al. 1995). The ductal epithelium is still not well organized, although the ducts can be readily visible with dense stroma around them. (Richter et al 2000). Slowly the stromal adipocytes repopulate the gland and are filled with fatty acids. They can be clearly seen interspersed among the decreasing alveolar units (Neville et al., 1998). At day 6 of involution the majority of alveoli are collapsed leaving small clusters of epithelial cells (Strange et al., 1992). At day 8-14 the ducts within the increased stroma can be clearly detected, and are only surrounded by small disorganized clumps of alveolar cells (Figure 2.1D). The process of involution takes up to 21 days before the organization of the gland resembles that of a non-pregnant mouse (Richter et al 2000).

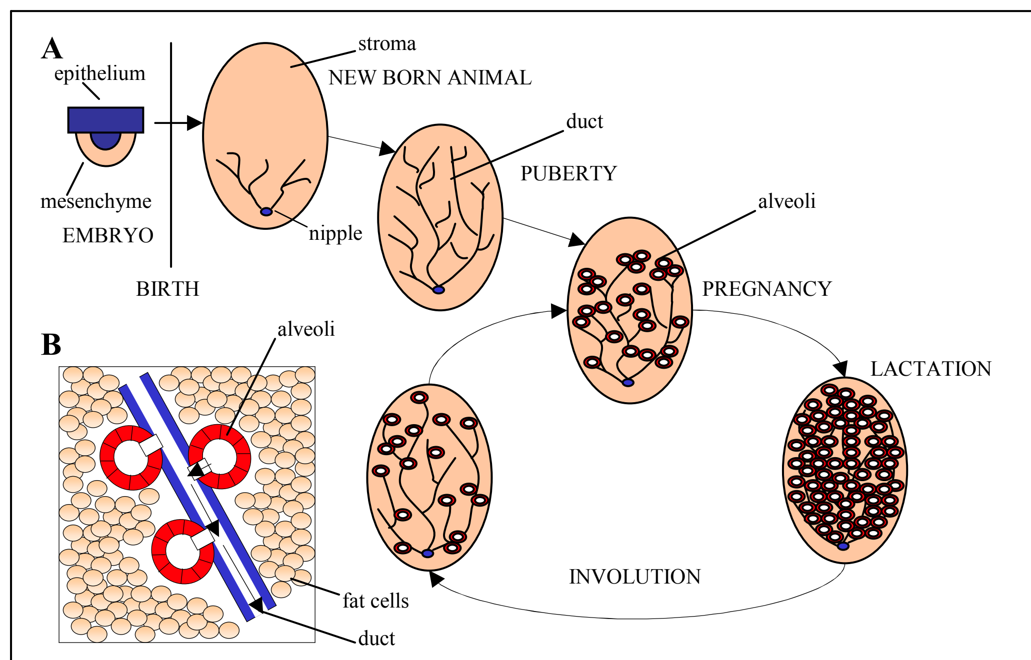

Figure 1.3 Schematic presentation of the mouse mammary gland development. (**A**) In the embryo the mammary bud is comprised of mesenchyme and epithelial cells. During puberty the ducts start to branch and finally fill the entire gland stroma. The alveolar compartment develops during pregnancy and at onset of lactation alveolis fill the whole gland stroma. The secretory compartment is eliminated after cessation of milk suckling by pups. During this involution period the alveoli disappear in an apoptotic way. The mammary gland enters this cycle, every time upon pregnancy. (**B**) Magnification in the mammary gland: the alveolar unit composed of secretory epithelial cells (red), luminal epithelial cells (blue), the adipocytes contributing to stroma surround the alveoli (pink). Milk is transported in direction of the arrows towards the nipple.

1.2 Signaling pathways playing a essential roles during mammary gland development

During evolution the mammary gland has adapted many signaling pathways from other organs in order to be able to regulate its own very complicated development cycle (Oftedal 2002). Some of the most important signaling pathways are regulated by prolactin, insulin and insulin like growth factors. The prolactin pathway is important for proliferation and differentiation of the alveolar epithelia during pregnancy (Ormandy et al., 1997). The insulin pathway is essential for the terminal end bud formation, ductal morphogenesis during puberty and for proper lactation (Hadsell and Bonnette, 2000).

1.2.1 Prolactin/Stat5 signaling in the mammary gland

The mammary gland development and the milk protein expression are severely inhibited in both prolactin and prolactin receptor knock-out mice. The ductal branching (wide main ducts and short site branches) and alveolar compartment (sparse lumina without milk inside) development during pregnancy are clearly reduced (Ormandy et al., 1997).

Prolactin (also called lactogenic hormone or luteotropic hormone) is a small peptide hormone of about 23 kDa and is expressed in many animal species (Cooke et al., 1981; Sinha, 1995). Prolactin binds to the extracellular part of the prolactin receptor (Bole-Feysot, 1998). The ligand-induced dimerization of the prolactin receptor leads to cross-phosphorylation and activation of the JAK2 kinases (Rui et al., 1994), which in turn phosphorylate the tyrosine residues in the C-terminal tail of the prolactin receptor, creating docking sites for proteins containing Src-homology (SH2) domains, including the signal transducer and activator of transcription 5 (Stat5) (Pezet et al., 1997). The proteins with an SH2-domain are activated by the JAK-kinases and induce various signaling pathways. Stat5 is the major transcription factor in the prolactin pathway, responsible for transduction of signaling activated by prolactin in the mammary gland (Liu et al., 1997).

Stat5 was originally identified as a mammary gland factor (MGF) in the lactating glands (Wakao et al., 1992). The MGF was then cloned from sheep mammary gland and found to be a new member of the Stat family (Wakao et al., 1994). Molecular cloning of the Stat5 locus from mice showed that there are two Stat5 genes coding for Stat5a or Stat5b which are 93% identical on the amino acid level (Liu et al., 1994).

Upon phosphorylation of the prolactin receptor Stat5 binds to the C-terminal tail and is then subsequently phosphorylated at specific tyrosine residues by JAK2. Upon phosphorylation Stat5 is released from the prolactin receptor and forms dimers with other phosphorylated Stat5 proteins through SH2 domain and finally after dimerization translocates to nucleus (Gouilleux et al., 1994). The activated Stat5 dimers bind to GAS (Gamma interferon Activation Site) elements in the promoter of several targets genes such as genes coding for milk protein and regulate their transcription (Wakao et al., 1994) (Figure 1.4).

Stat5 is expressed during all stages of mammary gland development with only small changes noticed between virgin, pregnant, lactating and post weaning mice (Liu et al., 1995). However, the kinetics of phosphorylation and activation of Stat5 is parallel to the terminal differentiation of mammary gland and correlates with the expression profile of the milk genes (Liu et al., 1996). Both Stat5a and Stat5b can induce the expression of β-casein in the mammary gland (Liu et al., 1997). In tissue culture the Stat5 binding was crucial for terminal differentiation of epithelial cells as indicated by expression of the milk protein β-casein (Schmitt-Ney et al., 1991).

The essential role for Stat5 in development and differentiation was demonstrated by gene targeting. Similar to prolactin receptor knock-out mice (Ormandy et al., 1997), the Stat5a null animals clearly showed impaired lobulo-alveolar development and failed to lactate. Interestingly, by deleting Stat5a the expression level and the tyrosine phosphorylation of Stat5b was reduced as well (Liu et al., 1997). The Stat5b knock-out mice also showed a reduced lobulo-alveolar development, but not as severe as for Stat5a. (Teglund et al., 1998). Stat5a/b null epithelium shows a perturbation of cell-cell contact and resembles undifferentiated ductal epithelium (Miyoshi et al 2001). The conditional inactivation of Stat5a/b genes also confirmed their potential role in epithelial cell physiology. In conclusion, from the knock-out studies it is now clear that Stat5 is essential for cell proliferation and differentiation during pregnancy as well as for the maintenance of alveolar compartment during lactation (Cui et al., 2004). Nevertheless, Stat5 is not the only player in terminal differentiation of the mammary gland. It is known from *in vitro* experiments that for the maximum expression of the milk protein β-casein, the mammary epithelial cells require the addition of insulin and dexamethasone in addition to prolactin (Henninghausen 1997).

This indicates that for full terminal differentiation of mammary epithelial cells the other pathways in addition to the prolactin/Stat5 signaling pathway are required.

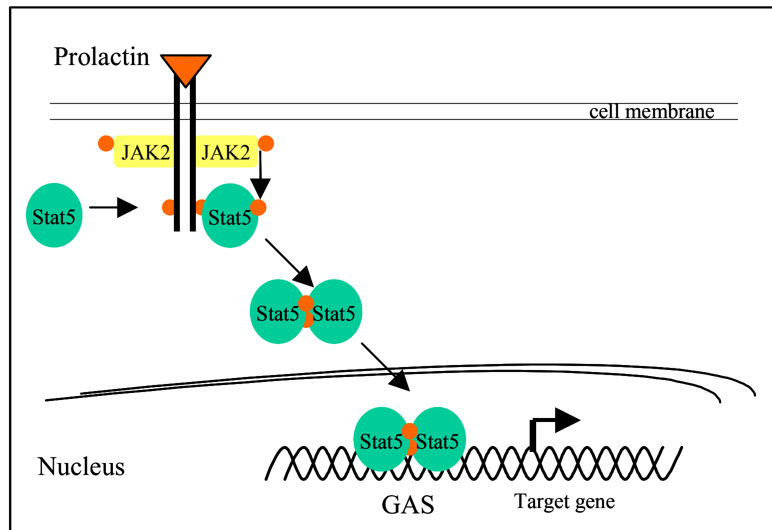

Figure 1.4 The Prolactin-Stat5 signal transduction pathway. Schematic presentation of the Stat5 pathway activated by prolactin in the mammary gland. The Stat pathway plays an essential role in mediating the response to prolactin in mammary epithelial cells. Prolactin activation leads to homodimerization of the prolactin receptor resulting in activation of the associated JAK2 kinase, which then phosphorylate the receptor. The phosphorylated tyrosine residues in the C-terminal tail of the receptor recruit Stat5. The tyrosine phosphorylation in the SH2 (Src-homology 2) domain of Stat5 by JAK2 kinase results in Stat5 dimerization, translocation to nucleus and finally to the activation of genes containing GAS (TTCCNGGAA) elements in their promoter.

1.2.2 The insulin/IGF pathway in mammary gland development

The importance of the insulin/IGF pathway in the regulation of mammary gland development and the expression of milk proteins has been investigated in many studies (Kleinberg, 1998; Rosen et al., 1999). Insulin is a polypeptide hormone produced in the pancreas. Insulin-like growth factors (IGFs) are polypeptides with high sequence similarity to insulin and are expressed mainly in the liver. Insulin and IGF can bind to the extracellular portion of the insulin and IGF-receptor respectively (Hadsell et al., 2000). Five distinct insulin and IGF-receptors have been described: the insulin receptor (IR) isoforms A (IR-A) and B (IR-B), the IGF-I receptor (IGF-IR) and IGF-IIR as well as a hybrid receptor (IR/IGF-IR)(Frasca et al., 1999). IR-A, IR-B and the IGF-IR can form heterodimers in the mammary gland, increasing the diversity of signals activated by insulin and IGFs during development of the gland (Bailyes et al., 1997).

In response to binding of the ligand to the extracellular receptor domain the intrinsic tyrosine kinase of the receptor is activated. This leads to autophosphorylation of tyrosine residues on the intracellular part of the receptor. The phosphorylated tyrosines serve as docking sites for signaling and adaptor proteins with an SH2 domain such as the SRC-homology-2-domain transforming protein (Shc) and the insulin receptor substrate proteins (IRS) (Butler et al 1998). Phosphorylation of Shc promotes the association with Grb2 (Growth factor receptor bound protein 2), which is tightly associated with SOS (son-of sevenless) a guanine nucleotide exchange factor. The Grb2-SOS complex transfers Insulin/IGF signaling to the Ras/Raf/MAP kinase pathway, which results in activation of nuclear transcription factors, mainly involved in the activation of genes regulating the cell proliferation (Skolnik et al., 1993). The IRS proteins act as adaptor proteins and serve as docking sites for multiple proteins including PI3K (Giorgetti et al., 1993) and allow for transferring the insulin/IGF signaling to PI3K/Akt/mTOR pathway, promoting cell growth during development (Pollak et al., 2004) (Figure 1.5).

In the mammary gland IGF-I cooperates with estrogens to promote full ductal development during puberty (Kleinberg, 1998). Studies using whole mammary organ cultures also confirm that IGF-I can stimulate extensive ductal development (Richter et al., 1999). Furthermore, the IGF-I knock-out mice have a significantly reduced ductal morphogenesis also indicating that IGF-I is an essential factor in terminal end bud formation *in vivo* (Kleinberg et al., 2000). Similarly, deletion of the IGF-I receptor also limits ductal development of the mammary epithelium (Hadsell et al., 2000). Apart from its role in ductal development IGF-I was demonstrated to act as a survival factor for mammary cells *in vivo* (Neuenschwander et al., 1996) as well as *in vitro* (Geier et al., 1992). However, for growth of mammary epithelial cells in cell culture usually insulin is used instead of IGF-I (Wood et al., 2000). *In vitro* insulin was shown to be able to stimulate expression of the milk protein β-casein gene even more potently than IGF-I (Prosser et al., 1987). In mammary epithelial cells expression of β-casein stimulated by the addition of insulin and IGF-I was associated with an anti-apoptotic effect of the ligands (Merlo et al., 1996).

Insulin can activate the transcription of C/EBPβ (MacDougald et al., 1995), which has a potent function in controlling the functional differentiation of mammary epithelial cells in the mammary gland (Robinson et al., 1998). The C/EBPβ has been shown to regulate the expression of milk protein β-casein (Raught et al., 1995).

Like Stat5, C/EBPβ is a transcription factor, which affects β-casein transcription by direct binding to multiple enhancer sites in the promoter of the β-casein gene (Doppler et al., 1995). The insulin effect on C/EBP expression is independent of MAP kinase signaling (Hemati et al., 1997), indicating that the PI3K/Akt/mTOR pathway may be involved in the regulation of C/EBP expression. Moreover, insulin may also influence the JAK/STAT pathway (Gual et al., 1998) and therefore may modulate prolactin signaling during proliferation and functional differentiation of alveolar mammary epithelial cells. Nevertheless, to unravel this mechanism more detailed studies have to be done in mammalian cells.

The mammalian target of rapamycin (mTOR) is an important downstream target of the insulin pathway. mTOR is thought to be involved in the regulation of mammary epithelial cell growth and differentiation induced by insulin signaling. It also may link signaling of insulin and prolactin pathway. Because of its important function in proliferation and differentiation of mammary epithelial cells the role of mTOR in cell signaling is explained in more detail below.

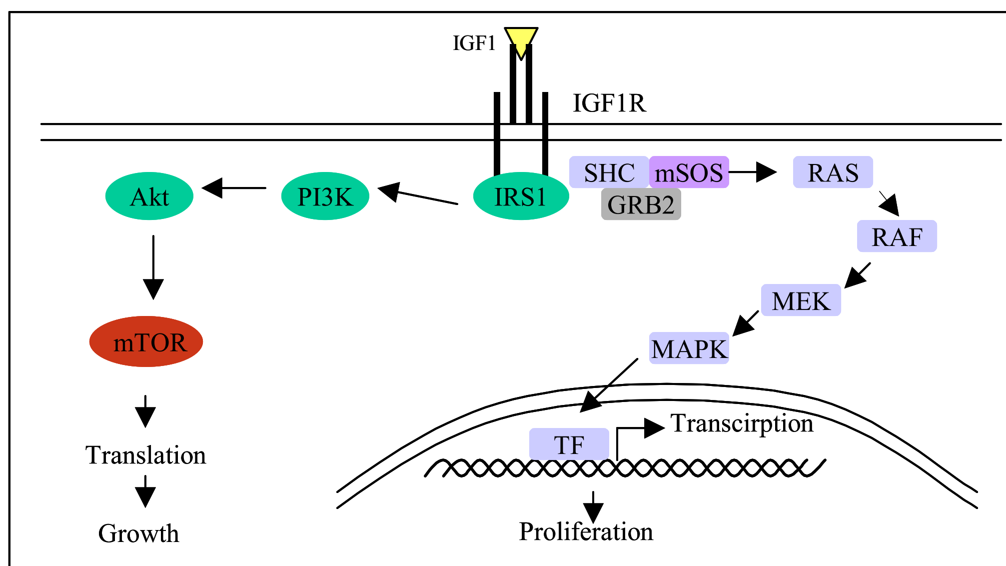

Figure 1.5 Signaling pathways induced by insulin. Activation of the IGF-1-Receptor by binding of its ligand insulin-like-growth-factor-1 (IGF1) leads to phosphorylation of the insulin receptor substrate-1 (IRS1) and activation of two important downstream signaling networks. The PI3K-Akt-mTOR pathway regulates translation and controls cell growth. The RAS/RAF/MAPK, regulating gene transcription by activating the transcription factors (TF), ultimately controls proliferation.

1.3 Target of Rapamycin

During development of the mammary gland the epithelial cells proliferate to form ducts and alveolar compartments. Finally cells differentiate and produce milk during lactation. The extensive protein expression during the mammary gland development requires high amounts of cellular energy. Protein synthesis can only be initiated when nutrients and growth factors are present in sufficient amounts. The target of rapamycin TOR plays a central role in sensoring nutrient and growth factor signals. As discussed above mTOR is a component of the insulin/IGF signaling pathway. Since the role of mTOR has been the topic of investigation in this study, the following chapter summarizes the most important features of mTOR including the signaling events involved in its regulation.

1.3.1 Discovery of TOR

TOR was originally identified in *Saccharomyces cerevisiae*. TOR was named as target of rapamycin due to the fact that rapamycin is a very specific inhibitor of TOR proteins. Rapamycin was originally discovered as an antifungal drug produced by *Streptomyces hygroscopicus*, a bacterial strain that was firstly isolated from Eastern Island soil (Vezina et all., 1975). Rapamycin has been shown to posses immunosuppressant and anti proliferative activity (Hidalgo and Rowinsky, 2000). Normally, if yeast cells are treated with rapamycin they are irreversibly arrested in the G1 phase of the cell cycle. Two genes TOR1 and TOR2 were identified during genetic screens of yeast for mutations making the cells resistant to anti-proliferative action of rapamycin (Heitman et al., 1991). The mammalian target of rapamycin (mTOR) was identified and cloned shortly after discovery of the two yeast homologs (Brown et al., 1994). The mTOR is also known as rapamycin target (RAPT1), rapamycin associated protein (FRAP), or sirolimus effector protein (SEP).

1.3.2 Structure of mTOR

TOR proteins are high molecular weight serine-threonine specific kinases that contain several distinct and conserved domains (Figure 1.6). Human, mouse and rat TOR proteins are 95% identical at the amino-acid level (Janus et al., 2005). The N-terminus of mTOR contains up to 20 tandem HEAT (huntingtin, EF3, A subunit of PP2A, TOR) repeats grouped into two blocks.

Tandem HEAT repeats are responsible for protein-protein interactions and are also present in many other proteins (Andrade et al., 1995). The HEAT repeats form two chains of 40 amino acids in length. The two chains contain a specific pattern of hydrophobic and hydrophilic residues together forming a pair of anti-parallel α-helices (Groves et al., 1999). The middle part of the protein contains a FAT-domain (FRAP-ataxia-telangiectasia mutated, transformation/transcription domain-associated protein) and a FRB domain. The FAT-domain is around 500 amino acids long and is only found in members of the phosphatidylinositol-3-kinase-related kinase (PIKK) family (Bosotti et al., 2000). The FAT-domains may have a similar function as HEAT repeats serving as a protein-protein interaction domain or as a scaffold for binding partners. The FRB (FKPB12-Rapamycin Binding) domain serves as a docking site for FKPB12 (12 kDa FK506-binding protein)-rapamycin blocking complex that specifically inhibits the activity of mTOR towards its downstream targets. In the C-terminal end region of mTOR a domain with high homology to the catalytic domain of PI3K is located. (Kunz et al. 1993). This catalytic domain (CD) with kinase activity phosphorylates Ser-Thr-Pro motifs present in interacting proteins (Brunn et al., 1997) as well as threonine residues flanked by hydrophobic amino acids (Brunett et al., 1998). Finally the very C-terminal end of the TOR protein contains a short sequence of about 35 amino acids termed the FATC (FAT C-terminus) domain. Like in all PIK related kinase family members this domain is only present in combination with the FAT domain. The FATC and FAT domains may interact with each other to regulate the catalytic activity of PIK-related kinases, including mTOR (Bossotti et al., 2000).

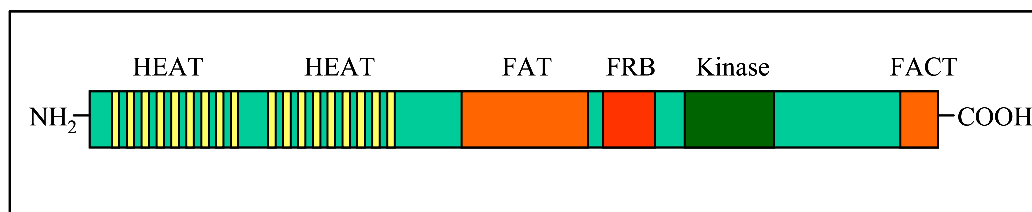

Figure 1.6 Schematic presentation of the domain structure of m TOR. The protein consists of up to 20 tandemly repeated HEAT – (huntingtin, EF3, A subunit of PP2A, TOR) motifs at the amino-terminus end, followed by FAT domain (FRAP-ataxia-telangiectasia mutated, transformation/transcription domain-associated protein) and FRB (FKPB12-Rapamycin Binding domain), and the CD (catalytic kinase domain) and FACT (FAT C-terminus) domains located near to carboxyl terminus. Adapted from Jacinto et al., 2003. Nature Review 4:117-226.

1.3.3 The function of the mTOR protein

Protein kinases are involved in the regulation of many important cellular processes including cell metabolism, cell motility, the cell cycle, repair of DNA damage, apoptotic signaling and respond to the micro-environment (Dancey and Sausville 2003). They mediate the cellular response to external stimulation via phosphorylation of hydroxyaminoacids (Hanks et al., 1988). TOR belongs to this large family of protein kinases and, accordingly, controls a large and diverse set of growth related processes including actin organization, transcription and translation in respond to nutrients and growth factor signaling (Schmelzle and Hall, 2000). To be able to regulate these diverse tasks, TOR proteins are very large and have several docking sites for interacting partners. This was confirmed by the fact that mTOR is highly complexed with other proteins in the cytoplasm (Schmelzle and Hall, 2000). TOR is known to be a central integrator of nutrient and growth factors signaling and coordinator of the cellular growth and cell cycle progression (Fingar and Blenis, 2004) (Figure 1.7).

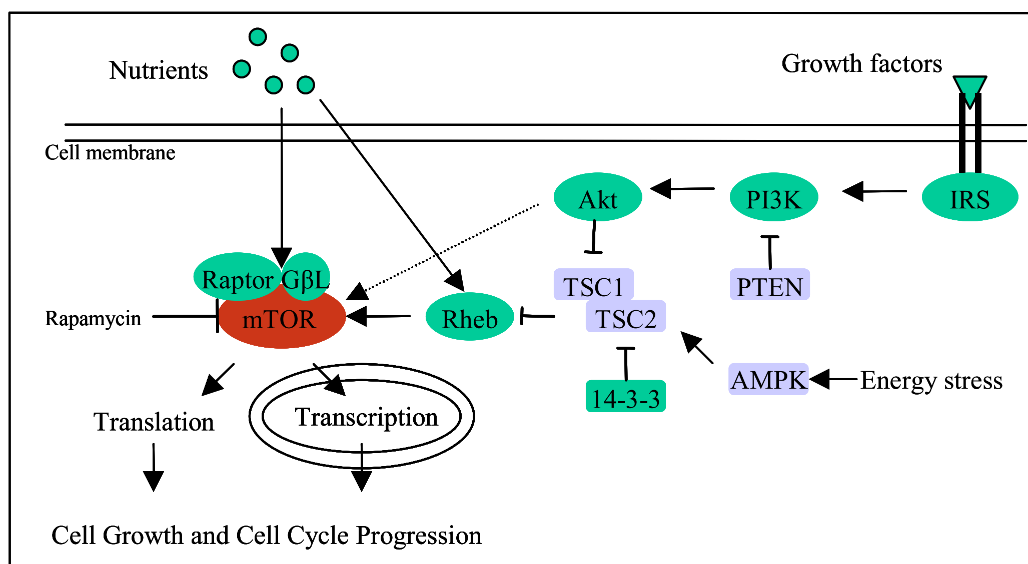

Figure 1.7 mTOR integrates signaling induced by nutrients, growth factors and energy metabolism leading to regulation of cell growth and cell cycle progression. Growth factors activate receptors and signal to PI3K, which can in turn be inhibited by PTEN. PI3K in turn activates PDK1 (not in figure) and Akt. Activated Akt phosphorylates the TSC1-TSC2 complex, which then becomes unstable. TSC2 dissociates from TSC1. TSC2 can also be inhibited by the 14-3-3 protein. Inactivated and dissociated TSC2 is not able to block Rheb, which then has a positive effect on mTOR activity toward its downstream targets involved in transcription and translation. Nutrients positively influence the Raptor-GβLmTOR complex, which also lead to mTOR activation and induction of transcription and cell growth. Energy stress activates the AMPK protein, which phosphorylates TSC2 causing its association with TSC1. The activated TSC complex blocks the Rheb GTPase activity and inhibits mTOR activity.

1.3.4 Regulation of mTOR activity

Mammary gland development requires nutrients and is controlled by the action of many growth factors. The role of mTOR in mammary gland has not been studied in detail although mTOR has been shown to be a central regulator of cellular growth and proliferation by linking the growth factor signaling and the nutrient availability. To understand the importance of mTOR in cellular signaling this chapter summarizes the molecules controlling the activity of mTOR.

1.3.4.1 The PI3K/Akt signaling pathway is an upstream regulator of mTOR

The insulin signaling is crucial for development and function of the mammary gland. The components of the insulin pathway PI3K and its downstream effector Akt act upstream of mTOR (Figure 1.7). PI3K/Akt signaling is activated by growth factors and miogenic stimuli (Janus et al., 2005). PI3K phosphorylates the 3-hydroxyl of the inositol ring of phosphoinositide phospholipids (PIP). The 3-phosphorylated phospholipides then serve as membrane tethers for proteins containing the pleckstrin homology (PH) region, such as Akt and Phosphoinositide-Dependent Protein Kinase 1 (PDK1). The PH-domain of Akt binds to PI3Ps which leads to the translocation of Akt to the plasma membrane. This enables PDK1 to directly contact Akt. PDK1 is responsible for the phosphorylation of Akt, which leads to its activation. The tumor suppressor phosphatase PTEN negatively regulates the activation of PI3K and Akt signaling by dephosphorylation of the phosphoinositol-3,4,5-triphosphate at the D-3 position of the inositol ring (Dancey, 2003). Ultimately, Akt phosphorylates Ser2448 of mTOR as shown by *in vitro* and *in vivo* experiments (Nave et al. 1999). There are several other studies confirming that mTOR is located downstream of the PI3K/Akt pathway. Insulin activates phosphorylation of mTOR at Ser2448 *in vivo* (Reynolds et al., 2002). Furthermore, overexpression of a constitutively active form of Akt in HEK-293 cells leads to phosphorylation of 4E-BP1, which is a down-stream target of mTOR. This effect could be influenced by rapamycin showing that mTOR mediates the effect of Akt on 4E-BP1. In the same study, overexpression of an inactive form of Akt inhibits insulin mediated phosphorylation of 4E-BP1 confirming vise-versa that Akt is transferring the signaling from insulin to mTOR (Gingras et al. 1998). The first genetic evidence that Akt acts upstream of mTOR in mammalian cells came from a study in which mice were used lacking two out of three forms of Akt. These mice showed a clear decrease in phosphorylation of 4E-BP1 (Peng et al. 2003).

1.3.4.2 TSC negatively regulates mTOR

Recent study indicates that tuberous sclerosis proteins (TSC1 and TSC2) are inhibitors of mTOR and antagonize the activation of mTOR by insulin and amino-acid (Gao et al. 2001; Gao et al., 2002). Two proteins TSC1 and TSC2 contribute to Tuberous Sclerosis Complex and only dimers are functional to control cell growth and proliferation. TSC1 also know as hamartin, is a 130 kDa protein with coiled-coil domains. TSC2 alias tuberin is a 200 kDa protein with coiled-coil domains and a carboxy-terminal region, which shares homology to the Rap GTPase-activating protein (GAP) (Scheffzek et al.1998). These proteins were first identified to play a role in Tuberous sclerosis, an autosomal dominant disorder characterized by the formation of hamartomas in various organs (Young and Povey 1998).

From studies in flies it became clear that Tsc2 is a critical target of Akt in mediating growth signals from the insulin pathway. Akt directly phosphorylates Tsc2 at conserved residues Ser 924 and Thr 1518. Phosphorylation of Tsc2 by Akt stimulated growth by inhibiting the formation of the functional Tsc1-Tsc2 complex (Potter et al., 2002). In mammalian cells Akt was also demonstrated to associate with the TSC complex in response to insulin, promoting the degradation of hamartin-tuberin complexes by phosphorylation of TSC2. (Dan et al., 2002). Mutation of the Akt phosphorylation sites make the Tsc2 insensitive to Akt signaling and increases the stability of the Tsc1-Tsc2 complex. The Akt growth signals were blocked also by expression of mutant Tsc2 lacking the Akt phosphorylation sites (Potter et al., 2002). Inactivation of the *Drosophila* homolog of TSC1 (Tsc1) protein dramatically increased the cell size and the proliferation rate and as a consequence, organ size. Overexpression of Tsc1 and Tsc1 alone had no effect, whereas co-expression of both Tsc1 and Tsc2 decrease cell size, cell number and organ size. (Potter et al., 2001).

It was shown that mammalian TSC1-TSC2 inhibits phosphorylation of S6K1 and 4E-BP1, which are down stream targets of mTOR. Function of TSC is negatively regulated by Akt in response to treatment with insulin. TSC2 is inactivated by Akt dependent phosphorylation, which destabilized TSC2 and disrupts its interaction with TSC1 (Inoki et al., 2002).

1.3.4.3 Rheb a positive regulator of mTOR is suppressed by TSC

TSC proteins are able to suppress mTOR signaling. However this suppression is not direct, but involves the Rheb proteins.

Rheb (Ras Homolog Enriched in Brain) is widely expressed as a small GTPase and was initially isolated as a Ras homolog from brain tissue (Yamagata et al., 1994). Mouse and human cells express two Rheb genes, Rheb1 and Rheb2 (Patel et al., 2003). A mutation in Rhb1 (Rheb related GTPase) in yeast *Schizosaccharomyces pombe* causes growth arrest and a starvation-like phenotype, indicating that Rheb proteins are involved in growth signaling in response to nutrients (Mach et al., 2000). In *Drosophila melanogaster,* Rheb is also required for cell-cycle progression and cell growth (Patel et al., 2003).

In recent studies Rheb was indicated to act upstream of mTOR in mammalian cells and to stimulate translation and cell growth. Rheb, positively influences mTOR signaling, since overexpression of Rheb activates S6K1 even during amino acid insufficiency via rapamycin sensitive mechanism. Moreover, Rheb is not able to activate a S6K1 mutant, which is unresponsive to mTOR mediated signals, confirming that Rheb is upstream of mTOR. (Tee et al., 2003). Rheb promotes also phosphorylation of another downstream target of mTOR; the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). This phosphorylation causes the dissociation of 4E-BP1 from eIF4E, which is completely sensitive to mTOR inhibitor rapamycin. This indicates that rapamycin blocks the positive action of Rheb on the mTOR pathway (Tabancay et al., 2003).

Overexpression of Rheb in HEK293 cells caused a significant increase in mTOR Ser2448 phosphorylation, whereas phosphorylation of Akt was not affected. These results indicate that Rheb activates mTOR directly (Inoki et al., 2003). In agreement with this idea, it was shown that over-expression of an inactive form of Rheb effectively blocks the activation of mTOR signaling pathway induced by growth factors and insulin (Tabancay et al., 2003).

Studies in *Drosophila* have shown that Rheb is a molecular target of the TSC tumor suppressors (Zhang et al., 2003).The C-terminal end of TSC contains a region that shares homology with the catalytic domain of GTPase-activating protein (GAP), suggesting that a GTPase might be the physiological target of TSC2 (Scheffzek et al., 1998). A study using mammalian cells showed that TSC2 indeed, has the GAP activity towards Rheb (Tee et al., 2003). Overexpression of TSC1-TSC2 heterodimers inhibits Rheb-mediated phosphorylation of downstream targets of mTOR confirming the model, where Rheb is a direct target of TSC. Both components act upstream of mTOR in nutrient signaling (Tee et al., 2003).

1.3.4.4 AMPK activates TSC2 and negatively regulates the mTOR pathway

The level of translation is regulated by several factors including growth factors, nutrients availability and the intracellular ATP level. The mTOR protein was suggested to be a homeostatic ATP sensor (Dennis et al., 2001) although a very drastic decrease in the ATP level is required to inhibit the activity of mTOR (Proud, 2002). The level of translation, however also responds to small variations in the ATP level. A small decrease in ATP results in a relatively large increase in the AMP level. This stimulates the activity of AMPK (AMP-activated protein kinase), which is a major sensor of cellular energy levels (Hardie et al., 1998; Hardie et al., 2003). TSC2 has been shown to be regulated by the cellular ATP level. Under energy starvation AMPK phosphorylates TSC2 and enhances its GAP activity towards Rheb, which is then deactivated preventing stimulation of the mTOR protein. The AMPK/TSC2 pathway preventing translation is acting opposite to the PI3K/Akt/TSC2 signaling pathway stimulating the mTOR and translation activity (Inoki et al., 2003; Hynes and Boulay, 2006).

1.3.4.5 Regulation of TSC by 14-3-3 proteins

Apart from the fact that TSC protein activity is inhibited by Akt upon insulin/growth factors stimulation, this protein is also negatively regulated by proteins sensoring nutrients. In this context, 14-3-3 proteins were shown to associate *in vivo* with the TSC complex if nutrients are available. Accordingly, the interaction between 14-3-3 and TSC2 is decreased under serum–starvation indicating that the complex formation is modulated by factors influencing the nutrients availability (Li et al., 2002; Shumway et al., 2003). 14-3-3 proteins belong to a family of highly related proteins with numerous isoforms which have been shown to directly associate with many cellular proteins in order to regulate their function (Fu et al., 2000). TSC2 was shown to associate with different 14-3-3 isoforms under physiological conditions. In contrast, TSC1 cannot interact with 14-3-3 as indicated in co-immunoprecipitation studies. Interestingly, the interaction between 14-3-3 and TSC2 is stronger than with the TSC1-TSC2 complex, although 14-3-3 has no effect on the tuberous complex formation between TSC1 and TSC2. Phosphorylation of a single residue (Ser1210) in TSC2 is required for the association with 14-3-3. Interestingly, this residue is not known as an Akt phosphorylation site suggesting that the phosphorylation the Ser1210 and binding of TSC2 to 14-3-3 are dependent on kinases other than Akt.

Moreover, mutations in all six predicted Akt phosphorylation sites (residues 939, 1086, 1088, 1378, 1422, 1756) of TSC2 still allowed binding to 14-3-3 at the same level as wild type TSC2 (Inoki et al., 2002). Also the inhibition of Akt with Wortmannin or the inhibition of the ERK pathway by PD90589 has no effect on the association of 14-3-3 with TSC2. Overexpression of 14-3-3 reduced the ability of the TSC1-TSC2 complex to negatively regulate S6 kinase phosphorylation. It enhanced the basal phosphorylation of 4E-BP1 by mTOR confirming that 14-3-3 negatively regulates the function of the TSC complex. (Li et al., 2002; Shumway et al., 2003). Apart from the fact that 14-3-3 influences mTOR signaling by binding to the inhibitor TSC2 of this pathway, 14-3-3 has also been shown to interact directly with mTOR and positively modulate mTOR action (Bertram et al., 1998; Mori et al. 2000). Therefore, 14-3-3 regulates the phosphorylation of S6K and 4E-BP1, which are two downstream components of the mTOR pathway by interacting with at least two: TSC2 and mTOR.

1.3.5 Components of the mTOR complex

The m TOR is a very large protein (289 kDa) with several distinct domains. These domains are known to be interaction sites for numerous proteins. As a consequence, mTOR forms a very large complex with other proteins in the cell (Bjornsti and Hughton, 2004). Some of the important components present in this complex will be introduced.

1.3.5.1 mTOR is complexed with Raptor and GβL

In several studies Raptor (regulatory associated protein of mTOR) was indicated to be an upstream protein that regulates the catalytic activity of mTOR and serves as a scaffold in the nutrient signaling pathway. Raptor is a high molecular weight protein of 150 kDa that binds to mTOR as well as to two mTOR downstream targets 4E-BP1 and S6 kinase. The binding of Raptor to mTOR is necessary for the phosphorylation of 4E-BP1 by mTOR. Inhibition of the expression of Raptor using siRNA reduced the mTOR catalysed phosphorylation of 4E-BP1 *in vitro* (Hara et al., 2002). The mTOR-Raptor complex is sensitive to rapamycin treatment causing disruption of the complex (Kim et al., 2002).

GβL (G protein β-subunit-like protein) is an additional subunit of the mTOR-raptor signaling complex. It is also a positive regulator of mTOR kinase activity (Kim et al., 2003). GβL is a small, 36-kDa protein expressed in many tissues (Rodgers et al., 2001). The structure of GβL consists almost entirely of seven WD-40 repeats with high sequence homology to those found in β subunits of G proteins (Rodgers et al., 2001). The GβL protein is conserved among all eukaryotes, including yeast, flies, worms, and plants (Ochotorena et al., 2001). The budding yeast homolog of GβL (Lst8p) also interacts with Tor1p and Tor2p (Loewith et al., 2002). Genetic studies with Lst8p confirmed that it plays a role in the regulation of cell growth (Roberg et al., 1997; Loewith et al., 2002), a process mainly governed by TOR proteins. GβL was shown to interact strongly with the C-terminal half of mTOR in particular with the mTOR kinase domain.

A decrease in the expression of GβL reduced the phosphorylation state of S6K1 *in vivo* to a similar extend as caused by a decrease in the expression of mTOR. Targeting mTOR or GβL with siRNA results in a comparable reduction in cell size (Kim et al., 2003). Co-expression of GβL and mTOR significantly increased the mTOR kinase activity towards S6K1 and 4E-BP1 and its autophosphorylation.

The exact mechanism by which GβL stimulates the mTOR kinase activity is not well understood. Probably, GβL contributes to the stability or folding of the mTOR kinase domain. GβL has a positive role in the mTOR pathway transmitting both growth factor and nutrient signaling to downstream effectors of mTOR. It is also known that the binding of GβL to mTOR and stimulation of the mTOR kinase activity does not require Raptor and is likely an important mechanism by which GβL positively regulates mTOR. The GβL and mTOR interaction unlike the raptor-mTOR association is not sensitive to nutrient conditions in the medium nor to the treatment with rapamycin. A decrease in the amount of GβL reduces the amount of raptor–mTOR complexes indicating that an interaction between GβL and mTOR is also necessary for the nutrient sensitive association of raptor with mTOR. If the amount of nutrients is low raptor interacts with mTOR and decreases the mTOR kinase activity by inhibiting GβL. Probably, under nutrient poor conditions raptor interacts directly with GβL which is constitutively docked to the mTOR kinase domain, and prevents the activation of mTOR by GβL. This is important to prevent cell growth signaling when nutrients are not available (Figure 1.8).

Rapamycin was shown to destabilize the interaction between raptor and mTOR regardless of nutrient availability (Kim et al 2002). Since the rapamycin-FKBP-12 complex binds directly to the N-terminal of the mTOR kinase domain, which is a docking site for GβL, it is suggested that rapamycin also perturbs the Raptor-mTOR interaction mediated by GβL (Kim et al., 2003). GβL, like raptor, also plays a role in the recognizing and recruiting mTOR substrates. GβL can interact directly with S6K1 and independent of mTOR and raptor (Kim et al., 2003).

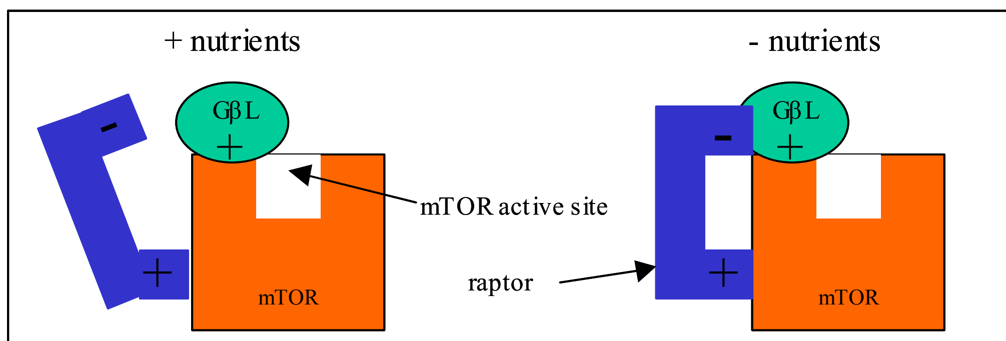

Figure 1.8 Schematic model for the regulation of mTOR activity by Raptor and GβL. mTOR interacts with Raptor in nutrient-rich conditions (+) and GβL positively regulates the mTOR activity. Raptor in nutrient poor condition interacts with GβL and blocks (–) positive action of GβL on mTOR kinase activity. Adapted from Kim et al., 2003.

1.3.5.2 Rictor is a component of the mTOR complex

Another protein associated with mTOR is rictor (rapamycin-insensitive companion of mTOR). Rictor is also a very large protein of 192 kDa. Unlike mTOR and raptor, this protein is not well conserved among eukaryotes. Complexes isolated with a rictor antibody only contain mTOR and GβL, but not raptor, indicating that the association of rictor with the mTOR-GβL complex is independent of raptor. Over-expression of raptor can, however, suppress the interaction between rictor and mTOR showing that rictor and raptor compete for binding to mTOR. The rictor-mTOR complex formation is not sensitive to rapamycin in contrast to raptor-mTOR interactions. This is shown by the fact that down-regulation of rictor does not decrease rapamycin sensitive phosphorylation of S6K. Purified rictor-mTOR complexes also do not phosphorylate S6K1 *in vitro*. Therefore rictor is unlikely to participate in rapamycin sensitive functions of mTOR. Recent studies suggest that rictor may be a substrate for mTOR since down-regulation of mTOR leads to a decrease in the phosphorylation of rictor. (Sarbassov et al., 2004).

Studies in yeast have shown that a rapamycin insensitive TOR complex controls the organization of the actin cytoskeleton through PKC (protein kinase C) (Loewith et al., 2002). Reduction in the expression of rictor leads to a decrease in the phosphorylation of PKCα (protein kinase C alpha) and to a perturbed cell morphology due to an altered actin cytoskeleton. In rictor depleted cells the pattern of actin staining resembles that of cells with reduced expression of mTOR (Sarbassov et al., 2004), indicating that rapamycin insensitive rictor-mTOR complexes are involved in the organization of the actin cytoskeleton via PKCα kinase. Activation of Akt requires phosphorylation of Thr308 by PDK1 (Stephens et al., 1998) and Ser473 by an unknown kinase. A recent study indicates that the rictor-mTOR complex is involved in the regulation of Akt and directly phosphorylated Ser473 *in vitro*. This is indicated by the fact that reduction of mTOR or rictor expression inhibits Akt downstream effectors. The phosphorylation of Ser473 is required for full activation of Akt. If only Thr308 of Akt is phosphorylated by PDK1 the activity of Akt is fivefold less in comparison with Akt which was previously phosphorylated on Ser473 by the rictor-mTOR complex prior to phosphorylation of Thr308 by PDK1 (Sarbassov et al., 2005). These studies indicate that not only Akt phosphorylate mTOR but there is a feedback loop from the rictor mTOR complex, which regulates the activity of Akt.

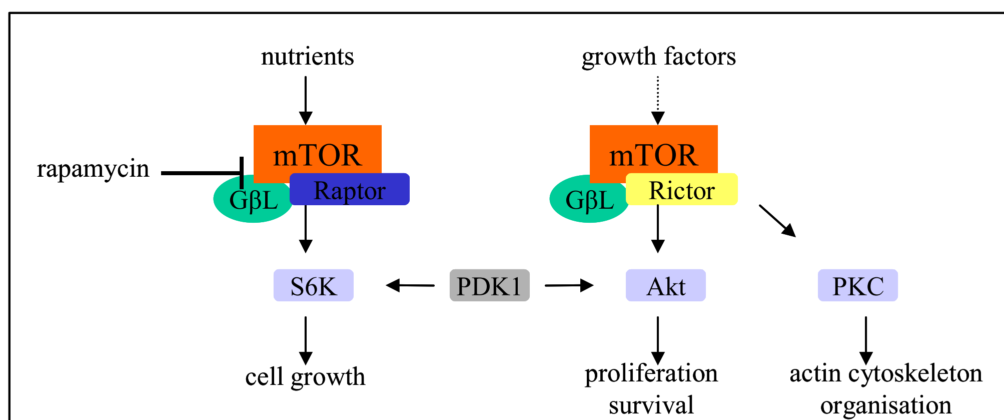

Figure 1.9 Regulation of Target of Rapamycin signaling by Raptor and Rictor protein. Sensitive to rapamycin treatment raptor-mTOR complexes regulate cell growth. Rapamycin insensitive rictor-mTOR complexes regulate proliferation survival as well as actin cytoskeleton organization. Adapted from Sarbassov et al., 2005.

1.3.6 Downstream effectors of the mTOR pathway

The development of the mammary gland requires a high expression level of proteins regulated by mTOR. Components of the protein translation system belong to downstream targets of the mTOR pathway. The mTOR pathway is involved in the positive stimulation of transcription activities of genes coding for proteins involved in ribosome biogenesis, activation of transcription of rRNA genes by RNA polymerase I (Pol I), transcription of ribosomal proteins by RNA polymerase II (Pol II) and transcription of tRNA and 5S unit by RNA polymerase III (Pol III) (Hannan et al., 2003). mTOR is known to influence protein expression through two mayor downstream targets: 4E-BP1 and S6K1, which are required for the recruitment of ribosomes to the mRNA and translational enhancement of mRNA, which have a terminal oligopyrimidine tract (TOP) at their 5' end (Hay and Sonenberg, 2004). The regulation of these targets of mTOR will be discussed in the following chapters.

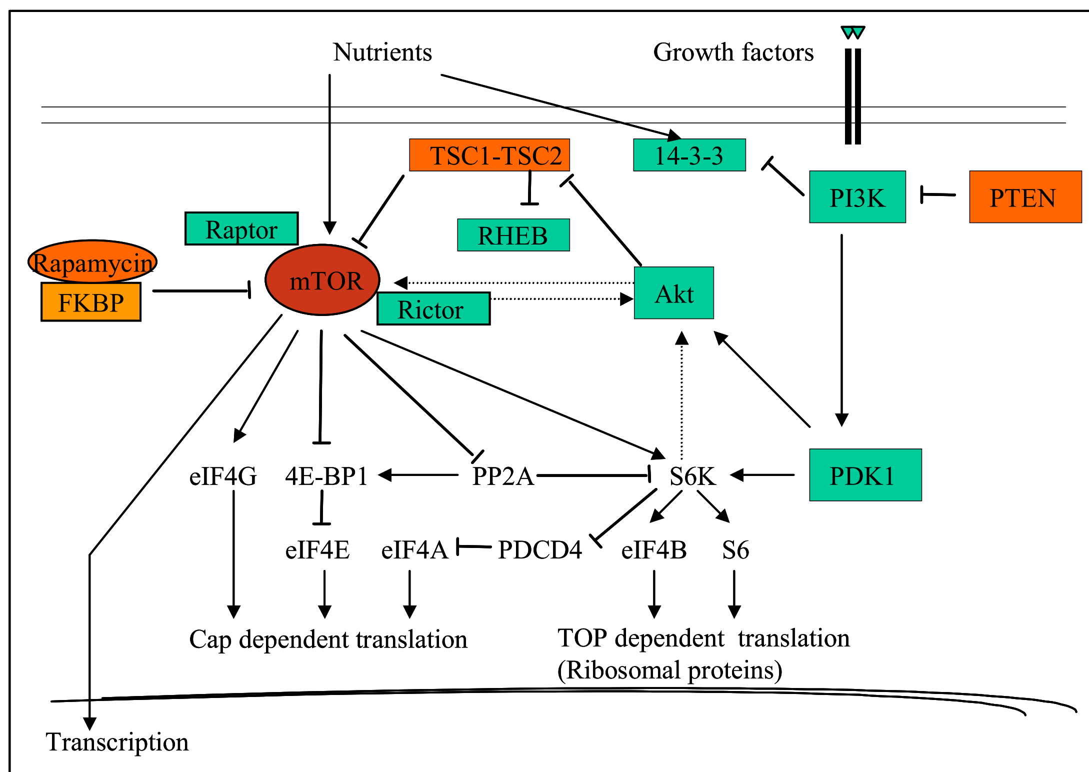

Figure 1.10 Regulation of mTOR and its downstream effectors in mammalian cells. Arrows indicate activation, bars indicate repression. mTOR is activated upon stimulation with nutrients and growth factors, which in turn leads to activation of transcription (genes coding components of protein biogenesis machinery) and translation controlled by mTOR signaling pathway. eIF4A, eukaryotic translation initiation factor 4A. eIF4E, eukaryotic translation initiation factor 4E. 4E-BP1, eIF4E-binding protein 1. eIF4G, eukaryotic translation initiation factor 4G. eIF4B, eukaryotic translation initiation factor 4B. S6K, ribosomal S6 protein kinase. PP2A, protein serine/threonine phosphatase type 2A. PDCD4, programmed cell death protein 4.

1.3.6.1 Phosphorylation of 4E-BP1 by mTOR leads to the activation of eIF4E

In mammalian cells the first step in translational initiation involves ribosome recruitment to the 5' end of the mRNA. The process of ribosome binding is regulated by several eukaryotic translation factors, which guide the ribosome to the 5' end of the mRNA. The nuclear-transcribed mRNA posses at 5' end the cap structure defined by m7GpppN, in which m represents a methyl group located at position 7 in guanine (G) connected with 5'-to-5' bond (ppp) to any following nucleotide (N). This sequence is specifically recognized by the eukaryotic translation initiation factor 4E (eIF4E) (Gingras et al., 1999). The 4E-binding proteins 4E-BPs are translation repressors and compete with eIF4G proteins (eukaryotic translation initiation factor 4G) for the same binding site in eIF4E. The eIF4G is a scaffold protein containing three domains, which interact with other translation initiation factors (Raught et al., 2000b).

The eIF4G is phosphorylated in response to stimulation with the growth factors insulin and serum, which all induce cell growth. The phosphorylation of these sites is sensitive to PI3K and mTOR inhibitors, indicating that the eIF4G is also regulated by the mTOR pathway (Raught et al., 2000). Binding of 4E-BPs to eIF4E leads to the inhibition of eIF4E (Marcotrigiano et al., 1999). The eIF4E can be activated by phosphorylation of 4E-BP1, which is a direct target of mTOR kinase. (Miron et al., 2001; Miron et al., 2003). The mammalian family of 4E-binding proteins consists of three low molecular weight homologs (Lin et al., 1994). This protein contains seven phosphorylation sites of which mTOR directly phosphorylates the priming sites Thr37 and Thr46 (Gingras et al., 2001). These two threonine phosphorylations are essential for subsequent phosphorylation of Thr70 and Ser65 as mutation of Thr37 and/or Thr46 to alanine prevents phosphorylation of Thr70 and Ser65 (Gingras et al., 2001). The phosphorylation of all four sites results in deactivation of 4E-BP1 and causes release of eIF4E from the inhibitory complex with 4E-BP1. Inhibition of mTOR leads to hypo-phosphorylation of 4E-BP1, which then associates tightly with eIF4E. Upon activation of mTOR, eIF4E is released from its inhibitor 4E-BP1 and is able to initiate the cap-dependent translation (Gingras et al., 2001). This will cause protein synthesis required for cell growth. The eIF4E protein cooperates with S6 Kinase and both pathways mediate a positive effect on cellular growth. In accordance, the overexpression of eIF4E was shown to increase cell size (Fingar et al., 2002).

mTOR has also been shown to control cell cycle progression through eIF4E and S6K1 (Fingar et al., 2004).

1.3.6.2 mTOR phosphorylates S6 kinase promoting translation

Mammalian cells contain two S6 kinase proteins (S6K1 and S6K2) which are very homologous. They are activated by phosphorylation at specific sites, which are conserved between the two proteins (Shima et al., 1998). Activation of S6 kinases is complex and requires interplay between several upstream kinases (Volarevic et al., 2001). In response to nutrition and hormonal stimulation 12 residues can be phosphorylated (Shah et al., 2000). TOR has been shown to phosphorylate only one site *in vitro*: the Thr389 in the regulatory domain (Dennis et al., 1996). Activated S6 kinases are able to promote the translation of so-called 5'-TOP mRNAs, which contain a short polypyrimidine sequence of 4-14 nucleotides next to the cap-motif at the 5' end. These mRNAs code for the components of the translation machinery, including elongation factors, poly-A-binding proteins and proteins contributing to ribosome complexes (Mayuhas and Hornstein, 2000). The S6 kinases are thought to control the rate of mRNA translation indirectly by phosphorylation of its downstream effectors. One of the most studied targets of S6 Kinases is the S6 protein, which is a component of the 40S small ribosomal subunit (Jefferies et al., 1997). The phosphorylation of S6 protein is required for the recruitment of the 40S ribosomes to 5'-TOP mRNA (Thomas, 2000). Phosphorylation of S6 by S6 Kinases correlates with an increase in the translation of mRNA possessing the 5'-TOP sequence (Jefferies et al., 1997). However, the S6 protein is certainly not the only target of S6 Kinases mediating an effect on the translation of TOP mRNA and cell growth. This was shown by the fact that there is no correlation between the regulation of the G1 phase by S6K1 and the phosphorylation status of the S6 protein (Fingar et al., 2004). Furthermore, in S6K1-/- embryonic cells the translation of 5'-TOP mRNA can still be activated in response to serum although the S6 protein remains unphosphorylated (Stolovich et al., 2002). Even in cells lacking both, S6K1 and S6K2, translation of 5'-TOP mRNA is activated in response to serum and, more importantly, the translation activation was still inhibited by rapamycin treatment (Pende et al. 2004). Therefore it is clear that other rapamycin sensitive targets of S6K1 are involved in the regulation of 5'-TOP mRNA translation. One of these targets is eIF4B.

1.3.6.3 mTOR regulates the eIF4B through S6K

eIF4B is an RNA binding factor required for efficient recruitment of ribosomes to the mRNA (Hershey and Merrick, 2000). This factor specifically activates the eukaryotic translation initiation factor 4A (eIF4A) protein (helicase/ATPase) involved in unwinding the secondary structures of translated mRNA (Rogers et al., 2002). eIF4B is phosphorylated in response to a variety of extracellular stimuli, such as insulin and serum, which promote cell growth and proliferation (Ducan and Hershey, 1985). One of the phosphorylation sites of eIF4B, Ser422, was shown to be specifically phosphorylated by S6 Kinase *in vitro* (Raught et al. 2004). *In vivo* studies show that phosphorylation of Ser422 by S6K is sensitive to the inhibition of PI3K, which is an upstream component of mTOR signaling pathway (Raugth et al., 2004). Thus, in addition to the S6 protein, eIF4B may be an important factor mediating several effects of mTOR/S6K signaling on translation, cell growth and proliferation.

1.3.6.4 mTOR activates degradation of PDCD4 through S6 kinase

The tumor suppressor programmed cell death protein 4 (PDCD4) is another downstream target of mTOR/S6K signaling pathway. Normally, PDCD4 blocks initiation of translation of mRNA by inhibiting the helicase activity of eIF4A. In response to growth factors the PDCD4 protein is phosphorylated on Ser67 by S6 kinase. This leads to subsequent ubiquitination of PDCD4 by the E3 ubiquitin ligase complex $SCF^{\beta TRCP}$ and as a consequence to rapid degradation of PDCD4 by the proteasome. The elimination of PDCD4 allows initiation of protein expression required for cell growth and proliferation (Dorrello et al., 2006). Thus, deregulation of mTOR/S6K/PDCD4 signaling pathway may lead to uncontrolled cell growth and ultimately to cancer development.

1.3.6.5 Phosphorylation loops in the mTOR/S6K signaling pathway

The direct phosphorylation of S6K by mTOR is not the only mechanism for regulation of translation. mTOR may regulate the action of S6K as well as 4E-BP1 indirectly by inhibiting the action of associated phosphatases. Indeed, rapamycin and amino acid deprivation activate the PP2A phosphatase, which causes the dephosphorylation of S6K1 as well as of 4E-BP1 ultimately, leading to the inhibition of translation. Activated mTOR phosphorylates and deactivates PP2A preventing the dephosphorylation of S6K1 and 4E-BP1 and inducing the activation of translation (Peterson et al., 1999).

Very recent studies indicate that not only mTOR phosphorylates S6K1, but S6K1 is also able to phosphorylate mTOR in a cell-free system (Holz et al., 2005). Overexpression of a rapamycin–Wortmannin resistant form of S6K1 leads to the constitutive phosphorylation of mTOR, whereas the down regulation of S6K1 expression reduces the phosphorylation of mTOR despite the full activity of Akt (Holz et al., 2005). Many data have shown that mTOR is directly phosphorylated by Akt at Ser2448 in response to mitogenic signals. Recent data show, however, that rapamycin blocks serum stimulated phosphorylation of mTOR at Ser2448 although this drug doesn't inhibit the activity of Akt. Interestingly, the S6K was shown to phosphorylate mTOR at Ser2448 *in vitro* and over-expression of the rapamycin-resistant S6K restored Ser2448 of phosphorylation in the rapamycin treated cells. Moreover, the S6K-dependent phosphorylation of Ser2448 was regulated by the cellular amino acid status through the TSC-Rheb pathway (Chiang et al., 2005). To obtain further insights in the regulation of the mTOR pathway (especially regulation by the Akt, TSC, Rheb, S6K) it has to be investigated in more detail. Cell culture models as well as *in vivo* studies can be used to unravel possible regulation mechanisms of the mTOR pathway. This is of importance since many of the components of the mTOR signaling are involved in cancer development.

1.4 The mTOR pathway and cancer development

Recent studies with cancer models indicate that signaling molecules acting up and down stream of mTOR are involved in essential processes regulating tumor growth. For example the upstream acting PI3K and Akt proto-oncogenes are frequently deregulated in tumors. More specifically, the catalytic subunit of Akt and PI3K are encoded by genes frequently found amplified in human cancers (Vivanco and Sawyers, 2003). In addition, PTEN is a tumor suppressor gene able to down-regulate the action of PI3K and Akt and loss-of-function-mutations in PTEN occur in human cancers with a frequency comparable to the rate of mutations found in the tumor suppressor gene p53 (Cantley et al., 1999). Hamartomas developing in any organ of the body are caused by mutation in TSC1 and TSC2 also acting upstream of mTOR (Mak and Yeung, 2004). Interestingly, mutations in or overexpression of mTOR have not been reported so far (Bjornsti and Houghton, 2004).

Deregulation of downstream targets of mTOR has also been shown to play a role in malignant transformation. For example, overexpression of eIF4E leads to deregulated growth and malignant transformation of cells *in vitro*. Elevated eIF4E levels are associated with solid tumor formation and can be found in breast, colon, head and neck cancers (DeBenedetti et al., 1999). On the other hand, the translation inhibitor 4E-BP1 has been found down-regulated in some types of tumors, which is also associated with cancer progression (Jiang et al., 2003).

These observations strongly indicate that the components of the translation initiation complex are important effectors of mTOR and their deregulation is involved in cancer formation. As shown the mTOR signaling pathway is also required for cell-cycle progression and inhibition of mTOR activity by rapamycin causes a G1 phase cell-cycle arrest (Jacinto and Hall, 2003). For these reasons the mTOR pathway is thought to be a valuable target for anti-cancer therapy. It is not surprising that rapamycin inhibits tumor growth and has been used in clinical studies (Houghton and Huang, 2004). Despite the significant antiproliferative activity of rapamycin it can not be easily used for cancer therapy because of stability and solubility problems. Therefore, three analogues of rapamycin were recently synthesized to obtain better pharmacokinetical properties. These analogs, CCI-779, RAD001 and AP23573 are inhibitors of mTOR and have similar growth inhibitory properties and are currently tested in clinical trials (Janus et al., 2005).

1.5 The role of bHLH proteins in mammary gland development

The development of the mammary gland is tightly controlled through the coordinated action of many signaling pathways that regulate the expression of genes involved in proliferation and differentiation. However, so far very little is known about the molecular mechanisms that regulate the action of transcription factors involved in this process. One of the group of transcription factors identified to play such an important role in development is the bHLH (Basic Helix-Loop-Helix) super-family. Generally, bHLH proteins are transcriptional regulators involved in cell proliferation and differentiation during many important developmental processes (Massari and Murre, 2000). The bHLH super family can be subdivided into four main groups, on the basis of the presence or absence of distinct functional domains like a DNA binding domain, LZ (leucine zipper) or PAS (Per-Arnt-Sim) domain (Figure 1.11). Alternatively, they can be grouped into seven classes, based on their tissue distribution, dimerization capabilities and DNA binding specificity (Table 1.1) (Massari and Murre, 2000).

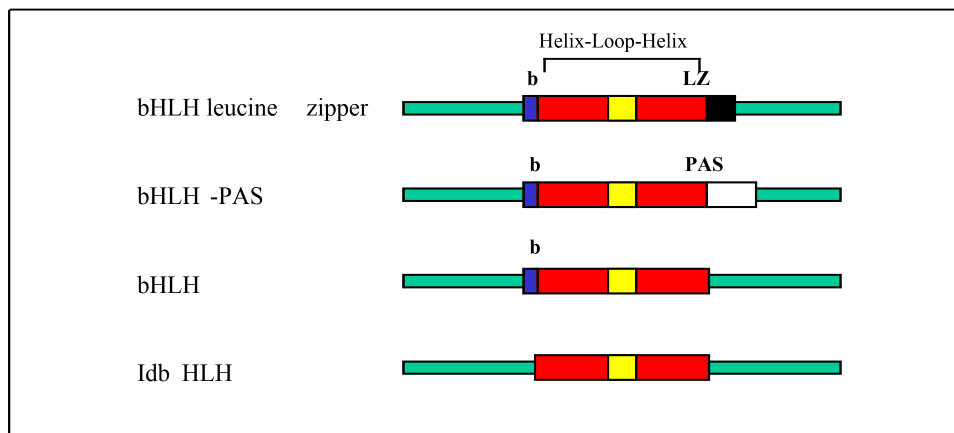

Figure 1.11 Schematic structure of different bHLH subfamilies. The bHLH transcription factor family can be divided into 4 groups based on their structure. The Idb class doesn't contain the basic-DNA binding domain (b) and therefore is not able to bind the DNA. HLH, helix-loop-helix. b, basic DNA binding domain. Per-Arnt-Sim homology (PAS) or leucine zipper (LZ) domain. Adapted from Norton J. D., 2000.

The highly conserved helix-loop-helix domain enables bHLH transcription factors to form homo or hetero dimers. The dimerization of bHLH proteins is absolutely required to achieve transcriptional activity (Murre et al., 1994). The basic domain is a common sequence motif, which mediates the binding of dimerized proteins to the target DNA sequence. They can bind certain sequence motifs in promoters such as E-boxes (CANNTG) or E-box-like sequences as homo or heterodimers and are able to regulate the transcription of target genes.

Tabele 1.1 Classification of the different members of bHLH Transcription Regulators Adapted from Desprez et al., 2003.

Class	Protein families	Function	Characteristics
I	E12, E47, HEB, E2-2 ITF-2 (E proteins)	Myogenesis, lymphogenesis Neurogenesis, sex determination, lymphomagenesis	Ubiquitous pattern of expression and capable of forming either homo or heterodimers
II	Myo D , Myogenin, NeuroD/BETA2 DHAND, eHAND Mash Twist Mist SCL/Tal1	Myogenesis Neurogenic differentiation, pancreatic development Cardiac morphogenesis Positive regulator of neurogenesis Inhibition of myogenic and neurogenic differentiation Exocytosis of serous secretions Involved in functional differentiation of mammary gland Hematopoiesis	Tissue-restricted pattern of expression and incapable of forming homodimers and preferentially heterodimerize with the E proteins Class I or with Class II proteins and can bind both canonical and noncanonical E-boxes
III	Myc TFE	Cell proliferation, differentiation Oncogenesis, apoptosis Transcription in immunoglobulin Heavy chain enhancer	Presence of a leucine zipper adjacent to the HLH motif
IV	Mad, Max Mxi	Interaction with Myc family proteins, regulators of cell proliferation	Dimerization with the Myc proteins or with each other
V	Idb Id1 and Id2	Inhibition of DNA binding, cell proliferation growth and differentiation in many type of cells. Regulators of growth invasion and terminal differentiation of mammary epithelial cells	Lack the basic DNA-bibding domain and act as dominant negative regulators of Class I and Class II bHLH proteins
VI	HES, HESR1	Notch signaling pathway, cell proliferation	Presence of proline in their basic region
VII	AHR, ARNT, Sim HIF	Biological responses to planar aromatic hydrocarbons Regulator of oxygen homeostasis	Presence of bHLH-PAS domain

Idb (Inhibitors of differentiation and DNA binding) proteins belong to the basic helix-loop-helix (bHLH) super-family. They act as negative regulators of bHLH DNA-binding proteins (Benezra et al., 1990). The Idb subclass is comprised of four members: Id1 to Id4. They have been shown to be implicated in the regulation of a variety of cellular processes including cell growth, proliferation and differentiation. All Idb proteins contain a HLH domain, allowing the formation of dimers with other bHLH proteins, but in contrast to other members of the bHLH super family they lack the basic DNA binding domain. In absence of Idb proteins the bHLH proteins form dimers, which can bind to specific DNA-elements and activate the transcription of proliferation or differentiation-associated genes. When Idb proteins are abundant the other bHLH proteins of class I and II preferentially form heterodimers with the Idb proteins. These heterodimers are not able to bind the DNA because the Idb proteins lack the DNA binding domain, preventing the transcription of their target genes (Figure 1.12). In this way the Idb proteins regulate transcription indirectly by inhibiting the interaction of bHLH proteins with promoters of various target genes.

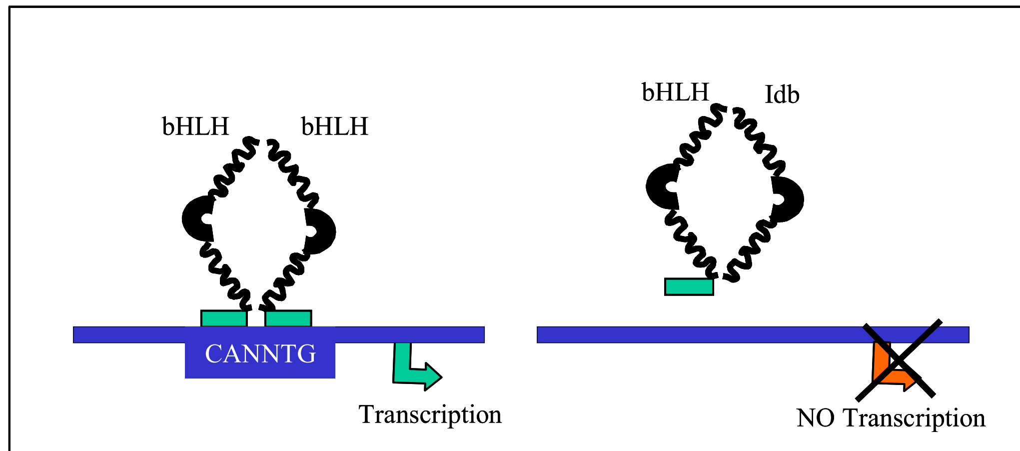

Figure 1.12 Schematic model of transcriptional regulation of bHLH transcription factors by Idb proteins. (**A**) The bHLH proteins can form homodimers as well as heterodimers with other members of the bHLH family. As a dimers the bHLH proteins can bind the DNA containing the sequence CANNTG and activate the transcription of target genes. (**B**). During development the Idbs are able to preferentially form dimers with the bHLH proteins. Since Idbs lack the DNA binding domain these heterodimer are not able to bind the DNA and activate the transcription. Adapted from Sikder Hashmat Cancer Cell 2003.

1.5.1 The role of Id1 in proliferation and invasion

It is known that Id1 is important for the regulation of proliferation, differentiation motility and programmed death of normal as well as of transformed mammary epithelial cells. Id1 mRNA is expressed at a high level in proliferating mouse mammary epithelial cells SCp2 *in vitro*, and its expression declines prior to differentiation or in quiescent cells. SCp2 cells transfected with an Id1 expressing vector grow more rapidly, whereas cells transfected with a vector leading to expression of Id1 anti-sense sequence grow more slowly compared to control cells. In addition constitutive over-expression of Id1 prevented the cells to functionally differentiate as measured by expression of β-casein (Desprez et al., 1995).

It is unclear how many genes are regulated by the Id1 protein, only a few targets have been described so far. A recent study reports cloning of a novel zinc finger protein, Zfp289, the expression of which is induced by Id1. The expression of Zfp289 correlates with proliferative stages of mammary epithelial cells *in vitro* as well as during mammary gland development *in vivo*. Therefore, Zfp289 is potentially an important mediator of the Id1 induced growth pathway in mammary epithelial cells (Singh et al., 2001). Other investigations showed that constitutive over-expression of Id1 can induce an invasive and migratory phenotype in non-transformed and non-tumorigenic murine mammary epithelial cells (SCp2).

The ability of Id1 over-expressing SCp2 cells to actively invade the extra cellular matrix (ECM) suggested that Id1 might induce the expression of ECM degrading proteases. Indeed a novel matrix metalloproteinase (MMP) was found to be secreted by Id1 over-expressing cells. Experiments with MMP inhibitors showed that this Id1-related metalloproteinase is indeed essential for the invasive phenotype of SCp2 cells. This MMP is also expressed during involution of the mammary gland, correlating with the expression of Id1. During involution this Id1 associated MMP may then participate in the remodelling of the mammary gland. Interestingly, this MMP was also detected in the medium of invasive breast cancer cells which highly express Id1 suggesting that Id1 regulates the invasive phenotype of breast cancer cells at least in part through transcriptional activation of the MMP gene. This idea is supported by the fact that other breast cancer cell lines expressing Id1 at a high level are also more invasive than those in which the Id1 gene is silenced (Desprez et al., 1998).

1.5.2 The role of Id2 during pregnancy and lactation

The Id2 protein has been shown to be an important transcription factor involved in the maintenance of the differentiated phenotype of mammary epithelial cells. This is shown by the fact that Id2 is expressed at a high level in fully differentiated SCp2 cells that express milk proteins. Unlike Id1 the expression level of Id2 is undetectable in proliferating cells. Over-expression of exogenous Id2 in undifferentiated SCp2 cells accelerates differentiation whereas silencing of Id2 by using anti-sense sequence blocks the expression of milk protein also confirming a role of Id2 in the functional differentiation of mammary epithelial cells. These *in vitro* data are supported by *in vivo* experiments. The mammary gland of Id2 knock-out female mice develops normally during embryogenesis and virgin mice have normal ductal trees, similar to those of heterozygous animals. At the day of delivery, however, the alveolar compartment is still poorly developed. The alveoli are small without clear milk accumulation. The mammary gland of the Id2-/- female mice resembled the gland of wild type mice at early pregnancy (Mori et al. 2000). From these *in vitro* and *in vivo* data it can be concluded that Id2 is involved in the regulation of functional differentiation of mammary epithelial cells and it regulates the development of the secretory alveolar compartment during pregnancy.

1.6 RNA interference as a tool for the analysis of genes involved in mammary gland development

Functional genomics and proteomics analysis help to uncover novel genes and proteins involved in the various steps of mammary gland development. To study the role of these genes more specifically and in more detail different techniques have been established. One popular method is the use of siRNA. With short interfering RNA it is possible to silence gene expression in a sequence specific manner in a process called RNA interference (Dykxhoorn et al., 2003).

RNA interference can be divided into two separate pathways (Figure 1.13). In the first case long double stranded RNA precursors are cut into typical short interfering RNA consisting of two 21 base pairs nucleotides, which form 19 base pairs duplexes, with 2-nt overhangs at the 3' ends and 5'-phosphates and 3'-hydroxyl groups (Elbashir et al., 2001) (Figure 1.13 A). This process is catalyzed by Dicer, which is a evolutionarily conserved member of the RNAse III enzyme family (Bernstein et al., 2001), and is an ATP dependent reaction (Zamore et al., 2000). Dicer guides the siRNA to a multiprotein RNA-inducing silencing complex (RISC). Although uptake of siRNA by RISC is independent of ATP the unwinding of siRNA duplexes requires ATP (Nykanen et al., 2001). The antisense strand then guides the RISC complex to its homologous target mRNA sequence (Schwarz et al., 2002). The target mRNA is endonucleolytically cleaved at a single site in the center of the duplex region between the guiding antisense siRNA and sense mRNA strand. Finally, this promotes the degradation of the cleaved mRNA. (Elbashir et al., 2001). In the case of the micro RNA (miRNA) pathway, Dicer produces the short 22 nt miRNA by cleavage of longer RNA precursors of 70 nt possessing imperfect hairpin structures (Figure 1.13C). Unlike the siRNAs the miRNAs are single stranded and are incorporated into the miRNA-protein complex (miRNP) (Hutvagner et al., 2002). The miRNA are known to bind to sites that share complementarity in the 3'-untranslated region (UTR) of the target mRNA, leading to suppression of mRNA translation and inhibition of protein synthesis (Pasquinelli and Ruvkun, 2002). In case of perfect pairing the miRNP complex is able to cleave the target mRNA (McManus and Sharp, 2002).

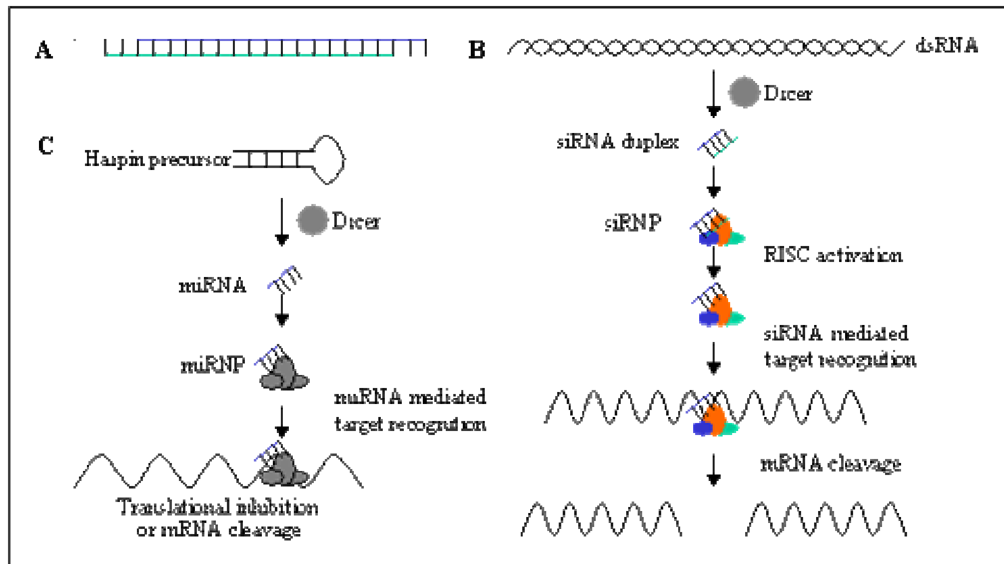

Figure 1.13 The RNA interference pathways. (**A**) Short interfering RNA 5'P ends and 19 nt duplex with 2-nt unpaired and unphosphorylated 3' ends. (**B**). The siRNA is processed by Dicer and in corporated to the siRNA-protein complex (siRNP) that lead to activation of RISC (RNA inducing silencing complex) which is guided by antisense RNA strand to target mRNA and cleaves it. (**C**) The miRNA are processed by Dicer form hairpin precursors and incorporated to miRNP-protein-complex (miRNP) that can inhibit translation or cleavage the mRNA.

1.6.1 Endogenous expression of siRNA

The siRNA molecules can be chemically synthesized and directly transfected into cells. Such siRNAs are relatively resistant to degradation. Nevertheless, in this way the knock-down of a gene is only transient, since with each cell division the siRNA is diluted. Soon the siRNA concentration is too low for effective inhibition of targeted gene expression. To achieve long lasting gene silencing the siRNA can be expressed from DNA vectors containing promoters for RNA polymerases for intracellular synthesis of siRNA (Dykxhoorn et al., 2003). The transient transfection of vectors expressing siRNA also has some limitation. For example the transfection reagents have to be tested for any negative effects on the cells. The number of cells used for the transfections, the total amount of vector DNA as well as the ratio vector: carrier has to be optimized for each cell line. There are many cells that are refractory to transfection including the mammary epithelial HC11 cell line. Therefore, an alternative to achieve stable siRNA expression in mammary epithelial cells is of interest to study the function of genes found to be involved in mammary gland development. The retrovirus based siRNA delivery system could be of interest for this purpose (Naldini et al., 1996; Brummelkamp et al., 2002).

1.6.2 The use of lentiviral vectors for stable siRNA expression in mammalian cells

Lentiviral vector system has been developed for stable expression of sequences encoding siRNA. These vectors are delivered into many types of cells *in vitro* and *in vivo*, with transduction efficiencies of almost 100% (Rubinson et al., 2003; Naldini et al., 1996). Stable expression of siRNA enables to study the effects of mRNA silencing over an extended period of time. This is important for analysis of genes involved in functional differentiation of mammary epithelial cells, a process which takes 10 days *in vitro*. Long term siRNA expression is also required if proteins with a long half-life are to be studied since in this case the effect of mRNA degradation on the protein level can be detected many hours even days after transduction with siRNA delivery vectors. Several members of the lentivirus family have been molecularly engineered for stable expression of a certain sequence of interest. These lentiviruses have a highly manipulable genome and sequences up to 8 kbp long can be inserted. They have a very low level of pro-inflammatory activity and most importantly they have the ability to stably integrate into the genome of dividing as well as non-dividing cells. These vectors have been successfully used for gene delivery into primary cells, liver, muscle, cardiac, neuronal cells and immune system dendritic cells, *in vitro* as well as *in vivo.* Several lentiviral based gene therapies are now in phase I clinical studies although there is still much concern about the fact that these vectors integrate randomly in the genome raising the probability to activate proto-oncogenes (Lever et al., 2004; Quinonez, 2002; Stewart et al., 2003).

1.7 Systems for cultivating mammary epithelial cells

During organogenesis the mammary gland develops from a small epithelial mammary anlage present in the embryo into a highly organized ductal tree of polarized epithelial cells in a mature female individual. During pregnancy the cells start to form the secretory compartment with alveoli. This process depends on the correct interaction between epithelial cells building the ducts and alveoli as well as the interaction of epithelial cells with surrounding adipocytes, fibroblasts and myoepithelial cells, which contribute to the mammary gland stroma. The role of genes involved in this process can be studied *in vivo*, but this method is time consuming and work intensive. Such studies do not allow simple and fast analysis of genes of interest. Therefore two dimensional cell culture conditions have been developed but not all fullfill the physiological needs of mammary epithelial cells necessary for development of the ductal tree and alveolar units. To solve such problems, many systems mimicking *in vivo* conditions have now been established over the years. The cells can be co-cultured with other mammary gland cell types, they can be seeded within a special matrix (collagen, matrigel, hydrogel) or transplanted into specially prepared stroma tissue, cleared fat pad, or can be grown as whole organ culture *in vitro*.

In three-dimensional culture systems (3D culture) mammary epithelial cells are seeded within collagen type I gel or matrigel which serves as an extracellular matrix that allows epithelial cells to arrange themselves into branching-like structures that resemble the ductal tree of the mammary gland. The cells can also form acinar structures similar to alveoli *in vivo* (Ip and Darcy, 1996).

Co-cultures with adipocytes have been shown to enhance the proliferation of mammary cells *in vitro*. For example primary epithelial isolated from pregnant mice and seeded together with adipocytes grew more rapidly than cells cultured alone on plastic (Levine and Stockdale, 1984). In response to the induction with lactogenic hormones the primary mammary cells co-cultured with fat cells were able to differentiate and to synthesize milk proteins (Levine and Stockdale, 1985). The epithelial cells can be cultivated in an *ex vivo* system as transplants into cleared fat pads. The epithelial ductal tree can be entirely removed from gland number four of three weeks old mice resulting in a cleared (from endogenous mammary epithelial cells) fat pad, which can be used as a transplantation site for normal and tumour epithelial cells but also for cells in which certain genes are silenced or over-expressed.

The epithelial cells can be grown and would respond to induction with lactogenic hormones in other than mammary fat tissue, for example they can be maintained in perenal, micrometrical and subcutaneous fat depots (Hoshino K. 1980). Other study has shown that mammary epithelial proliferation can also be supported by brown fat matrix (Hoshino, 1967).

The epithelial tissues are structures of polarized and specialized cells, strictly organized by cell-cell contact and attached to basement membrane. The disruption of the epithelial architecture leads to deregulation of proliferation, differentiation and survival, resulting in tumour development. The 3D cultures have been used to study the role of cancer genes and pathways in disorganization of normal epithelial architecture in biologically relevant environment (Debnath and Brugge, 2005).

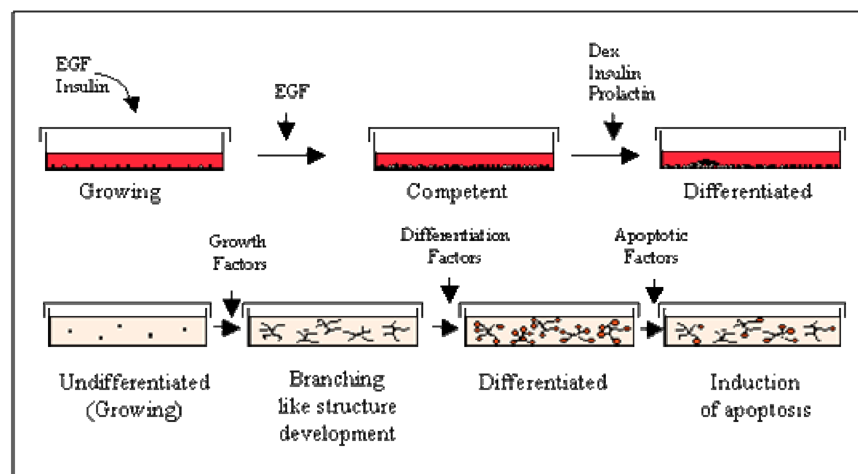

Figure 1.14 Schematic representation and comparison of mammary epithelial cell culture systems in *vitro* (**A**) Mammary epithelial cells cultivated on plastic dishes. The cells are grown in presence of insulin and EGF until they are confluent. After 2-4 days in medium containing only EGF the cells are competent and can be induced for functional differentiation with lactogenic hormone mix (Dexamethasone, Insulin, Prolactin) After 4 days of lactogenic stimulation the cells express the milk protein β-casein (indicator of terminal differentiation). (**B**) When cultivated in 3D context the cells can morphologically differentiate and form ducts and alveolar like structures that resemble branching and alveolar development in *vivo*.

The described three dimensional system using collagen or matrigel will be used to study the influence of mTOR inhibition by rapamycin as well as silencing and/or over-expressing of candidate genes using lentiviral vectors on the morphological differentiation of mammary epithelial cells *in vitro*.

1.8 The aims of this work

The mammary gland is a perfect model to study the molecular mechanisms that govern processes like organogenesis and organism development. The proliferation and differentiation of mammary epithelial cells, which lead to the formation of complicated glandular structure composed of ducts and secretory alveolar units are controlled by many pathways that activate transcription of hundreds of genes. Although the major pathways involved have been described, many aspects of the gene transcription profile regulation during mammary gland development are unknown. For this reason the aim of this work was the identification of novel signaling pathways, genes and proteins involved in proliferation and differentiation of mammary epithelial cells during the mammary gland development. This required the establishment of a system that allows the fast validation of candidate genes and proteins identified to play a possible role in this process. These candidate genes were identified in studies using the micro-array technology. For these experiments the expression patterns between proliferating and differentiated mammary epithelial cells were compared. This led to the identification of genes and proteins involved in the process of proliferation and/or differentiation of mammary epithelial cells. Some interesting candidates were selected and characterized in this work. As shown in the introduction the mTOR pathway is involved in the regulation of many important cellular processes influencing cell growth. The role of mTOR in mammary epithelial cell proliferation and differentiation as well as in mammary gland development has not been investigated in detail. Therefore, in this study the potent drug rapamycin, which is a very specific inhibitor of mTOR was applied to investigate the role of the mTOR pathway during proliferation and differentiation of mammary epithelial cells.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Acrylamid	Roth
Agarose	PeqLab
Albumin, bovine serum fraction V	Sigma
Ampicillin	AppliChem
Aprotinin	Sigma
APS	Sigma
Beta-mercaptoethanol	Roth
Bradford-Reagent	BIO-RAD
Bromophenol blue	Fluka
$CaCl_2$	Sigma
Chloroquine	Sigma
Chloroform	Roth
DMSO (Dimethyl Sulfoxide)	Merck
DTT (Dithiothreitol)	Sigma
ECL-reagent 1 and 2	AmershamPharma
EDTA (Ethylene diamine tetraacetic acid)	Roth
EGTA (Ethylene glycol tetraacetic acid)	Roth
Ethanol	Roth
Ethidium Bromide	Sigma
Eosin	Roth
Formaldehyde	Roth
Glycerol	Roth
HCl	Roth
HEPES	Sigma
Isopropanol	Roth
KCl	Roth
Leupeptin	Sigma, Steinheim
$MgCl_2$	Fluka
Milk powder	AppliChem
Na_2HPO_4	Sigma
NaCl	Roth
NaOH	AppliChem
Pefabloc	Sigma
Pepstatin	Sigma
Polybrene	Sigma
Polyethylenimine (PEI)	Sigma
SDS (Sodium dodecyl sulfate)	Roth
Sucrose	Roth
TEMED	Sigma
Tris	AppliChem
Triton X-100	Fulka
Tween 20	Fluka

2.1.2 Commercial kits and reagents

GeneAmp® - RNA PCR Core Kit	Applied Biosystems
IQ™ SYBR® Green Supermix	Bio-Rad
Lipofectamine™	Invitrogen
Mammalian Transfection Kit	Stratagene
mRNA Midi Kit	Qiagen
NUCLEOBOND® Plasmid Isolation Kit	Macherey-Nagel
NucleoSpin® Plasmid Isolataion Kit	Macherey-Nagel
NucleoSpin® RNA II Kit	Macherey-Nagel
peqGOLD TriFast™	peqLab
Ponceau S Solution	Sigma
Proliferation Kit II	Roche
Protein Assay Dye Reagent Concentrate	Bio-Rad
RNeasy® Midi Kit	Qiagen
QIAguick® Spin Kit	Qiagen
Trizol®	Gibco

2.1.3 Enzymes

AmpliTaq®DNA Polymerase	Roche
*Bgl*II, *Eco*RI, *Hinc*II, *Sac*II, *Sna*BI, *Sma*I, *Xba*I, *Xho*I	New England Biolabs
Calf Intestinal Phosphatase (CIP)	New England Biolabs
RNaseOUT™ Recombinant RNase Inhibitor	Invitrogen
SuperScript™ II Reverse Transcriptase	Invitrogen
T4 DNA Ligase	New England Biolabs

2.1.4 Material used in cell culture

Actinomycin D	Sigma
Collagen Type I from rat tail	BD Biosciences
Collagenase	Roche
Dexamethasone	Sigma
DMEM with 4.5 g/l Glucose w/o L-glutamine	BioWhittaker
DMSO, cell culture tested	Sigma
DPBS (1x) w/o Ca and Mg	PAA Labortories
EGF (Epidermal Growth Factor)	Becton Dickinson
F-12 (HAM) Nutrient Mixture with L-Glutamine	Gibco
FBS (Foetal Bovine Serum)	Gibco
Fetuin	Sigma
Hydrocortisone	Sigma
Insulin	Sigma
L-Glutamine 200 mM (100X) in 0.85% NaCl Solution	BioWhittaker
Matrigel™ Basement Membrane Matrix	BD Biosciences
Opti-MEM® Medium	Invitrogen
PEN-STREP (10.000U Penicillin/ml 10.000 µg Streptomycin/ml)	BioWhittaker
Prolactin (Luteotropic hormone)	Sigma
Rapamycin	Calbiochem
RPMI 1640 w/o L-Glutamine	BioWhittaker
TRYPSIN-EDTA(1x) in HBSS w/o Ca&Mg w/EDTA	Gibco

2.1.5 Markers

1 kb DNA-Ladder	New England BioLabs
100 bp DNA-Ladder	New England BioLabs
Precision Plus Protein™ Standards Dual Color	Bio-Rad

2.1.6 Plasmids

Name	**Specification**	**Reference**
pSuper	Mammalian expression vector with H1-RNA gene promoter allowing intracellular synthesis of siRNA transcript by polymerase III	Oligoengine
pSuper-A115	siRNA sequence A115 against Id1 upon regulation of H1 promoter	This work
pSuper-B131	siRNA sequence B131 against Id1 upon regulation of H1 promoter	This work
pSuper-C152	siRNA sequence C152 against Id1 upon regulation of H1 promoter	This work
pSuper-7	siRNA sequence 7 against Id2 upon regulation of H1 promoter	This work
pSuper-9	siRNA sequence 9 against Id2 upon regulation of H1 promoter	This work
pSEW	Lentiviral vector with SFFV promoter allowing stable intracellular expression of proteins of interest	Grez, M. Frankfurt am Main
pSEW-A115	siRNA sequence A115 against Id1 upon regulation of subcloned H1 promoter	This work
pSEW-B131	siRNA sequence B131 against Id1 upon regulation of subcloned H1 promoter	This work
pSEW-C152	siRNA sequence C152 against Id1 upon regulation of subcloned H1 promoter	This work
pSEW-7	siRNA sequence 7 against Id2 upon regulation of subcloned H1 promoter	This work
pSEW-9	siRNA sequence 9 against Id2 upon regulation of subcloned H1 promoter	This work
pCMVSport-6	Mammalian expression vector with CMV promoter allowing intracellular expression of proteins of interest	Invitrogen
pCMVSport6-Id1	Id1 protein coding sequence upon regulation of CMV promoter	This work
pCMVSport6-Id2	Id2 protein coding sequence upon regulation of CMV promoter	This work
pSiEW	Lentiviral vector with SFFV promoter allowing stable intracellular expression of proteins of interest	Grez, M. Frankfurt am Main
pSiEW-Id1	Id1 protein coding sequence upon regulation of SFFV promoter	This work
pSiEW-Id2	Id2 protein coding sequence upon regulation of SFFV promoter	This work

2.1.7 Antibodies

Epitope	Dilution	Origin	Manufacturer
β-casein	1:1000	Rabbit, polyclonal	Kindly provided by Barash, I.
β-tubulin	1:1000	Mouse, monoclonal	Sigma (C-20)
Id1	1:500	Rabbit, polyclonal	Santa Cruz (C-20)
Id2	1:500	Rabbit, polyclonal	Santa Cruz (C-20)
Phospho-mTOR	1:1000	Rabbit, polyclonal	Cell Signaling (Ser2448)
Phospho Stat5a/b	1:1000	Rabbit, polyclonal	UpState (Tyr694/699)
Stat5a/b	1:1000	Rabbit, polyclonal	Santa Cruz (N-20)
Anti-mouse Ig-HRP	1:5000	Sheep, polyclonal	Amersham Bioscience
Anti-rabbit Ig-HRP	1:5000	Donkey, polyclonal	Amersham Bioscience

2.1.8 Primers

2.1.8.1 Primers used for generation of siRNA

Name	Sequence 5' to 3'
mId1_A_up	GAT CCC CAA GGT GGA GAT CCT GCA GCA TTT CAA GAG AAT GCT GCA GGA TCT CCA CCT TTT TTT GGA AA
mId1_A_low	AGC TTT TCC AAA AAA AGG TGG AGA TCC TGC AGC ATT CTC TTG AAÀ TGC TGC AGG ATC TCC ACC TTG GG
mId1_B_up	GAT CCC CAA CGG CGA GAT CAG TGC CTT GTT CAA GAG ACA AGG CAC TGA TCT CGC CGT TTT TTT GGA AA
mId1_B_low	AGC TTT TCC AAA AAA ACG GCG AGA TCA GTG CCT TGT CTC TTG AAC AAG GCA CTG ATC TCG CCG TTG GG
mId1_C_up	GAT CCC CAA CGT CCT GCT CTA CGA CAT GTT CAA GAG ACA TGT CGT AGA GCA GGA CGT TTT TTT GGA AA
mId1_C_low	AGC TTT TCC AAA AAA ACG TCC TGC TCT ACG ACA TGT CTC TTG AAC ATG TCG TAG AGC AGG ACG TTG GG
Id2-7_up	GAT CCC CGG ACT GTG ATA CCG TTA TTT TCA AGA GAA ATA ACG GTA TCA CAG TCC TTT TTG GAA A
Id2-7_low	AGC TTT TCC AAA AAG GAC TGT GAT ACC GTT ATT TCT CTT GAA AAT AAC GGT ATC ACA GTC CGG G
Id2-9_up	GAT CCC CGT GAA CCT TGT GGA CTC TTT TCA AGA GAA AGA GTC CAC AAG GTT CAC TTT TTG GAA A
Id2-9_low	AGC TTT TCC AAA AAG TGA ACC TTG TGG ACT CTT TCT CTT GAA AAG AGT CCA CAA GGT TCA CGG G

2.1.8.2 Primers for amplification of Idb gene with SacII enzyme adaptor.

Name	Sequence 5' to 3'
Id1-SacII-up	TCC CCG CGG ATG AAG GTC GCC AGT GGC AG
Id1-SacII-lp	TCC CCG CGG TCA GCG ACA CAA GAT GCG AT
Id2-SacII-up	TCC CCG CGG ATG AAA GCC TTC AGT CCG GT
Id2-SacII-lp	TCC CCG CGG TTA GCC ACA GAG TAC TTT GC

2.1.8.3 Primers used for Real-Time PCR

Name	**Sequence 5' to 3'**
18S_up	CGT TGG TGT GGG GAG TGA ATG GTG
18S_lp	GCG TGG GGG TTG GCG GAA AGA GAA
β-casein_up	TCA CTC CAG CAT CCA GTC ACA
β-casein_lp	GGC CCA AGA GAT GGC ACC A
Id1_up	TGG TCT GTC GGA GCA AAG C
Id1_lp	GCA GCC GTT CAT GTC GTA GAG
Id2_up	ACT CGC ATC CCA CTA TCG TCA GC
Id2_lp	TGA CCA CCC TGA ACA CGG ACA T
Cited4_up	GGC CGA CCA CCT GAT GCT C
Cited4_lp	ATG CCC GGG CCT GCG TAT
Nedd8_up	CAG TGG CAA GCA AAT GAA TGA TGA
Nedd8_lp	CACTGC CCA AGA CCA CCT C
Ltf_up	ATG CTG GAG CCT TGA GGT GTC TGA
Ltf_lp	GGT CCC TTT CGG CTT TAT TTG GTA
Atp5l_up	CAA CCC CTG CTG AAA TCC CTA CA
Atp5l_lp	GGC CAC CAA ACCATT CAG CAC AG
Nkd2_up	GAG CAG GCC AGG CAA GAA CAT CAT
Nkd2_lp	GTA TAG CGT GGG CAT CCG ACT CTT
Hdac2_up	TCA ATT GGG CTG GAG GAC TAC A
Hdac2_lp	TTC GAG GAT GGC AAG CAC AAT
Sap30_up	GAA GGT GAA GAT CGA GCT GGA
Sap30_lp	CCT TCT GTT CCG AAC GCT CT
Esg1_up	GAG CCA GGC CAT GTT TGA GC
Esg1_lp	TAG CCC GAA TCT TGT TGT TTT GAG
Rae1_up	CAG CAA CCA GGC CAT TCA GA
Rae1_lp	CAG CTC CCG GTC ATC ACA C
Nap1l1_up	GGG CGT GGG ACA GTT CGT
Nap1l1_up	CGC CTC AGC ATC ATC ATC CA
Hsp105_up	CCA GGC TAA GCA GGC ATA CAT
Hsp105_lp	ACA CTT TCG GTC GTT CCT CAG
Pdlim1_up	CCC AAC TGG CCT CTA CTC ATC
Pdlim1_lp	ACC ACG GGC CTG CTG TTT
Nol5_up	TAG GCC AGT CAA GTC CCA AAC AC
Nol5_lp	TTC ACC AAA AGC GTC GTA TCT G
Biklk_up	TCA CCA ACC TAC GGG AAA ACA
Biklk_lp	AAG AAG ACC AGC AGC ACC ATC
Kpna2_up	GAT GGT TCA GCT TTC CGA GAC T
Kpna2_lp	CCA CAT GCC AAG GTA GAC AGA

2.1.8.4 Primers used for RT-PCR

Name	Sequence 5' to 3'
β-casein-up	ACT ACA TTT ACT GTA TCC TCT GA
β-casein-lp	GTG CTA CTT GCT GCA GAA AGT ACA G
GAPDH-up	GTG AAG GTC GGT GTG AAC GGA TTT GGC CGT
GAPDH-lp	CCA CCA CCC TGT TGC TGT AG

2.1.9 Labware

Company	Item
Abgene	Thermo-Fast®, 0.2 ml Semi Shirted 96-well PCR plate
BD Biosciences Becton Dickinson Labware	Falcon® Polypropylene Conical Tube BLUE MAX™ 50 ml Falcon® Polypropylene Conical Tube BLUE MAX™ Jr 15 ml Polypropylene Round-Bottom Tube 14 ml Polypropylene Round-Bottom Tube 5 ml Falcon® ADVANTAGE™ Serological Pipet 2, 5, 10, and 25 ml Microlance™ 0.9 x 40 mm Sterile needle Plastipak™ Sterile syringe 1 ml
Bio-Rad	Mini Protean Short Plates Mini Protean Long Plates ICycler iQ™ Optical Tape
Brand	Plastibrand™ 0.5 ml, 1.25 ml, 2.5 ml, and 5 ml dispenser-tips
B/Braun	Omnican®30 0.33 x 12 mm 0,3 ml syringe
Corning Incorporated Costar®	STRIPETTE® 5 ml, 10 ml, and 25 ml serological pipet Sterile Cell Lifter Sterile 12, 24 and 96 well cell culture flat bottom plate with lid
Dahlhausen	2 ml sterile syringe
Fujifilm	Fuji Medical X-Ray Film 18 x 24 cm 100 NIF
Greiner Bio-One	CELLSTAR® Tissue Culture Dish 94,0/16 mm Vented, Sterile
Millipore	Millex™ (0.22 µm pore size filter)
Ratiolab	10 µl, 20 µl, 200 µl and 1 ml Aeroject® xL tips
Schleicher-Schuell	PROTRAN®BA83 Nitrocellulose membrane (0.2µm pore size)

2.1.10 Eukaryotic cell lines

Cell line	Description	Ref/ATCC-No.
HC11	mouse mammary epithelial cell line established *in vitro*	Ball et al., 1998
PMEC	mouse primary mammary epithelial cells	Freshly Isolated
HEK 293T	humane embryonic kidney cells transformed with SV40 large-T antigen.	CRL-11268

2.1.11 Bacterial strains

Strain	Genotype	Supplier
TOP10	F- mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu),7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen
Stbl2	F– mcrA (mcrBC-hsdRMS-mrr) recA1 endA1 lon gyrA96 thi supE44 relA1– (lac-proAB)	Invitrogen
XL-1 blue	SupE44, hsdR17, recA1, endA1, gryA46, thi, relA1, Lac–, F´(proAB$^+$, lacI^q, lacZ, ΔM15, Tn110 (tet^r))	Invitrogen

2.1.12 Culture media

2.1.12.1 Media for cultivating bacterial cells

Bacterial cells were grown in LB medium with addition of 100 µg/ml ampicillin in a shaker at 200 rpm and 37˚C overnight. Bacterial strains are stored in –80˚C in LB medium with addition of 50% glycerol.

LB medium	Bacto-Trypton 10 g/l Bacto Yeast Extract 5 g/l NaCl 5g/l, pH 7.5 established with 5N NaOH, autoclave.
SOC-medium	Bacto-Trypton 20 g/l Bacto Yeast Extract 5 g/l NaCl 0,5 g/l KCl 2.5 mM pH 7.0 established with 5 N NaOH, autoclave

2.1.12.2 Medium for cultivating HEK 293T cells

DMEM growth medium	DMEM (Dulecco´s modified Eagle medium) 10% FCS, deactivated 30 min in 56˚C, 2 mM L-Glutamine 100 U/ml Penicillin 100 µg/ml Streptomycin

2.1.12.3 Media for HC11 cells (mouse mammary epithelial cells)

Growth medium	RPMI 1640 10% FCS, heat-inactivated 100 U/ml Penicillin 100 µg/ml Streptomycin 2 mM L-Glutamine 10 ng/ml EGF 5 µg/ml Insulin
Starvation medium	RPMI 1640 10% FCS, heat-inactivated 100 U/ml Penicillin 100 µg/ml Streptomycin 2 mM L-Glutamine 10 ng/ml EGF
Induction medium	RPMI 1640 10% FCS, heat-inactivated 100 U/ml Penicillin 100 µg/ml Streptomycin 2 mM L-Glutamine 5 µg/ml Prolactin 5 µg/ml Insulin 10^{-7}M Dexamethasone

2.1.12.4 Media required for isolation and cultivation of mouse primary mammary epithelial cells

Digestion Medium:	DMEM/F12 Collagenase Hyaluronidase Gentamicin Penicillin Streptomycin	1:1 (v:v) 2 mg/ml 100 U/ml 50 µg/ml 100 U/ml 100 µg/ml
2x Plating Medium	F12-medium Insulin Hydrocortisone EGF Gentamicin Penicillin Streptomycin	 10 µg/ml 2 µg/ml 10 ng/ml 100 µg/ml 200 U/ml 200 µg/ml
Growth Medium	F12-medium Insulin Hydrocortisone EGF FCS, heat inactivated Gentamicin Penicillin Streptomycin	 5 µg/ml 1 µg/ml 5 ng/ml 5% 50 µg/ml 100 U/ml 100 µg/ml
Differentiation Medium	DMEM/F12 Insulin Hydrocortisone Prolactin Gentamicin Penicillin Streptomycin	1:1 (v:v) 5 µg/ml 1 µg/ml 3 µg/ml 50 µg/ml 100 U/ml 100 µg/ml

2.1.13 Buffers and solutions

Anode Buffer I	300 mM Tris-HCl pH 10.4, 20% (v/v) Methanol
Anode Buffer II	25 mM Tris-HCl pH 10.4, 20% (v/v) Methanol
Blocking buffer	1 x TBS 0.1% Tween-20 with 5% w/v BSA (Bovine Serum Albumin)
Buffer A (Nuclear fractionation)	10 mM HEPES, 0.5 M Sucrose, 50 mM NaCl, 0.25 mM EGTA, 1m M EDTA, 0.5 mM Spermidine, 0.5% Triton X-100, 0.5 mM PMSF, 0.5 µg/ml Leupeptin, 0.7 µg/ml Pepstatin, 1 µg/ml Aprotinin, 40 µg/ml Bestatin, 1 µg/ml Trypsin
Buffer B (Nuclear fractionation)	10 mM HEPES pH 8.0, 25% Glycerol, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM Spermidine, 0.25 mM DTT, 0.5 mM PMSF, 0.5 µg/ml Leupeptin, 0.7 µg/ml Pepstatin, 1 µg/ml Aprotinin, 40 µg/ml Bestatin, 1 µg/ml Trypsin
DNA Gel loading Buffer (6x):	15% Ficoll, 25 mM EDTA, Bromphenolblue
Formalin fixative	10% Fromaldehyde (40% stock solution),10% glacial acid, 80% ethanol (70% stock solution)
Kathode Buffer:	25 mM Tris-HCl pH 10.4, 20% (v/v) Methanol, 40 mM ε-Aminocapron saure
Luciferase Lysis Buffer:	1% Trton X-100, 25 mM Glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT (added fresh before use)
Luciferin Substrate	2,97 g Glycylglycin 2.7 g ATP add 50 mg Luciferin add 900 ml sterile H_2O adjust to pH 7.8, store in dark
PBS (1x):	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 pH 7.4
RIPA:	50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Nonidet P40, 0.5% Na-deoxycholate, 0.1% SDS. Add fresh before use: 100 µl/ml Pefabloc, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin 1 µg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF
SDS-PAGE Running Buffer	25 mM Tris, 250 mM Glycin, 0,1% SDS
SDS-PAGE Loading Buffer (5x)	100 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 20% Glycerol, 0,2% Bromophenolblue
Solution 1 (preparation of bacterial competent cells)	100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15% (v/v)Glycerol, pH 5.8 (HCl), sterilized by filtration.
Solution 2 (preparation of bacterial competent cells)	10 mM MOPS pH 7.0, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% Glycerol (v/v), pH 7.0 (NaOH), sterilized by filtration.
TAE-Buffer:	40 mM Tris (pH 8.0), 2 mM EDTA
TBS (1x)	10 mM Tris (pH 7.5), 150 mM NaCl
TBS-T wash buffer	TBS with addition of 0,1% Tween 20
Tris Buffer	Tris with different molar concentrations and pH

2.2 METHODS

The standard methods were described on the basis of *Molecular Cloning* (Sambrook et al 1989), if not indicated by other reference in the text.

2.2.1 MOLECULAR BIOLOGY METHODS

2.2.1.1 Preparation of transformation competent bacterial cells

XL1-blue bacterial cells were incubated in 500 ml of LB medium and grown until the OD_{600} reached range 0.55-0.6. The bacterial culture was centrifuged at 2000 rpm for 10 minutes at 4°C and the resulting supernatant was discarded. The bacterial cell pellet was resuspended in 200 ml ice-cold solution 1. The obtained cell suspension was incubated on ice for 1 hour. Then the bacterial cells were centrifuged at 2000 rpm for 5 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 15 ml ice-cold solution 2. Aliquots of 200 µl volume each were prepared, which were shock frozen in liquid nitrogen. The aliquots of competent bacterial cells were stored at –80°C until needed for transformation with plasmid DNA.

2.2.1.2 Transformation of bacterial cells

A frozen aliquot of competent bacterial cells ($CaCl_2$ treated) was thawn on ice. For each transformation 100 µl was transferred to a sterile cold 1.5 ml tube using chilled micropipette tip. For each transformation sample 50 ng of plasmid DNA (in a volume of 10 µl or less) was added to the cells and the content of the tube was mixed by gently swirling. The tubes were incubated on ice for 30 minutes. Then the tubes were transferred to a heat block preheated to 42°C and incubated for exactly 90 seconds. After this incubation, the tubes were immediately transferred to an ice bath and allowed to cool down for 1-2 min. Then 900 µl LB medium without antibiotics was added to each sample tube and the transformed bacteria were incubated in a thermomixer with gentle mixing at 37°C for 1 h. Finally, 100 µl (up to 200 µl per 10 cm plate can be used) of transformed bacterial cells was plated onto an LB agar plate containing the appropriate antibiotic (normally 100 µg Amp per 1 ml LB medium). The plates were incubated at room temperature until the liquid had been absorbed. The plates were then inverted and incubated at 37°C for 12-16 hours.

The next day the colonies that were able to grow on the plates, were used to isolate the transformed plasmid DNA.

2.2.1.3 Plasmid DNA Mini Preparation

For the isolation of a small amount (<10 μg) of plasmid DNA the NucleoSpin® Plasmid Isolation Kit MACHEREY-NAGEL was used. The bacterial cells transformed with plasmid were cultivated in 5 ml LB medium at 37°C with constant shaking (200-250 rpm) overnight (>12h incubation is recommended). To pellet the cells, 1.5 ml of bacterial culture was centrifuged for 30 s at 11000 x g in a standard bench top microcentrifuge. The resulting supernatant was removed and 250 μl buffer A1 was added to the tube. The cell pellet was resuspended by vigorous vortexing. To lyse the cells 250 μl buffer A2 was added and the tube content was mixed gently by inverting the tube 6-8 times. The sample was incubated at room temperature for 5 min. After incubation, 300 μl buffer A3 was added and the contents were mixed gently by inverting it 6 to 8 times. The obtained lysate was centrifuged for 5-10 minutes at 11000 x g at room temperature. The resulting supernatant was loaded on a column, and placed in a 2 ml sterile collecting tube. The tube with the column was centrifuged for 1 minute at 11000 x g at room temperature and the flow-through was discarded. To wash the column 600 μl buffer A4 was added and the column was centrifuged for 1 min at 11000 x g at room temperature. The resulting flow-through was discarded. To dry the silica membrane completely the column was centrifuged for an additional 2 minutes at 11000 x g at room temperature. Finally to elute the plasmid DNA the column was placed in a sterile 1.5 ml microcentrifuge tube and 50 μl buffer AE was added. The column was incubated for 1 min at room temperature and then centrifuged for 1 min at 11000 x g at room temperature. The flow-through containing the plasmid DNA was collected in a properly labeled sterile tube. The isolated plasmid DNA was stored at –20°C until needed for future applications.

2.2.1.4 Plasmid DNA Maxi Preparation

For the isolation of large amount (>100 μg) of plasmid DNA the NUCLEOBOND® Plasmid Isolation Kit MACHEREY-NAGEL was used. The bacterial cells were cultivated in 500 ml of LB medium at 37°C with constant shaking (200-250 rpm) overnight (>12h incubation is recommended).

The bacterial culture was harvested by centrifugation at 3000 x g for 5-10 min at 4°C. The pellet of bacterial cells was resuspended in 12 ml buffer S1. The obtained suspension was transferred to a 50 ml conical tube. To lyse the cells 12 ml buffer S2 was added and immediately after closing the tube, the suspension was mixed gently by inverting the tube 6-8 times and incubated at room temperature for 5 min. Then, 12 ml of buffer S3 was added and the sample was immediately mixed by gently inverting the tube 6-8 times until a homogeneous suspension was formed. To complete precipitation of proteins and genomics DNA the suspension was incubated on ice for 5 min. The lysate was loaded onto pre-wetted Whatman filter and the flow-through was collected. The clear filtrated lysate was loaded onto a collection column equilibrated with 6 ml buffer N2. The column was than washed twice with 16 ml buffer N3. Finally the plasmid DNA was eluted with 15 ml of buffer N5. To reduce the volume the purified plasmid DNA was precipitated by adding 5 ml isopropanol, pre-equilibrated to room temperature. The tube was immediately centrifuged for 30 min at high speed (>15000 x g at 4°C). The obtained plasmid DNA pellet was washed with 5 ml of ice cold 70% ethanol and centrifuged for 10 min at 15000 x g at 4°C. The DNA pellet was briefly dried for 5 minutes at room temperature and re-dissolved in a 1 x TE buffer or sterile water before the plasmid DNA was stored at –20°C for further applications.

2.2.1.5 DNA concentration measurement

The DNA samples were unfrozen at room temperature and after that the tubes were immediately transferred on ice bath to prevent any DNA degradation. To prepare dilution of DNA samples for each measurement the 995 µl of DNase-free water and 5 µl of the sample DNA were added to properly labeled microcentrifuge tube (the final DNA dilution factor is 1 to 200). The tubes were closed and mixed by shaking on vortex machine. The DNA concentrations were measured by OD_{260} using spectrophotometer. The calculated concentrations were written down on the tubes with the DNA stock. The DNA samples were stored at -20°C for further applications.

2.2.1.6 RNA concentration measurement

RNase free laboratory equipment was used when working with RNA. To prevent degradation of RNA and inhibit eventually action of RNase all measurement steps were done at low temperature.

RNA samples were incubated until they unfreeze completely on ice bath. To prepare RNA sample dilution for measurement 99 µl of RNase free water and 1 µl of isolated RNA were transferred to microcentrifuge tube. The tubes were closed and the diluted RNA was mixed by short shaking on a vortex machine. The RNA sample concentration was measured by OD_{260} with spectrophotometer. A water resistant pen was used to write on the tubes the calculated RNA concentrations. The RNA samples were stored at -80°C for further applications.

2.2.1.7 Sequencing of nucleic acids

The DNA sequencing was performed by LabGroup Dietrich at the Georg-Speyer-Haus with Taq-Man Sequencing System. For each sample around 50 ng of DNA was transferred to a properly labeled 1.5 ml microcentrifuge tube using DNase-free pipette tips. Additionally the primers for sequencing the DNA fragment of interest were added (final primer concentration 100µM). The tubes were filled with HLPC water to a final volume of 15 µl. The obtained DNA sequences were analyzed manually or using a special computer program (DNA Star).

2.2.1.8 Gel Electrophoresis of DNA

The electrophoresis tank was filled with 1 x TAE electrophoresis buffer. The open ends of the plastic tray were sealed with adhesive tape to form a mold which was then set on even part of bench. Usually 1-2% (w/v) agarose was dissolved in electrophoresis buffer, depending on the particular size of the fragments expected in the separated DNA samples. The agarose suspension was boiled in a microwave oven until the agarose was completely dissolved. Ethidium bromide was added to a final concentration of 0.5 µg/ml after cooling the agarose. The agarose solution was poured into the mold and an appropriate comb was putted to the mold to form sample slots. The gel was incubated for 30-45 minutes at room temperature until it was completely set. A small amount of electrophoresis buffer was poured on the top of gel to enhance easy removal of the comb. The prepared gel was mounted in the electrophoresis tank and was covered with the electrophoresis buffer. The DNA samples were mixed with 6 x gel loading buffer. The size standards were loaded on both the right and left sides of the gel. The samples were loaded into the slots of the submerged gel. The lid of the gel tank was closed and the electrical leads were attached to power supply. The voltage of 1-5V/cm was applied.

When the DNA samples or dyes had migrated a sufficient distance toward the anode, the electric current was turned off and the leads and lid were removed from the gel tank. The gel was examined and photographed under UV light.

2.2.1.9 Extraction of DNA fragments from agarose gels

For the isolation of DNA fragments from agarose gels the QIAquick Gel Extraction Kit (QIAGEN) was used. The DNA fragment of interest was cut out from the agarose gel with clean, sharp scalpel. To minimize the size of the gel slice any extra agarose was removed. The gel slice was transferred into a microcentrifuge tube and weighed. Then 3 volumes of QG buffer were added to 1 volume of gel (100 mg gel = 100 µl buffer). The gel sample was incubated at 50°C for 10 minutes and mixed by vortexing every 3 minutes during incubation. When the gel slice had dissolved completely, one gel volume of isopropanol was added to the sample and mixed. The dissolved gel sample was applied onto the QIAquick spin column and centrifuged for 1 minute at room temperature. The flow-through was discarded and 0.75 ml of buffer PE was added to the QIAquick spin column and centrifuged at 13000 rpm for 1 minute at room temperature to wash the membrane. The flow-through was discarded and the column was placed back in the same micro-centrifuge collection tube and centrifuged at 13000 rpm for 1 minute at room temperature to dry the membrane completely. Finally, the column was placed in fresh microcentrifuge 1.5 ml tube and the DNA was eluted by adding 50 µl of buffer EB or sterile H_2O to the center of the column membrane followed by incubation of the sample for 1 minute at room temperature. The column was centrifuged at 13000 rpm for 1 minute at room temperature and the obtained DNA was stored the at –20°C for further applications.

2.2.1.10 Digestion of DNA with restriction endonuclease enzymes

Restriction enzymes and buffers were obtained from New England BioLabs. The DNA and all necessary buffers were unfrozen at room temperature. To a sterile microcentrifuge an appropriate amount of plasmid DNA was added. Generally, 1/10 volume of optimal restriction buffer (as required for the enzyme used), and 10 U of each enzyme per 1 µg of plasmid DNA were also added to the tube. If necessary 1/100 volume of BSA solution was included. The tube was full-filled with sterile water to the required final volume of 100 µl and incubated, depending on reaction, for 4 to 8 h at 37°C.

To deactivate the enzymes the sample was incubated additionally at 65°C for 10 minutes. An 5 µl aliquot of digestion mix was taken and resolved on 1% agarose gel to check the extension of digestion. As a control non-digested plasmid DNA sample was used. Properly digested DNA, was purified using special column based kit (Qiagen). The purified linear DNA was finally dissolved in sterile water. The concentration of DNA was estimated with spectrophotometer. The digested DNA sample was stored at –20°C for further applications.

2.2.1.11 Dephosphorylation of 5' ends of DNA

Removal of 5'-phosphate groups prevents self-ligation and circularization of linearized plasmids. The reasonable amount of plasmid DNA was digested and purified. The dephosphorylation reaction mixture was performed by adding to a microcentrifuge tube 10 µl of 10 x reaction buffer and 0.5 U of calf intestinal phosphatase (CIP, New England Biolabs) per 1 µg of linearized plazmid DNA. The reaction was adjusted to 100 µl with sterile water. The samples were incubated at 37°C for 45-60 minutes. To inactivate the CIP enzyme, EDTA (pH 8.0) and EGTA to final concentration 5 mM and 10 mM respectively were added to tube. The sample was mixed and incubated at 65°C for 30 minutes. After the reaction, the tube was cooled to room temperature for 5 minutes. The DNA was cleaned using column kit (Qiagen). The dephosphorylated DNA was stored in small aliquots at –20°C.

2.2.1.12 Ligation of DNA fragments into plasmids

Dephosphorylated plasmid DNA and a DNA insert were mixed with a molar ratio of 1:2 in a sterile 1.5 ml microcentrifuge tube. Additionally 40 U of T4-DNA-Ligase (New England Biolabs) and 2 µl of 10 x ligation buffer were added to the tube and finally, the reaction was adjusted to a total volume of 20 µl with sterile water. The ligation reaction was incubated for 2 to 3 hours at 16°C or overnight at 4°C. After ligation the tube was briefly centrifuged to down the contents of the tube and the whole ligation mix was used for transformation of 100 µl competent bacterial cells.

2.2.1.13 Fast total RNA purification from cultured cells.

For fast isolation of total RNA from cell cultures the NucleoSpin® RNA II isolation Kit (MACHEREY-NAGEL) was used. The cells of interest were seeded in 10 cm dish and cultivated in CO_2-incubator at 37°C with proper medium and growth regulators for the desired time. The medium was removed and 700 µl buffer RA1 mixed with 7 µl β-mercaptoethanol was added to the plate. The cells were scraped from the plate with a cell lifter and the obtained cell lysate was transferred to a 2 ml tube. To reduce the viscosity the lysate was passed 5-10 times through a 0,9 mm needle (20 gauge) fitted to a 1 ml syringe. To clear the sample by filtration the lysate was applied onto NucleoSpin® Filter Unit and centrifuged for 1 min at 11000 x g. After centrifugation the NucleoSpin® Filter unit was discarded and 700 µl 70% ethanol was added to the homogenized lysate and the sample was mixed by vortexing and. transferred onto a NucleoSpin® RNA II column placed in microcentrifuge 2 ml tube. The column was centrifuged for 30 s at 8000 x g at room temperature. Next 350 µl of MDB (Desalting Buffer) was applied to the column and the tube was centrifuged at 11000 x g for 1 min to dry the membrane. To remove the DNA from the RNA sample a DNase reaction mixture was prepared in a sterile microcentrifuge tube according to the manual. 95 µl DNase reaction mixture was directly applied onto center of the silica membrane of the column and the tube was incubated at room temperature for 15 min. After incubation the membrane was washed with 200 µl RA2 and centrifuged for 30 seconds at 8000 x g. The column was placed into a new 2 ml collecting tube and 600 µl buffer RA3 was added on the column. After centrifugation for 30 seconds at 8000 x g the flow-through was discarded and again 250 µl buffer RA3 was added to the column. To dry the silica membrane completely the column was centrifuged for 2 min at 8000 x g. To elute the RNA the column was placed into a nuclease-free 1.5 ml microcentrifuge tube and 60 µl RNase-free H_2O was added to the membrane. The column was centrifuged at 11000 x g for 1 min. The obtained total RNA sample was stored at –80°C for further applications.

2.2.1.14 Reverse Transcription of mRNA

To analyze the level of gene expression in Real-Time PCR the mRNA of interest has to be reverse-transcribed to cDNA. The reaction can be catalyzed by the reverse transcriptase. For the preparation of reverse transcription reactions the SuperScript™ II Kit (Invitrogen) was used. To a 1.5 ml RNase-free microcentrifuge tube 10 µg of the mRNA sample and 1 µl of 100 pM AncT-primer (Anchored oligo-dT primer) with the sequence 5'-$T_{(20)}$VN-3' (V can be any nucleotide except T, N can be any nucleotide) was added.

The reaction was adjusted to a final volume of 10 µl with RNase-free water. The tube content was then gently mixed by pipetting up and down. To denaturate the mRNA the tube was incubated in a heating block at 70°C for 10 minutes and after that the sample was placed on ice to cool down and was finally centrifuged briefly in a microcentrifuge. Then the following components were added: 4 µl 5 x First-Strand Reaction buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, 1 µl RNaseOUT™ (Ribonuclease Inhibitor), 1 µl SuperScript™ II RNase H^- Reverse Transcriptase and 1 µl RNase-free water. The final reaction volume is 20 µl. The tube was then incubated in a heating block at 42°C for 1h. At this temperature the enzyme reverse transcribe the mRNA into cDNA. To inactivate the reaction the tube was heated at 70°C for 15 minutes. After the reaction had cooled down on ice, the tube was shortly spun in a microcentrifuge. Finally to prepare 1:10 dilution of obtained cDNA sample (used for Real-Time PCR reactions) 80 µl of DNase-free water was added to the tube and the content was gently mixed. The prepared cDNA dilution was stored in –20°C for further applications.

2.2.1.15 Real-Time PCR

Real-Time PCR reaction is dependent on many factors that have to be optimized for before reliable results can be obtained. One of the most important factors is the design of a primer pair that anneals very specifically to its target sequence in the template cDNA. The following rules were used to design optimal primer pairs for successful amplification of target sequences in the cDNA of interest using Real-Time PCR. The oligos should be between 18 and 25 bp in length, with a G+C content between 40 and 60%. There should be an equal distribution of all four bases along the sequence. Long repeats of single bases should be avoided.

The difference between the amounts of oligo-nucleotides in a primer pair should not exceed 3 bp. The amplification product should be around 90-110 base pairs. In this study the Real-Time PCR reaction had been performed using the iCycler iQ™ Real-Time Detection System or The MyiQ™ Single-Color Real-Time PCR Detection System. Both were purchased from Bio-Rad. The instruments allowed analysis of 96 PCR reactions at once. A ready-to-use master mix from Bio-Rad was used for preparing Real-Time PCR reactions. This iQ™SYBR® Green Supermix is a 2 x solution that has been optimized for Real-Time PCR applications.

It contains all necessary reagents and only the cDNA sample and a primer pair have to be added to tube. The cDNA obtained from the reverse transcriptase reaction was diluted once more 1:5 and 1:50 in DNase-free water. The PCR reaction was performed always in triplicate for each cDNA sample and primer pair. Also the control reaction that contains all reagents except the cDNA template was included. For each reaction following components were added to a microcentrifuge tube: 12.5 µl 2 x Supermix, 0.5 µl of each primer (final concentration should be 100 nM-500 nM), 8.5 µl DNase-free water and 3 µl diluted cDNA (1:5 or 1:50). The prepared reactions were transferred into 96 plate and the dish was cover with optical tape. Finally the plate was carefully placed in the thermoblock of Real-Time PCR machine.

The Real-Time PCR reactions were run according to the protocol:

Cycle 1.	1x	95°C for 5 min.
Cycle 2.	40x	
	Step 1	95°C for 30 sec.
	Step 2	59°C for 30 sec.
Cycle 3.	100x	
	Step1	50°C for 10 sec.
	Step2	increase set-point temperature after Step1 by 0.5 °C
Cycle 4.	1x	4°C hold

During Real-Time PCR reaction the amount of amplified fragment is constantly measured with help of specific fluorescence dye SYBR Green. The SYBR Green binds to double stranded DNA and gives 100 fold brighter fluorescence than alone. This allows for non-specific visualization of amplified products in real time while running the reaction. The fluorescence data are presented as amplification plot (Figure 2.1A). To confirm that the fluorescence observed comes from one expected amplification product, the number of amplified products can be visualized by displaying the melting curve. Multiple peaks would indicate the presence of multiple amplification products.

In this case the primers have to be redesigned and tested again for their target specificity. In presented example presence of one peak indicates that primers are specific for the target conforming that only one sequence is amplified (2.1B). The amplification plot displays the relative fluorescence values for each sample at every cycle. The reaction cycle number when the reaction start to come into the logarithmic phase of amplification curve is called the Ct value (threshold cycle). By comparing the Ct value of two samples (Ct_1 and Ct_2) it is possible to calculate the relative expression level of the investigated gene in the samples.

For example one cycle difference would mean a 2 fold relative increase in DNA concentration according to the following formula where Relative Fold Expression is $2^{\Delta Ct}$ where delta Ct is the difference in cycle amplification number between two compared samples ($\Delta Ct = Ct_1 - Ct_2$).

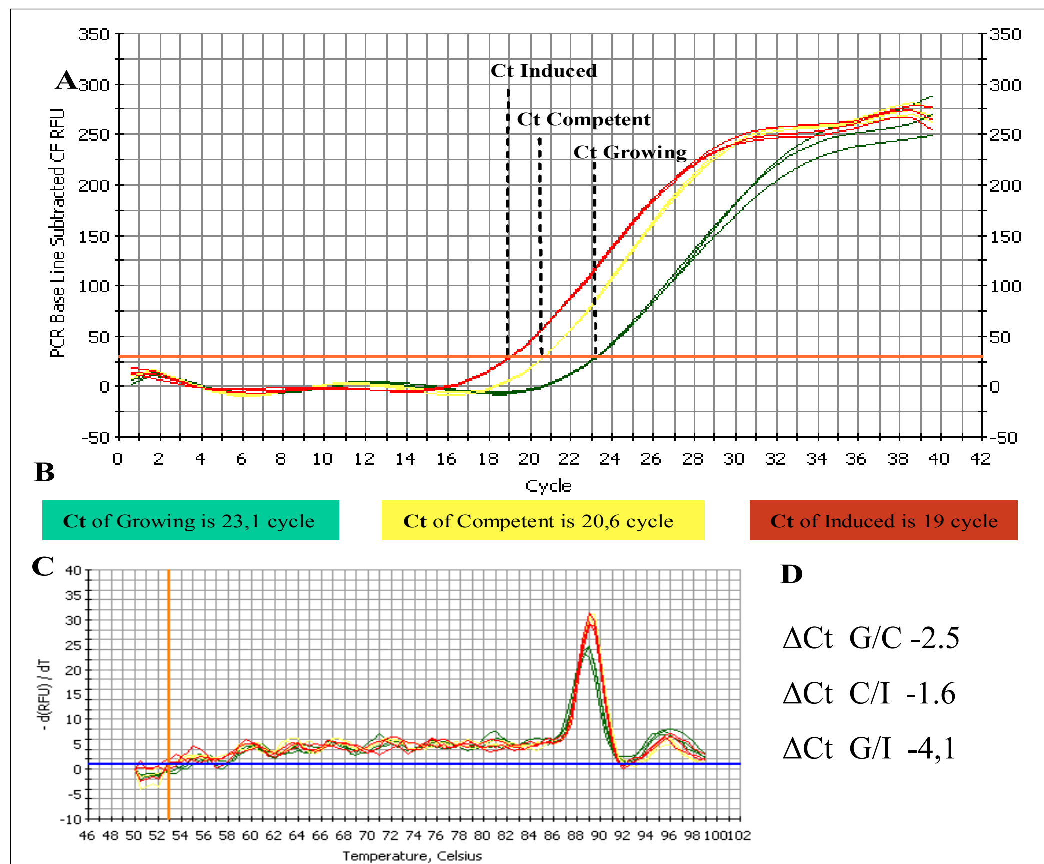

Figure 2.1 Analysis of Real-Time PCR data obtained with primers amplifying the Id2 gene. The RNA samples isolated from growing competent and induced HC11 cells were reversed transcribed. The cDNAs were used as templates for Real-Time PCR reactions run with primer pairs specific for a region in the Idb2 gene. (A) Amplification plot (B) Calculated average Ct values for 3 tested stages. Each reaction is performed in triplicate. (C) Melting curve graph a single peak indicates amplification of only one sequence. (D) Calculation of delta Ct value between growing competent and induced cells. The Relative fold expression of candidate gene in compared stages are calculated than according to formula $2^{\Delta Ct}$ where delta C_t is the difference in cycle amplification number between two compared samples ($\Delta C_t = Ct_1 - Ct_2$).

2..2.1.16 Addition of restriction sites to cloned sequence using PCR

Primers were designed (with restriction sites for *Sac*II) for the amplification of a DNA fragment encoding a sequence of the target protein. The following PCR mix for each amplified fragment was prepared: 1 µl dNTPs (10 mM), 0.5 µl Forward primer (100 pmol), 0.5 µl reverse primer (100 pmol), 0.5 µl Native AmpliTaq-Polymerase (Invitrogen), 5 µl 10x AmpliTaq-Buffer, 1 µl $MgCl_2$ (50 mM) and 10 ng template DNA. Sterile water was added to final volume of 50 µl.

The PCR was run using following protocol and lid temperature was set to 95°C to prevent evaporation of water from the reaction

Step 1. 95°C 3 minutes
Step 2. 95°C 30 seconds
Step 3. 62°C 30 seconds
Step 4. 58°C 30 seconds
Step 5. 54°C 30 seconds
Step 6. 51°C 30 seconds
Step 7. 72°C 1 minute back to step 2 and repeat 30-40 times
Step 8. 72°C 10 minutes
Step 9. 4°C hold

The size of the product was determined by running 2 µl of the PCR reaction on 1% agarose gel. The obtained product of the PCR reaction was purified using column kit (Qiagen). The purified fragments were eluted in water and store –20°C for further applications

2.2.1.17 Extraction of total RNA from mammalian cell

The cells were grown in 10 cm dish in CO_2-incubator at 37°C in medium containing the appropriate regulators for the desired time. At the time point of interest the medium was completely removed and 1.5 ml of pegGOLD TriFast^{TM} (Peqlab) reagent was added to each plate, which was then incubated at 37°C for 2-5 minutes. The cells were disrupted by pipetting them up and down with 1 ml tips. The cell lysate was collected in a 2 ml microcentrifuge tube and incubated for 5 minutes at room temperature. The tube was centrifuged at 12000 x g and 4°C for 10 minutes and the resulting supernatant was transferred to a new 2 ml microcentrifuge tube. To remove proteins 300 µl chloroform was added to the sample and the tube was shaken vigorously for 15 seconds on a vortex. After a 3 minute incubation at room temperature the tubes were centrifuged at 12000 x g at room temperature for 5 minutes.

The obtained clear upper phase was transferred to a new 1.5 ml tube and 750 µl isopropanol was added to each sample. The tubes were then gently shaken and incubated for 5 minutes at room temperature. After the tubes were centrifuged at 12000 x g at 4°C for 10 minutes, the supernatant was removed and the white RNA pellet was washed with 1 ml of 75% ethanol (for RNA the ethanol solution was diluted with RNase-free water). The tube was centrifuged at 12000 x g and 4°C for 10 minutes and the washing step with ethanol was repeated once more. After the second wash the ethanol was carefully removed and the RNA pellet was dried at room temperature for 5 minutes. Then 50 µl of RNase-free water was added to the pellet and the tubes were incubated for 5 minutes at 60°C or until the RNA pellet was completely dissolved. The isolated RNA samples were stored at -80°C for further applications.

2.2.18 Isolation of RNA and proteins from tissues

The mammary gland tissue samples were homogenized in Trizol® Reagent (Gibco, 1 ml per 50-100 mg of tissue) using a power homogenizer. The tissue volume did not exceed 10% of the volume of Trizol® Reagent used for homogenization. The samples were incubated for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml chloroform was added (per 1 ml Trizol® Reagent) and the tubes were vigorously shaken by hand for 15 seconds and incubated for 5 min at room temperature. The samples were centrifuged at 12000 x g and 4°C for 15 minutes and the resulting colorless upper aqueous phase was transferred to fresh tube. The lower red organic phase and white interphase were stored if DNA or protein isolation was desired. After adding 0.5 ml isopropyl alcohol, the sample was incubated for 10 minutes at room temperature. The tubes were centrifuge at 12000 x g for 10 minutes at room temperature and the resulting supernatant was removed. The RNA pellet was washed by vortexing with 1 ml of 75% ethanol. The samples were centrifuged at 7500 x g at 4°C for 5 minutes. Finally, the RNA pellet was briefly dried for 5-10 minutes at room temperature and dissolved in RNase-free water by passing the solution a few times through pipette tip and incubating for 10 minutes at 55°C. The red organic and the white interphase were mixed with 300 µl 100% ethanol (per 1 ml of Trizol® Reagent). The samples were incubated at 30°C for 2-3 minutes.

To sediment the DNA the tubes were centrifuged at 2000 x g at 4°C for 5 minutes. The resulting phenol-ethanol supernatant contains proteins. To precipitate the proteins 1.5 ml isopropyl alcohol was added to the phenol-ethanol supernatant and the samples were incubated for 10 minutes at room temperature. The resulting protein precipitate was sedimented by centrifugation at 12000 x g at 4°C for 10 minutes. The supernatant was removed and the protein pellet was washed 3 times in 2 ml of a wash solution (0.3 M guanidine hydrochloride in 95% ethanol).

The protein pellet was incubated in the wash solution for 20 minutes at room temperature and centrifuged at 7500 x g and 4°C for 5 minutes. After a final wash, 2 ml 95% ethanol was added and mixed with the samples by vortexing. The protein suspension in ethanol was stored for 20 minutes at room temperature and centrifuged at 7500 x g and 4°C for 5 minutes. The protein pellet was dried for 5-10 minutes at room temperature and dissolved in 1% SDS by pipetting. The remaining insoluble material was sedimented by centrifugation at 10000 x g for 10 minutes at 4°C. The obtained supernatant containing proteins was transferred to fresh micro centrifuge tube. The protein samples were stored at –20°C for further applications.

2.2.2 VIROLOGY METHODS

2.2.2.1 Generation of viral particles

5 x 10^6 HEK 293T cells were seeded in a 10 cm culture dish and 10 ml DMEM growth medium was added to each dish and the cells were cultivate in CO_2-incubator at 37°C over night so that at the time of transfection the monolayer of cells were 80% confluent. All steps from now were performed in the L2 lab. The HEK 293T were transfected with three lentiviral plasmid using the calcium phosphate transfection Method. Per 10 cm plate the following plasmids were used: 10 µg of pSiEW or pSEW) construct, 6.5 µg of the packaging construct pCMVRΔ8.91 as well as 3.5 µg of the envelope construct pMD2G.VSVG. The transfected cells were incubate in CO_2-incubator at 37°C for 16 hours. The transfection efficiency was verified under fluorescence microscope. The transfected cells expressed the GFP protein encoded by pSEW or pSiEW. After successful transfection (50 to 70% GFP positive cells) the plate was used to collect the lentiviral particles. The medium was carefully aspirated off and discarded as infectious material. Next, 5 ml of fresh medium was added to each culture dish and the cells were incubated in CO_2-incubator at 37°C for 16-24 h.

The next day the culture medium was collected in 50 ml conical tubes and centrifuged at 1000 rpm for 5 minutes at room temperature to remove the cell contaminations. The obtained supernatant was filtered through a nitrocellulose filter (0,22 µm pore size). Finally, 1 ml aliquots of filtrated supernatant containing lentiviral particles were prepared and stored at -80°C for further applications.

2.2.2.2 Titration of lentiviral particles containing supernatants

The one day before titration 1 x 10^5 293T cells were seeded in each well of a 24 well plate in a DMEM growth medium and were cultivated in CO_2-incubator at 37°C over night. All steps from now were performed in a laboratory suitable for working with infectious material under laminar flow hood (L2 lab). Serial dilutions (1:10 to 1:10000) of the obtained viral supernatants in the growth medium were prepared. The culture medium was carefully aspirated from the 24-well plate and 250 µl fresh growth medium containing polybrene to final concentration 16 µg/ml was immediately added to each well. Next 250 µl of the prepared serial dilutions were added to each well changing the tips each time between adding different dilutions. The plate was centrifuged at 2500 rpm for 90 minutes at 32°C and incubated for 2.5 hours in CO_2-incubator at 37°C. Then the medium was slowly aspirated and replaced carefully with fresh growth medium. The cells were cultured for next 24 to 48h in CO_2-incubator at 37°C. The next day the medium was removed and the cells were washed with 500 µl 1x PBS. The PBS was completely removed and 50 µl Trypsin-EDTA solution was added to each well and the plate was incubated for 3 minutes on a thermoplate at 37°C to detach the cells from the plate bottom. Then 500µl of PBS was added to each well and the cells suspension was collected into 5 ml round bottom tubes (Becton Dickinson). The tubes were centrifuge at 1000 rpm for 5 minutes at room temperature. The resulting supernatant was removed and 250 µl of fixative solution (4% formaldehyde in 1x PBS) was added to each cell pellet and the tubes were vortexed. Samples can be stored at 4°C up to 2 weeks. The samples were analyzed using FACS. For proper calculation only values corresponding to less than 20% of positive cells were taken for account. The viral titer was calculated using the following formula:
Transducing Units/ml (TU/ml) = (%positive cells/100%) x Nb of cells x dilution.
Nb of cells is a number of cells at the day of fixation.
The MOI (multiplicity of infection) was calculated using the following formula:
MOI= TU/ml divided by number of transduced cells.

2.2.2.3 Transduction of epithelial cells with lentiviral particles

10^5 cells were seeded per well of 6-well multi-dish so that the plate reached 70% confluency at the time of transduction with lentiviral particles. 3 ml of growth medium was added to each well and the cultures were incubated in a CO_2-incubator at 37°C over night. All steps from now on were performed under laminar flow hood in laboratory suitable for working with infectious material (L2 lab). To each well following components were added: 1 ml of supernatant containing proper amount of lentiviral particles, 2 ml of medium without antibiotics, 30 µl polybrene (stock 400 µg/ml). The plate was centrifuged at 2500 rpm for 30 minutes at 32°C.

The cells were then incubated with the lentiviral particles in a CO_2-incubator at 37°C for the following 8 hours. The medium was discarded (infectious material) and fresh growth medium was added to each well. After 24 hours incubation in CO_2-incubator at 37°C the medium was removed again and the fresh growth medium was added to the transduced cells. The cells were grown in CO_2-incubator at 37°C for another 24 hours. Next day the medium was aspirated and the cells were washed with 1x PBS. Finally 3 ml of warm growth medium was added to each well. The cells can be manipulated under L1 cell culture condition.

2.2.3 PROTEIN BIOCHEMISTRY METHODS

2.2.3.1 Isolation of proteins using RIPA-Buffer

Mammalian cells were cultivated in a 10 cm dish in a CO_2-incubator at 37°C in proper medium and regulators for the desired time. The medium was aspirated and the adherent cells were washed twice with ice-cold 1 x PBS. After the last washing the PBS solution was completely removed. The cells were lysed by adding 500 µl ice-cold RIPA-buffer and immediately scraped from the plate with a cell lifter. Then the cell extract was transferred to 1.5 ml microcentrifuge tube. The suspension was gently mixed and incubated on ice for 15 minutes. After centrifugating the cell lysate for 15 minutes at 14000 x g at 4°C, the resulting supernatant was immediately transferred to a fresh 1.5 ml microcentrifuge tube. The isolated proteins were stored at –80°C for further applications.

2.2.3.2 Determination of the protein concentration

To prevent degradation of the proteins samples were unfrozen on ice. A commercially available solution (Protein Assay Dye Reagent Concentrate, Bio-Rad) for determination of the protein concentration was used. This solution was diluted 1:5 in water. To measure the protein concentration a dilution of each sample was prepared by adding 5 µl of protein extract and 995 µl of diluted reagent. The mixtures were incubated for 3 minutes at room temperature. The protein concentrations were measured at wave length of 260 nm using a spectrophotometer (Bio-Rad). The protein concentrations were calculated according to a BSA-standard.

2.2.3.3 SDS-polyacrylamid-gel-electrophorese

Two glass plates were assembled according to the instruction of the manufacturers (Bio-Rad). A separating gel was prepared with an acrylamide concentration necessary for optimal separation of the proteins of interest. The acrylamide solution was poured between the assembled glass plates and immediately covered with isopropanol. The gel polymerized at room temperature for approximately 30 minutes. The isopropanol was removed and the top of the gel was dried with a strip of Whatmann paper. The stacking gel was prepare and poured on top of the separating gel. A comb was inserted and the stacking gel polymerized for approximately 30 minutes at room temperature. The comb was removed and the polymerized gel was place into the electrophoresis tank. The tank was filled with SDS-PAGE running buffer until the wells of gel were submerged. The isolated proteins (40–80 µg) were diluted in a proper volume of 4 x loading buffer in a 1.5 ml microcentrifuge tube. The samples were heated to 95-100°C for 5 minutes and centrifuged for 30 seconds. Then they were carefully loaded on the gel using a long thin pipette tip. The chamber was closed with a lid and electrodes were connected to the power supply. The gel was run with 50 mA at room temperature until the tracking dye had reached 0.5 cm from the bottom of the gel. The power supply was turned off and the gel was removed form glass plates with the help of a spatula. The unnecessary stacking gel was cut off with a scalpel. Subsequently, the gel was used for western blotting or Coomassie brilliant blue staining.

2.2.3.4 Electro-transfer of proteins onto nitrocellulose membranes

To analyze the proteins separated by SDS-electrophoresis they were electro-transferred onto a nitrocellulose membrane (Schleicher&Schuell) using the Semi-Dry-Blotting apparatus (BIO-RAD). Therefore a "sandwich" construction was prepared in the following way. Four filter papers cut in the size of the gel (3 MM-Whatmann paper) were soaked in anode buffer I and were placed on the anode. Two filter papers (3 MM-Whatmann) were soaked in anode buffer II and placed on the top pf the first papers. A piece of nitrocellulose membrane (0.2 µm pore) was wetted in water, washed in anode buffer II and placed on top of the paper Stack. Then the separating gel was rinsed in cathode buffer and placed on the top of the membrane.
Finally, four filter (3 MM-Whatmann) papers soaked in cathode buffer were placed on the top of the gel. The blotting chamber was closed and the proteins were transferred for 1 h with 1 mA per cm^2 membrane. Proper transfer was determined by staining the membrane with Ponceau S solution (Sigma).

2.2.3.5 Verification of protein transfer on nitrocellulose membranes

The nitrocellulose membrane was transferred from the blotting chamber to a flat dish containing 10 ml Ponceau S solution (Sigma). The membrane was incubated for 5 minutes on an orbital shaker or until the proteins bands became visible. Finally, the membrane was washed twice with 15 ml of distillated water and analyzed before processing for immuno-blotting.

2.2.3.6 Detection of proteins on a western blot

After the transfer of proteins from gel to a nitrocellulose membrane, the membrane was washed with 10 ml of TBS-T wash buffer for 5 minutes at room temperature and then incubated in 10 ml of blocking buffer (5% BSA in TBS-T) for 30 minutes at room temperature. Then it was washed three times with 15 ml of TBS-T for 5 minutes each and the membrane was incubated with primary antibody (1:1000 dilution in 10 ml blocking buffer) with gentle agitation overnight at 4°C. The next day the membrane was washed three times with 15 ml of TBS-T for 5 minutes each and incubated with HRP-conjugated secondary antibody (1:5000 dilution in 10 ml of blocking buffer) with gentle agitation for 1 hour at room temperature.

The membrane was then washed three times with 15 ml of TBS-T for 5 minutes each and finally incubated with 1 ml of ECL solution (Amersham) for 1 minute at room temperature. The developing solution was removed and the membrane (do not let it dry completely) was placed into a plastic cover and exposured to an X-ray film. The film was developed using an automated developing machine.

2.2.3.7 Stripping of nitrocellulose membranes

To remove primary and secondary antibodies bound to a western blot the membrane was put into 50 ml conical tube and washed once with 1x TBS-T for 5 minutes to remove the ECL solution. The TBS-T wash buffer was completely removed and 10 ml stripping buffer was added. Than the tube was securely closed and the membrane was incubated at 55°C in rotating oven for 30 minutes.
The stripping solution was discarded and the membrane was washed two times with 20 ml sterile H_2O for 10 minutes. Finally, the membrane was washed in 10 ml of TBS-T for 10 minutes. Subsequently, the membrane can be blocked with blocking buffer and used again for detection of another protein of interest.

2.2.3.8 Preparation of nuclear protein extracts

The cells of interest were washed 5 ml 1x PBS containing 1 mM EDTA and the wash solution was completely aspirated. Then 1 ml of PBS containing 1 mM EDTA was added to the dish and the cells were carefully scraped from the plate with cell lifter cells. The obtained cell suspension was transferred to 1.5 ml microcentrifuge tube and centrifuged at 1000 rpm for 10 minutes at room temperature. The cell pellet was re-suspended in ice cold buffer A (800 µl per 10 cm dish, or 10 volumes of buffer per 1 volume of cell pellet) to disrupt the cell membranes. The nuclei were collected by centrifugation at 4000 rpm for 5 minutes at 4°C. The supernatant containing the cytoplasmic fraction was stored at -80°C. To wash the nuclear fraction 800 µl fresh ice cold buffer A was added to the pellet and the nuclei were collected again by centrifugation at 4000 rpm for 5 minutes at 4°C. The resulting nuclear pellet was re-suspended by adding 300 µl of buffer B (per 10cm dish or $1x10^8$cells) and vortexing. Then the nuclear suspension was incubated for 30 minutes at 4°C. Finally, the disrupted nuclei were centrifuged at 12000 rpm for 10 minutes at 4°C and the obtained supernatant containing nuclear proteins was stored at –80°C for future applications.

2.2.4 CELL CULTURE METHODS

2.2.4.1 In *vitro* differentiation of mammary epithelial cells

HC11 is a mammary epithelial cell line that can differentiate in *vitro*. When induced with lactogenic hormones the cells can functionally differentiate and express milk proteins for example β-casein. The cells were seeded into 10 cm sterile plastic dish and cultivated in RPMI 1640 growth medium in the 5% CO_2 incubator (98% humidity) at 37°C for 2 days or until the plate was confluent. Then the growth medium was aspirated from the plate and the cells were washed with warm 1 x PBS. The starvation medium was added to the dish and the cells were incubated for the next 2-4 days. After 4 days the starvation medium was removed from the plate and the cells were washed twice with warm 1 x PBS. Finally, the cells were induced for differentiation with induction medium with lactogenic hormones and cultured for the next 4 days. Accumulation of β-casein mRNA was detected using Real-Time PCR after 8-24 h of lactogenic stimulation and expression of β-casein protein was observed after 4 days of induction with lactogenic hormones.

2.2.4.2 Splitting mammalian adherent cells

The medium as well as 1 x trypsin-EDTA and 1 x DPBS solutions were pre-warmed to 37°C in water bath. One 10 cm plate containing a confluent monolayer of adherent cells was chosen and placed under a laminar flow hood. All manipulations with cells (open plate) were performed under sterile condition preventing contamination with bacteria, fungi and mycoplasm. The growth medium was aspirated from the plate and the cells were washed with 1x DPBS by agitating the plate carefully. The wash solution was completely removed and 1 ml 1x trypsin-EDTA solution was added to the plate making sure that the entire surface of the cell monolayer was covered. The dish was then incubated on a 37°C thermo-plate for 3-5 minutes. In the meantime it was checked if the cells started to detach from the bottom of the plate under a phase contrast microscope. Then 9 ml of warm growth medium containing 10% FCS (it will neutralize activity of trypsin) was added to the plate and the content was mixed by pipetting up and down with serological pipette. The cell suspension was transferred to 15 ml conical tube and centrifuged at 1000 rpm for 3 minutes at room temperature.

The resulting supernatant was aspirated and the obtained cells pellet was resuspended in 10 ml fresh growth medium. 1 ml of the cell suspension was added to a 10 cm plate containing 9 ml of fresh warm growth medium (1:10 dilution). The cells were then distributed evenly over entire plate surface by gently steering. The cells were cultivated in the 5% CO_2 incubator (98% humidity) at 37°C until the plate was 90% confluent, than the cells were split again.

2.2.4.3 Isolation and culture of primary mammary epithelial cells from mice

The isolation of primary mammary epithelial cells was performed in a sterile laminar flow hood to prevent the cells from contamination with bacterial or fungi. The mouse was anesthetized by an overdose of chloroform. The skin of the animal was sterilized by washing its body with 70% ethanol. The mouse was pinned out on sterile dissection board. The thoracic (number 2 and number 3), abdominal (number 4) and inguinal (number 5) mammary glands were removed using sterile dissection tools and the glands were transferred into 25 ml DMEM/F12 (100 U/ml Penicillin 100 µg/ml Streptomycin 50 µg/ml Gentamicin) to keep them moist until all glands from the mice were collected. Under sterile condition the isolated glands were placed on a glass slide and all muscles and lymph nodes were carefully removed. The gland tissue was chopped into very small pieces using sterile scalpel blades. The minced tissue was transferred into a round bottom Erlenmeyer flask containing digestion medium and the tissue suspension was incubated for 1-3 hours or until the tissue was thoroughly digested at 37°C on a shaker set to 125 rpm. When the tissue was completely digested it appeared as a cloudy homogeneous solution. The digested tissue mixture was transferred into a 50 ml conical tube and centrifuged at 1000 rpm (200 x g) for 5 minutes. The resulting supernatant was removed and the cells were resuspended in cold F-12 medium (containing 50 µg/ml Gentamicin) and centrifuged again at 1000 rpm for 5 minutes. The obtained cell pellet was than resuspended in 5 ml of 2 x plating medium. Finally, around 2.5-5.0 x 10^5 cells per cm^2 of the plate were seeded on the 10 cm dish containing 5 ml serum-fetuin mix (20% fetal calf serum, 1 mg/ml fetuin in F-12 medium). After 24 h incubation in 37°C the culture medium was aspirated and replaced with fresh warm growth medium.

For proper growth and differentiation of primary mammary epithelial cells *in vitro* the cells require the addition of extracellular matrix. Rat Tail Collagen Type I and Matrigel™ used in this study were purchased from Becton Dickinson Biosciences. To coat the plastic 10 cm dishes with a thin collagen layer the gel was diluted in ice-cold 1 x DPBS to a final concentration 80 µg gel/ml. 100 µl of prepared collagen dilution was used to cover 1 cm^2 of the dish surface. The gel was allowed to polymerize overnight at 4°C.

The next day the gel was washed two times with ice-cold 1 x DPBS and once with F-12 medium and finally the collagen layer was covered with 5 ml serum-fetuin mix (20% fetal calf serum, 1 mg/ml fetuin in F-12 medium). Plates were then ready for use. To cover plastic dish with a thin layer of Matrigel™, the stock of Matrigel™ had to be thawed at 2-8°C overnight. Matrigel™ gels rapidly at room temperature so pre-cooled plates and pipettes tips were used and the gel stock was always kept on ice. The gel stock was initially diluted 1:2 in serum-free F-12 medium. 50 µl of prepared matrigel dilution was used to cover 1 cm^2 of plastic dish. The plates coated with the matrigel dilution were then incubated at 37°C for 30 minutes. The plate was then ready and was used immediately.

To induce functional differentiation of the primary epithelial cells they were cultivated on the plates coated with extracellular matrix in primary mammary epithelial cells growth medium until they reached confluency. Then the cells were induced for functional differentiation by adding a lactogenic hormone mix containing prolactin (5 µg/ml), insulin (5 µg/ml), hydrocortisone (1 µg/ml). The increase in β-casein mRNA was measured after 8 h and the expression β-casein protein 4 days after stimulation with lactogenic hormones.

2.2.4.4 Freezing and thawing mammalian cells

The medium as well as 1 x trypsin-EDTA and 1 x DPBS solutions were pre-warmed in a water bath set to 37°C. All subsequent steps were performed in a laminar flow hood. One 10 cm plate containing a confluent monolayer of adherent cells was used to make frozen cell stocks. The medium was removed from the plate and the cells were washed with warm 1 x DPBS. The washing solution was completely aspirated and 1 ml trypsin-EDTA solution was added to the plate making sure that entire surface of the cell monolayer was covered.

The dish was incubated on a thermo-plate at 37°C for 3-5 minutes or until the cells started to detach from the plate (if cells are not adherent the trypsin is not needed).To inactivate the trypsin 9 ml medium containing 10% FCS was added to the plate and the plate content was mixed by gentle pipetting up and down. The cell suspension was then transferred to a 15 ml conical tube, and centrifuged at no more than 1000 rpm for 3 minutes at room temperature. The resulting supernatant was removed and the cell pellet was resuspended in 1 ml freezing buffer (5% DMSO-95% FCS). Finally, the cells were transferred to 2 ml labeled cryo tubes and were stored at -80°C. For long-term storage of stocks the cells were frozen in liquid nitrogen.

To cultivate the cells from frozen stocks the frozen cells were treated according to the following protocol. The frozen cells were melted quickly by incubating the vial in a water bath set to 37°C. The cell aliquot was transfer to 15 ml conical tube and 9 ml of growth medium was added drop-by-drop to the cells, preventing osmotic shock. To remove the DMSO the tube was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed. The obtained cell pellet was resuspended in 10 ml of warm growth medium and seeded into culture dish.

2.2.4.5 Three-dimensional culture of mammary epithelial cells

The cells were cultured in a CO_2 incubator at 37°C in sterile 10 cm dish with 10 ml of growth medium. When the plate reached 90% confluency the cells were detached with trypsin solution and the cell suspension was transferred in a 15-ml conical tube and spun down using microcentrifuge at 1000 rpm for 3 min at RT. The cell number per ml was then estimated using a special cell counter chamber (Marienfeld). After counting the cells suspension was diluted in RPMI 1640 medium containing 10% FCS to a cell density of 5×10^4 cells/ml in a sterile 15 ml conical tube. 100 µl of this cell suspension was transferred into fresh sterile 1.5 ml microcentrifuge tubes and mixed with an equal volume (100 µl) of ice-cold collagen (Becton Dickinson). The cells were mixed with the collagen by gently pipetting up and down, very carefully without generating air bubbles. Quickly 100 µl of the performed collagen/cells suspension was dispensed as a drop into the center of each well of a 12 well plate without leaving air bubbles on the top of the gels. The collagen was then let to polymerize at room temperature for 10 min (the gel turns milky) without shaking or moving as leading to homogenous polymerization.

The plate was then placed in the incubator (37°C) for at least 30 min to let the gel completely polymerize. After the polymerization was completed 500 µl of growth medium was carefully and slowly added to each well. The medium should not be blown onto the gel as this might detach or destroy it. The medium was refreshed every other day. Three dimensional growth was observed using a contrast phase or fluorescence microscope.

2.2.4.6 Proliferation assay

For this assay the XTT-based proliferation kit II (Roche) was used. 5 x 10^3 mammalian cells were seeded in a flat 96-well plate. For each sample drug concentration and time point triplicates were prepared. To each well 100 µl of warm growth medium was added and the cells were cultivated in CO_2 incubator at 37°C for desired time. At the time point of interest the XTT reagent solution and the activation solution (Roche) were defrosted immediately prior to use in a 37°C bath and swirled gently until clear solutions were obtained. To prepare the reaction solution, 100 µl of activation solution were mixed with 5 ml XTT reagent. The reagent solution allows for detection of metabolic active cells. 50 µl of the freshly prepared reaction solution was than added to each well and the plate was incubated in the CO_2 incubator for 2-5 hours at 37°C. After this incubation the plate was gently shaken to equally distribute the dye in the wells. The absorbance of the samples was measured with a spectrophotometer (ELISA reader, Molecular Devices Corporation) at a wavelength of 490 nm.

2.2.4.7 Luciferase-Assay

The cells were cultivated in 10 cm dish in a CO_2-incubator at 37°C with proper medium and regulators. At the time point of interest the medium was removed and the cells were washed two times with ice-cold 1x PBS. After the second wash the PBS solution was completely aspirated. Just before use the luciferase lysis buffer was prepared by adding 1/1000 volume of 1M DTT. Then, 300 µl of ice cold luciferase lysis was added to the plate. The cells were scraped off and transferred to a 1.5 microcentrifuge tube. The sample was stored on ice for 10 min and mixed from time to time during the incubation.

The sample was centrifuged for 10 minutes at full speed at 4°C in a microcentrifuge and the resulting supernatants were transferred to a new 1.5 ml microcentrifuge tube. 10 μl of supernatant was dropped into well of a 96-well plate, for each sample triplicates were prepared. Then, luciferase substrate was added to each sample and the luminescence was immediately measured with an integration time of 10-15 seconds in a microplate luminometer (Berthold Technologies).

2.2.4.8 Transfection of mammalian cells with Lipofectamine

To transfect the HC11 cells with plasmid DNA the commercially available transfection reagent Lipofectamine™ (Invitrogen) was used. The day before transfection the adherent HC11 cells were plated in a 12-well plate in 1 ml of growth medium without antibiotics so that at time of transfection the plate would be 90% confluent. The cells were than cultivated in a CO_2 incubator over-night at 37°C. The next day 1.6 μg of plasmid DNA and 100 μl of Opti-MEM® Medium (Invitrogen) or other serum-free medium was gently mixed by pipetting up and down in a 1.5 ml microcentrifuge tube. Next, 4 μl of Lipofectamine™ was diluted in 100 μl of Opti-MEM® Medium (or other medium without serum) and the suspension was incubated for 5 minutes at room temperature.

The diluted DNA sample was combined with the diluted Lipofectamine™ and the tube was incubated for 20 minutes at room temperature to allow DNA-Lipofectamine complexes to form. Finally, 100 μl of DNA-Lipofecamine complexes were spread into each well drop-by-drop and then mixed with the medium by gently rocking the plate back and forth. The transfected cells were placed in the 5% CO_2 incubator (98% humidity) at 37°C and incubated for 24-48 hours until they were ready to be analyzed for expression of the transfected gene.

2.2.4.9 Transfection with Polyethylenimine

To test the different transfection reagents in mammary epithelial cells the PEI (Polyethylenimine, Sigma) was used. One day before transfection the HC11 cells were seeded in 10 cm plate so that the plate should be 80% confluent the next day. To prepare the DNA-PEI suspension mix for 10 cm dish Solution A containing 16 μg DNA dissolved in 240 μl 1x PBS was mixed with Solution B containing 45 μl 10 mM PEI in 240 μl 1x PBS and vortexed.

The suspension was incubated for 15 minutes at room temperature. In the meantime the medium was aspirated from the plate and the cells were washed with 5 ml of 1 x PBS and finally 5 ml of fresh growth medium was added to the plate. The prepared DNA-PEI suspension was very carefully added drop-by-drop around the whole surface of the cells monolayer. The transfected cells were incubated in the 5% CO_2 incubator (98% humidity) at 37°C for 3 hours. The medium was then aspirated from the plate and replaced with 10 ml fresh and warm growth medium. After 24-48 h of incubation the transfection efficiency (the percent of GFP positive cells) was monitored under the fluorescence microscope.

2.2.4.10 Transfection with Calcium Phosphate

To transfect lentiviral plasmids mostly the calcium phosphate transfection method was used. The day before transfection $5x10^6$ adherent HEK 293T cells were seeded per 10 cm plate, so at the time of transfection the plate is 80% confluent. 10 ml DMEM growth medium was added to the plate and the cells were cultivated in CO_2-incubator at 37°C over night. Two hours before transfection the medium was aspirated from the plate and replaced with 5 ml fresh DMEM growth medium. For the production of viral particles a transfection mix was prepared containing, 10 µg of vector pSiEW or pSEW encoding siRNA or gene of interest, and 6.5 µg of core packaging plasmid pCMVRΔ 8.91 as well as 3,5 µg of plasmid pMD2-VSVG encoding envelope components. All plasmids were added to 450 µl of dpH_2O (Sigma) in a 10 ml sterile round bottom conical tube. Finally, 50 µl of 2.5M $CaCl_2$ were added to the tube. To precipitate the plasmid DNA 500 µl of 2 x HBS solution, were added drop-by-drop (one drop per sec.) while blowing air through the prepared DNA-$CaCl_2$ suspension with a 2 ml serological sterile pipette mounted in automatic air pipettor. After adding of 500 µl 2x HBS solution, the obtained 1 ml transfection mix was bubbled for additional 1 minute and than incubated for 15 min at room temperature. In the meantime 1 µl of 100 mM chloroquine was added to the HEK 293T cells cultured in the 5 ml of medium. The precipitated DNA solution (1 ml) was slowly added to the cells. The transfected HEK 293T cells were incubated in CO_2-incubator at 37°C over nigh but no longer than 16 h. The next day the medium was aspirated and replaced with fresh DMEM growth medium. The transfection efficiency was monitored by checking the expression of the GFP protein under fluorescence microscope.

The culture medium containing the lentiviral particles was collected for following 48 hours. After filtration and virus titration the medium was used for transduction of mammary epithelial cells.

2.2.5 HISTOCHEMICAL METHODS

2.2.5.1 Preparation of mammary gland whole mounts

For staining of mammary glands, abdominal gland (number 4) was removed from treated and control mice. The glass slides were labeled with specimen code using a diamond-tipped pencil. The isolated mammary glands were spread on labeled glass slides making sure that the edges of the glands were completely stretched. The glands were dried for 10 min at room temperature so they attached the glass. The slides with the mammary gland were then fixed for 16 to 24 hours in formalin fixative. The next day to re-hydrate the gland tissue the slides were washed in the 70% ethanol for 15 minutes and after that, transferred to 50% ethanol and incubated for 15 minutes. Finally, the slides were washed in 25% ethanol for 15 minutes. The glands tissue was stained with Hematoxylin solution (colors basophilic structures with blue-purple hue) over night and than eosin (colors eosinophilic structures bright pink) for 2 hours. After staining the slides were washed in 70%, and then 95% ethanol for 15 minutes in each concentration. The glands were mounted and covered with cover-slip. The gland structure were analyzed under light microscope.

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2.3 INSTRUMENTATION

Manufactures	Instrument name
Beckman	GPKR Centrifuge Avanti^{TM} J-20 Megacentrifuge L8-80M Ultracentrifuge
Berthold Technologies	MICROLUMAT LB96P luminometer
Bio-Rad	ICycler iQ^{TM} Thermal Cycler My iQ^{TM} Thermal Cycler Single SmartSpec^{TM} 3000 spectrophotometer SmartSpec^{TM}Plus spectrophotometer GelDoc2000 gel scan system TRANS-BLOT® Semi-dry transfer cell Molecular Imager®FX western blot scanner
Brand	Handy Step® Electronic automatic dispenser
Eppendorf	Thermomixer 5436 Thermomixer Compact Centrifuge 5417C Centrifuge 5417R
Gilsion	1000μl, 200μl, 20μl,10μl and 2μl pipetmans
Heidolph	Polymax 2040 shaking plate
Heraeus Instruments	Heraeus SEPATECH Megafuge 1.0 Heraeus SEPATECH Megafuge 1.0R HERA Safe Typ HS 12/2 cell culture hood CO_2 Incubator Typ BB6220CU Heraeus -80°C freezer Model No 75007693
Herolab	TRANSILLUMINATOR UVP Model: TM-20
Hettich	UNIVERSAL 16R centrifuge
Janke&Kunkel IKA®-Labortechnik	ULTRA-TURRAX T25 ultramixer for tissue IKA-COMBIMAG RET magnetic mixer VIBROFIX UF1 ELECTRONIC vortex
Julabo Labortechnik	JULABO VC Typ VC/3 CIBA heater for water bath JULABO 12B Typ VC/3 BRUE.12/20B water bath
Las Laborapparate	PRÄZITHERM Typ PZ60 thermoplate in cell culture
Marienfeld	Cell counter chamber 0.100 mm x 0.0025 mm^2
Mettler Toledo	METTLER PM4800 DeltaRange® balance METTLER AE 240 balance
Molecular Devices Corporation	SPECTRAmax^{TM}-Microplate Spectrophotometer System
MS Laborgeräte	Optimax Typ TR film developing machine ΦpH Meter
New Brunswick Scientific	Incubator shaker G25
Nikon	Super High pressure mercury lamp power supply HB-10104-AF Power Supply white light TE-PSE100 Nikon ECLIPSE TE300 fluorescent microscope Nikon DIAPHOT PHASE CONTRAST ELWD 0.3 No.133819
Perkin Elmer	Gene Amp PCR System 9600
Prutscher	Laboratory hood
Revco Scientific Inc.	-80°C Freezer ULT1786-5-V14
Scotsman	Ice machine
Sony®	Progressive 3CCD Color Video Camera DXC-91100P
Whatman Biometra®	T3 Thermocycler Advanced Multi Block Thermocycler Oven chamber used for stripping of cellulose membranes
WILL WETZLAR GELMAN INSTRUMENT	Wilovert® No.96792 microscope GELAIRE Laminar air flow Class100 cell culture hood GELAIRE® FLOW LaboratoriesTyp BSB 6A cell culture hood
WTB Labortechnik	CO_2- Incubators for cell culture, controller version E045.06.984

3. RESULTS

The proper development of the mammary gland requires a tight coordination of expression of many genes involved in proliferation and differentiation. The aim of this work was to identify novel genes and proteins as well as signaling pathways involved in the development of the mouse mammary gland and to find a possible correlation between the pathway components and their downstream targets that are activated during proliferation and/or differentiation of mammary epithelial cells. In this study rapamycin has been used to specifically inhibit the mTOR pathway to analyze its role in mammary gland proliferation and differentiation (Chapter 3.1 to 3.3). Furthermore, a genomic approach was used to identify novel genes differently expressed during mouse mammary epithelial cells proliferation and differentiation (Chapter 3.4).

3.1 The role of mTOR signaling in mammary epithelial cells

During development of the mammary gland the epithelial cells first extensively proliferate in order to form ducts and alveoli. Finally the cells differentiate, they become functional and start to secrete milk proteins. The mTOR signaling pathway has been shown to be implicated in the regulation of many crucial cellular processes. However, the role of the mTOR pathway in mammary gland development has not been studied before. In order to study the role of the mTOR pathway in growth and functional differentiation of mammary epithelial cells *in vitro* HC11 cells were used. This is an established mammary epithelial cell line able to proliferate and differentiate *in vitro*. These cells were originally isolated from a mid-pregnant mouse and have been optimized for *in vitro* cultivation. The HC11 cells proliferate well on plastic dishes and when they are induced with a mix of lactogenic hormones (Dexamethasone, Insulin and Prolactin) they are able to express the milk protein β-casein. This indicates that these cells are able to terminally differentiate *in vitro*.

To study the role of mTOR signaling during proliferation, morphological and terminal differentiation of mammary epithelial cells *in vitro,* the HC11 cells were treated with rapamycin. This is a very specific inhibitor of mTOR, which can prevent the activation of downstream targets by mTOR.

3.1.1 Activation of mTOR in proliferating and differentiating HC11 cells

To study the role of mTOR signaling during proliferation and differentiation of mammary epithelial cells, the amount of phosphorylated mTOR in HC11 cells was analyzed. Therefore, the HC11 cells were cultivated in growth medium for 2 days, then the medium was changed to starvation medium and the cells were cultivated for additional 4 days. At this stage the cells reach the competent stage and can be induced with lactogenic hormones (DIP) for the next 4 days. This will lead to the functional differentiation of HC11 cells. Every day and from all three stages protein extracts were prepared with RIPA buffer (growing (proliferation) stage, competent stage, and induced stage). The total protein extracts were than used for western blot analysis with an anti-mTOR antibody specific for mTOR phosphorylated at Ser2488. To verify proper differentiation of the mammary epithelial cells the blots were also incubated with an antibody specific for β-casein. High expression of the milk protein β-casein indicates that the induction of terminal differentiation was successful. To check equal loading of the samples, the membrane was stripped and re-probed with anti β-tubulin antibody.

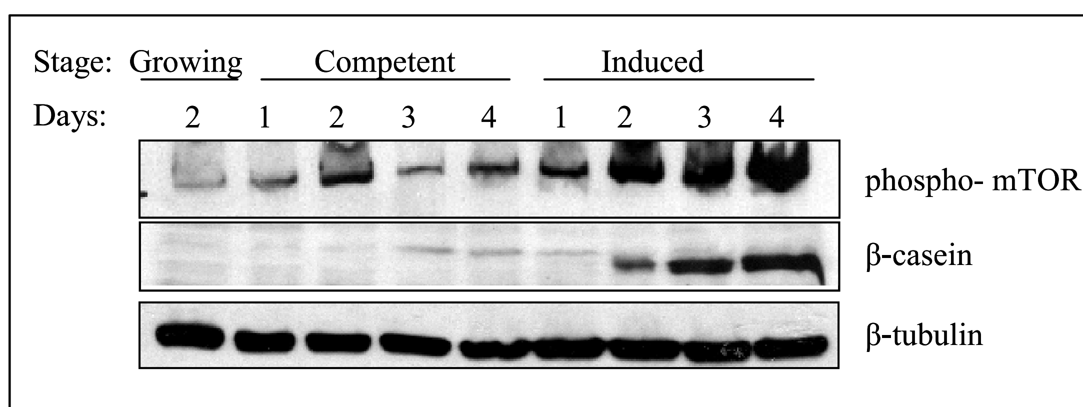

Figure 3.1 Phosphorylation of mTOR in mammary epithelial cells. The HC11 cells were grown for 2 days, brought to competency for 4 days and than induced for differentiation with lactogenic hormones (DIP). Protein extracts from each stage (growing, starvation (competent), and induced) were isolated and western immuno-blotted with anti Phospho Ser2488-mTOR antibody. To confirm functional differentiation of the treated cells, blots were also incubated with β-casein antibody. To confirm equal loading of the samples the blots were stripped and reprobed with anti β–tubulin antibody.

The western blot (Figure 3.1) shows that the described treatment of the cells indeed induces the expression of β-casein. This shows that it is possible to mimic the differentiation of mammary epithelial cells by using HC11 cells and lactogenic hormones. It also shows that mTOR is active at a low level in proliferating (growing) and competent cells (days 1-4).

However, during induction with lactogenic hormones the amount of phosphorylated mTOR increases. This experiment indicates that mTOR signaling is mainly involved in the regulation of differentiation of mammary epithelial cells.

3.1.2 Influence of mTOR on expression of β-casein during differentiation of mammary epithelial cells

As shown above, HC11 is a mammary epithelial cell line which is able differentiate upon stimulation with lactogenic hormones. As shown in Figure 3.1 the successful induction of differentiation can be monitored by checking the expression of the milk protein β-casein. It is commonly used as a marker of functionally differentiated mammary epithelial cells *in vitro*. The previous experiments showed that mTOR is strongly activated upon induction with lactogenic hormones and therefore, may play an important role during differentiation of HC11 cells. To confirm this idea the activity of mTOR was inhibited by the addition of rapamycin (1 or 10 nM) during starvation and/or DIP induction. As a control equivalent amounts of DMSO (drug solvent) were added. Protein extracts of HC11 cells in different stages were analyzed in a western blot for the presence of β-casein protein.

The first 3 lanes Figure 3.2A show that β-casein expression is induced if cells are treated with DIP. However, if cells are treated with rapamycin and induced with lactogenic hormones they are not able to synthesize β-casein protein and therefore are not functionally differentiated. Rapamycin treatment prevents the expression of β-casein if the drug was added 4 days before induction of differentiation (lane 8) or just before activation with lactogenic hormones (lane 6). The control cells treated with DMSO (lanes 7 and 9) expressed β-casein at a level comparable with untreated differentiated cells (lane 3). This experiment was performed using 1 or 10 nM rapamycin and both treatments gave the same results.

The western blot experiment with extracts from rapamycin treated HC11 cells has shown that mTOR influences the amount of β-casein on the protein level. Although mTOR is known to regulate the activity of components of the translation machinery it was of interest to investigate if rapamycin treatment also influences the transcription of the milk gene by reducing the level of β-casein mRNA synthesis. Therefore, total RNA was isolated from the same HC11 cells as described in figure 3.2A.

A semi-quantitative reverse transcription PCR reaction was performed with primers specific for β-casein. Primers amplifying GAPDH were used as an internal control to verify the use of equal amounts of mRNA in each reaction.

The PCR products were analyzed on an agarose gel and as shown in Figure 3.2B rapamycin treatment (lanes 6 and 8) clearly prevents the accumulation of β-casein mRNA during induction of HC11 cells for differentiation. From mRNA extracts of untreated and induced HC11 cells (lane 3) or control cells treated with DMSO (lanes 7 and 9) it was possible to amplify the β-casein cDNA. The reverse transcription PCR experiment shows that inhibition of mTOR down regulates the transcription or influences mRNA stability of the β-casein gene. The results confirm that mTOR signaling is essential for functional differentiation of HC11 cells as shown by the expression of β-casein.

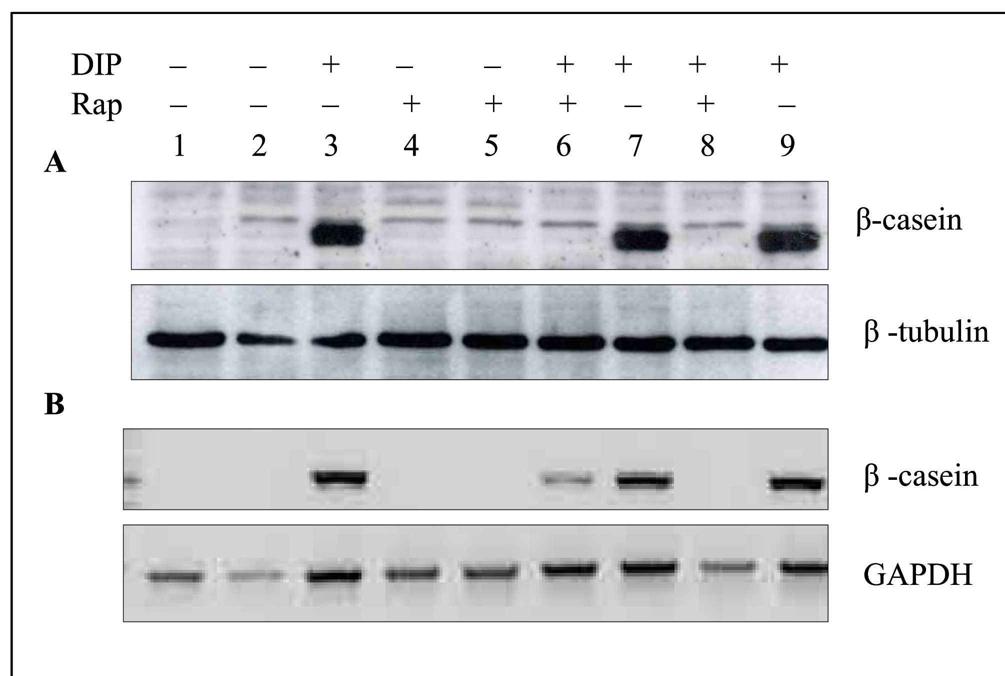

Figure 3.2 Rapamycin blocks the expression of the milk protein β-casein during induction of differentiation in HC11. (A) Total protein was extracted from growing (proliferating) (lane1) competent (lanes 2,4,5) and differentiated (lanes 3,6,7,8,9) HC11 cells treated with rapamycin or as a negative control DMSO. As indicated the HC11 were treated with rapamycin 4 days before the induction (lane 8) or the drug was added just before activation with the lactogenic hormones (lane 6) The protein concentration were estimated by spectrophotomerty. 40 µg of each protein extract was loaded and separated on 12% SDS-PAGE gel. The gel was blotted on a nitro-celulose membrane which in turn was incubated with anti β-casein antibody. To control equal loading of the sample the membrane was stripped and reprobed with an antibody specific for β-tubulin. **(B)** The RNA was isolated from growing competent and cells induced for differentiation treated with rapamycin or DMSO. The RNA samples were used in reverse transcription PCR run with primers specific for β-casein or GAPDH as a control. The reaction products were separated on 1.2% agarose gel and visualized by EtBr staining.

3.1.3 Effect of rapamycin on β-casein mRNA stability

The inhibition of mTOR with rapamycin has a clear effect on the β-casein expression. The down-regulation of β-casein mRNA could be caused by the fact that mTOR influences the post-transcriptional processing of mRNA making it less stable. To test this theory the effect of rapamycin on β-casein mRNA stability was studied in HC11 cells. To block transcription of mRNA a potent inhibitor of transcription actinomycin D, was used in the following experiment. HC11 cells were grown to competent stage and were than induced for differentiation with lactogenic hormones. After 16 hours of stimulation a mixture of actinomycin D and rapamycin or actinomycin D and DMSO was added to the medium. The control cells were treated with a mixture of ethanol (solvent for actinomycin D) and rapamycin or equivalent mixture of ethanol and DMSO, so the inhibitory effects of actinomycin D could be compared to the control. The addition of actinomycin D blocks the transcription process and the gradual degradation of mRNA can be measured over time. Therefore total RNA was isolated at several time points after drugs treatment. The amount of β-casein transcripts was analyzed using Real-Time PCR at each time point.

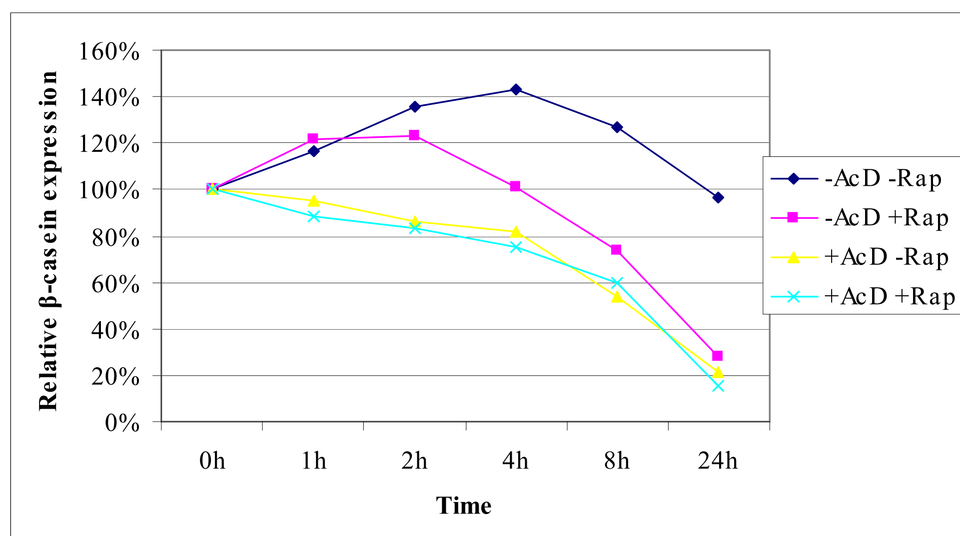

Figure 3.3 The influence of mTOR on the stability of β-casein mRNA The HC11 cells were induced for differentiation in presence of combinations of rapamycin (10 nM) and Actinomycin D (1μg/ml) and their solvents ethanol and DMSO. Total RNA was isolated at indicated time points after treatment. The reverse transcription of mRNA was followed by a Real-Time PCR performed with primers specific for β-casein cDNA.

The obtained cDNA samples were used in a Real-Time PCR reaction performed with a primer pair specific for β-casein. The results obtained in these Real-Time PCR reactions were normalized against 18S ribosomal mRNA. The obtained data are presented as graph in Figure 3.3. The actinomycin D effectively inhibited the transcription of the β-casein gene as seen in treated cells (yellow and light blue curve on the graph). Rapamycin had no de-stabilizing effect on β-casein mRNA in actinomycin treated cells (light blue curve) when compared with cells treated with Actinomycin D and DMSO (yellow curve). An effect of rapamycin was only visible when transcription was active (dark blue and pink). After around 1h the cells treated with rapamycin (pink curve) stopped accumulating β-casein mRNA when compared with control (dark blue curve). These results indicate that mTOR has no influence on the stability of the β-casein mRNA, since the inhibition of mTOR by using rapamycin did not change the amount of β-casein mRNA. The question why β-casein mRNA is reduced upon mTOR inhibition is therefore still open.

3.1.4 Influence of mTOR on the morphology of mammary epithelial cells

Several mammary epithelial cell lines have been shown to differentiate morphologically when seeded in three dimensional (3D) culture systems. Upon hormonal stimulation, the cells form structures similar to the glandular organization of the mammary gland *in vivo*. To test the influence of mTOR on the morphology of differentiated mammary epithelial cells it was necessary to investigate the normal morphology of HC11 cells in 2D and 3D culture systems.

3.1.4.1 The morphology of HC11 cells in 2D and 3D cultures

To study morphological changes during differentiation the HC11 cells were seeded either on plastic dishes (2D) or in a collagen gel (3D). Collagen served as a three dimensional matrix for cell growth.

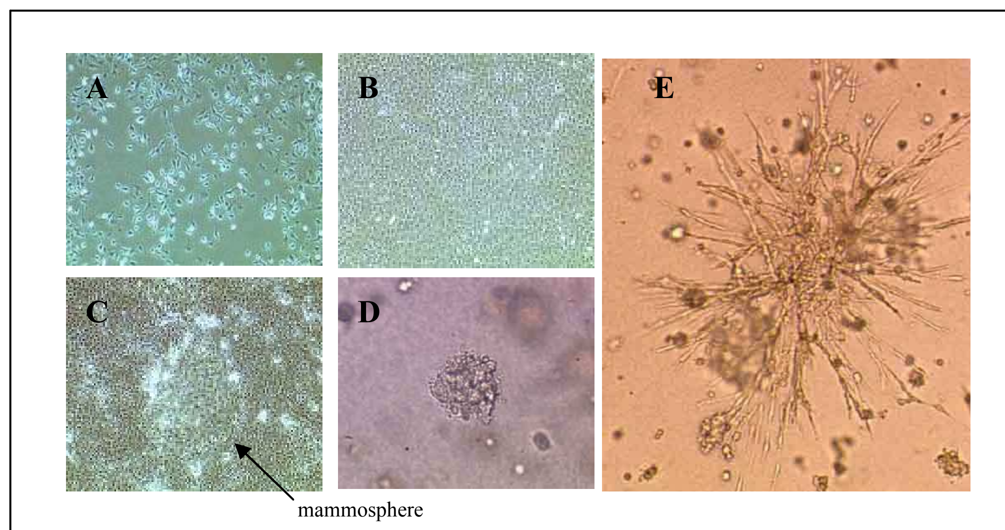

Figure 3.4 Comparison of HC11 cultured on plastic and in collagen gel. (A) HC11 cells growing for 24 h on plastic dish. (B) HC11 cells in confluent stage after 4 days. (C) HC11 cells induced for differentiation with lactogenic hormones for 4 days, a mammosphere (arrow) present in the middle of picture. (D) HC11 cells growing three dimensionally for 4 days in collagen. (E) Branching structures formed by HC11 cells induced for morphological differentiation in collagen after 8 days.

The cultures on plastic and in collagen gels were analyzed in a phase contrast microscope at several time points (Figure 3.4). In dishes, the cells proliferate (A) until they fill all available space and the plate become confluent (B). The HC11 cells are very compact, forming a tight monolayer. When induced for differentiation with lactogenic hormones the HC11 cells occasionally form “domes” also called mammospheres. In 3D cultures the growing cells first clumped together and formed globular structures (D). When induced for differentiation the cells formed branch-like structures (E). These branches resemble thin ducts seen in the mammary gland.

3.1.4.2 Influence of mTOR on the morphology of HC11 cells in 3D culture

The previous experiment showed that HC11 cells, like other mammary epithelial cell lines, when seeded in a three dimensional matrix are able to build structures that are similar to ductal structures formed by epithelial cells during mammary gland development. As matrix for 3D cell culture the rat tail collagen type I or matrigel® are commonly used. To test the effect of mTOR on the formation of branch-like structures the HC11 cells were seeded within collagen or matrigel. After 2 days the growth medium was changed to starvation medium and cells were cultured for next 4 days to obtain competent cells. Then, induction medium with fresh lactogenic hormones were added to the cells for next 8 days of culture with or without rapamycin. After the incubation the gels were analyzed under the light microscope.

Untreated HC11 cells are able to form duct-like structures in collagen and matrigel. The morphology resembles that of the ductal tree in mammary gland. In contrast, HC11 cells treated with Rapamycin were not able to form any structures neither in collagen nor in matrigel. These results indicate that mTOR plays an important role in the morphological differentiation of mammary epithelial cells.

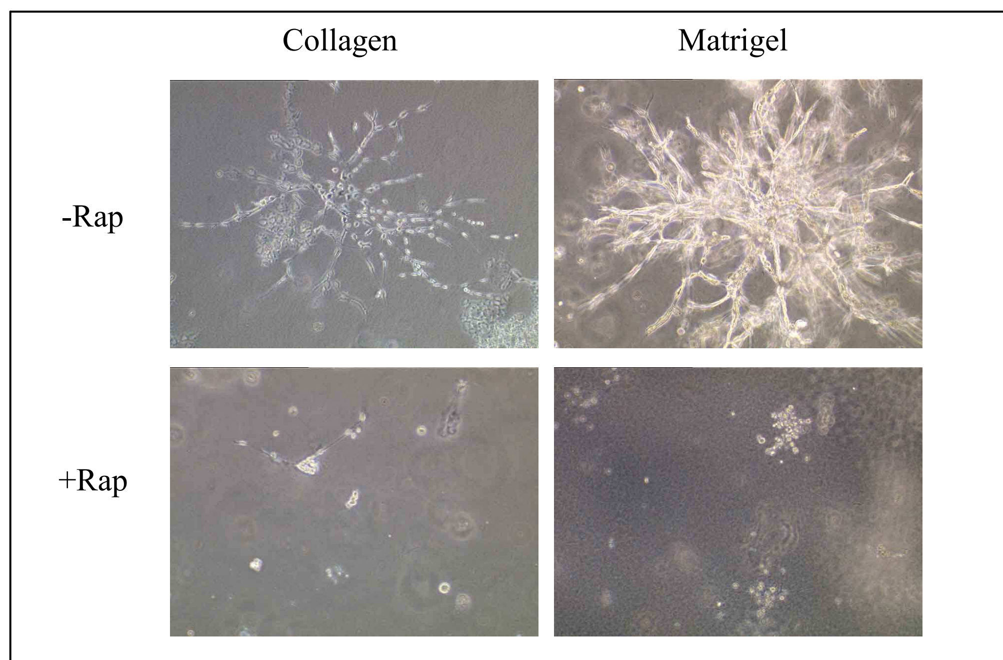

Figure 3.5 Rapamycin inhibits the morphological differentiation of HC11 cells in 3D cultures. Around 2000 cells were seeded in collagen or matrigel in 12-well plate and were stimulated for differentiation with lactogenic hormones in the absence (DMSO control) or presence of rapamycin (10 nM). After 8 days of stimulation the gels were analyzed under the phase contrast microscope.

3.1.5 Influence of mTOR inhibition on the proliferation of HC11 cells

The analysis of HC11 cells in 3D cultures indicated that mTOR plays an important role in the regulation of morphological differentiation of mammary epithelial cells. Inhibition of mTOR signaling in HC11 cells by rapamycin evidently reduced the formation of branch-like structures in collagen as well as in matrigel®. The rapamycin treated cells possibly proliferate slower than controls and therefore the effect of mTOR inhibition on the proliferation of HC11 cells was analyzed. Possibly, the lack of branch-like structures is due to the negative effect of rapamycin on the ability of mammary epithelial cells to proliferate. Therefore, HC11 cells were seeded in 96-well plates in growth medium. After overnight incubation rapamycin or DMSO (control) was added to the culture medium and the cells were incubated for the next 96 hours.

The amount of proliferating cells was measured every 24 hours starting from the time point when the drug was added (0 h). For this, an XTT assay was used, which detects metabolically active cells.

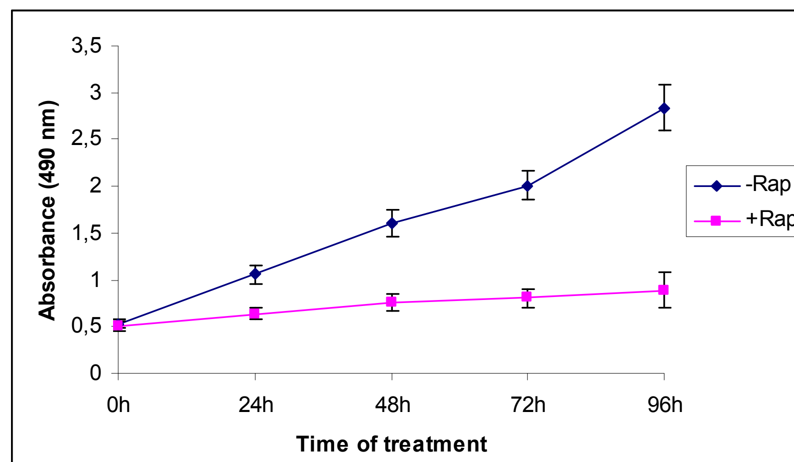

Figure 3.6 Rapamycin reduces proliferation of mammary epithelial cells. Around 5000 cells were seeded per one well of 96-well plate in growth medium and cultivated in presence of Rapamycin (10nM) or DMSO (control) for 96 hours. The amounts of living and metabolic active cells were measured at indicated time points with XTT proliferation assay.

As shown in Figure 3.6 the inhibition of mTOR by rapamycin has a negative effect on the proliferation of mammary epithelial cells. The control cells were able to proliferate much faster so the difference between the amounts of proliferating control and treated cells visibly increased with time. The obtained results indicated that the observed reduction in the development of branch-like structures in 3D cultures was at least partly due to the inhibition of cell proliferation. The next question to be answered was, which downstream targets of mTOR are involved in the regulation of proliferation and differentiation.

3.1.6 The influence of mTOR on the Stat5 signaling pathway

The HC11 cells treated with rapamycin were not able to express milk proteins indicating that mTOR is essential for the activation of pathways allowing the terminal differentiation of mammary epithelial cells. It was therefore interesting to investigate how mTOR controls these pathways. The prolactin pathway has been shown to be essential for the transcriptional activation of the milk protein genes (Rosen and Wyszomierski 1999). The prolactin hormone binds to the prolactin receptor (PR), which leads to the activation of the associated Jak2 kinase and in turn to the phosphorylation of Stat5.

Phosphorylated Stat5 proteins dimerize and translocate to the nucleus. Stat5 binds to GAS-elements in the promoter of various targets genes and activates their transcription. The promoter of the β-casein gene contains such GAS elements and it has been shown that Stat5 binding is essential for its transcriptional activation. To test if mTOR influences the Stat5 signaling pathway the phosphorylation, translocation and transactivation activity of Stat5 upon rapamycin treatment were investigated. The results are summarized in chapter 3.1.6.1-3.1.6.3.

3.1.6.1 The influence of mTOR on the phosphorylation of Stat5

To study the role of mTOR on the Stat5 signaling pathway the phosphorylation of Stat5 was investigated in HC11 cells. The HC11 cells were cultivated to the competent stage and were than induced for differentiation with lactogenic hormones including prolactin in presence or absence of rapamycin. The proteins were isolated after different time points of lactogenic stimulation. The isolated protein samples were analyzed in a western blot with an antibody specific for the phosphorylated form of Stat5. To verify equal loading of the samples the membranes were stripped and reprobed with an antibody specific for total Stat5.

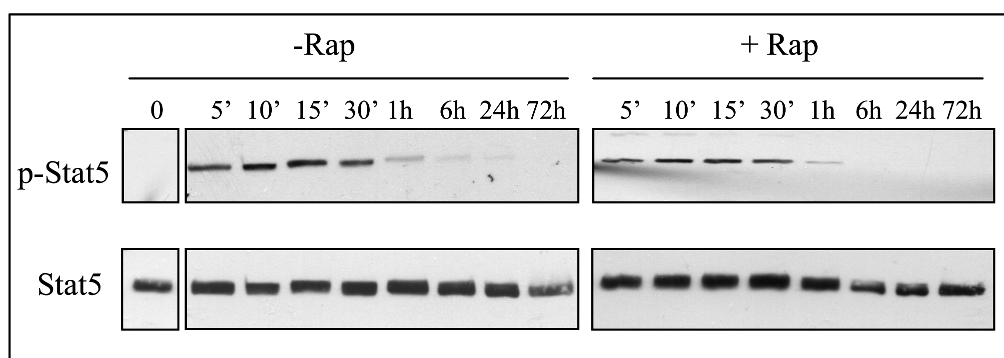

Figure 3.7 Phosphorylation of Stat5 is not influenced by rapamycin treatment. The HC11 cells were grown to the competent stage and were than induced with lactogenic hormones (DIP) in presence of rapamycin or DMSO (control). The protein extracts were isolated at indicated time points after lactogenic stimulation and western blotted and incubated with an anti phospho Stat5 antibody. To verify even protein loading the membranes were stripped and reprobed with an antibody specific for total Stat5 protein.

The western blot analysis in Figure 3.7 shows that phosphorylation of Stat5 is successfully induced by the addition of lactogenic hormones to the culture medium. Accordingly, in the non-induced cells (time point 0, competent stage) phosphorylated Stat5 was undetectable.

Comparison of the phosphorylation pattern of Stat5 of rapamycin treated cells with untreated cells did not show any differences. The kinetics as well as the amount of Stat5 phosphorylation is not influenced by rapamycin treatment. In both rapamycin treated and control cells phosphorylated Stat5 is present within 5 minutes after DIP induction and phosphorylation lasts over 30 minutes. After that the activation of Stat5 is gradually lost over time. Therefore it was concluded that mTOR does not influence the prolactin pathway on the level of Stat5 phosphorylation.

3.1.6.2 Influence on nuclear translocation of Stat5

Although mTOR has no influence on the activation of the Stat5 signaling pathway, it was possible that mTOR effects dimer formation preventing translocation of the Stat5 into the nucleus. Because only total protein extracts were used in the previous experiment such a translocation effect would be undetectable. Therefore, it was crucial to separate the nuclear and cytoplasmic cell fractions and investigate the translocation kinetics of Stat5 upon rapamycin treatment. To perform the experiment the HC11 cells were again cultivated to reach the competent stage and were than induced for differentiation with lactogenic hormones (DIP) in presence or absence of rapamycin (10 nM). Nuclear and cytoplasmic proteins were separated using a nuclear fractionation method described in Material and Methods (chapter 2.2). Proteins were isolated at several time points after lactogenic induction. The obtained cellular fractions were analyzed on a western blot with an anti Stat5 antibody.

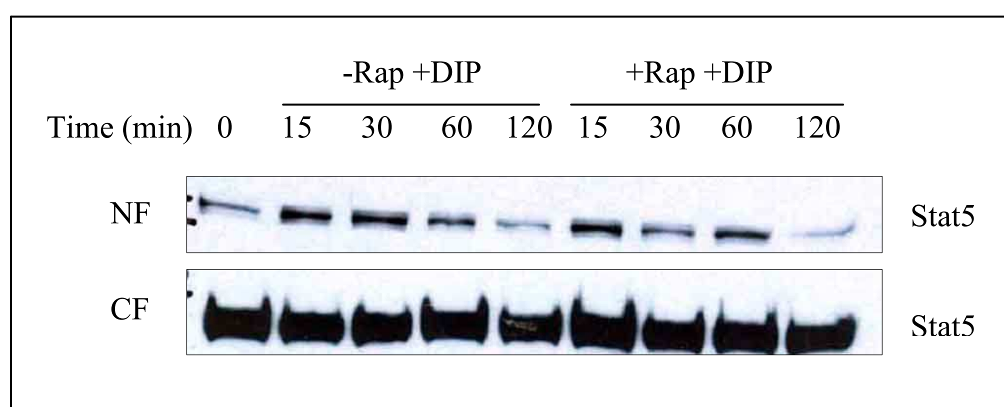

Figure 3.8 Rapamycin has no effect on the translocation of Stat5 into the nucleus. The HC11 cells were grown to reach competency and than induced for differentiation with lactogenic hormones (DIP). Nuclear and cytoplasmic proteins were isolated at indicated time points after induction. The blots of nuclear and cytoplasmic proteins were incubated with an anti-Stat5 antibody. CF-cytoplasmic fraction; NF- nuclear fraction.

As seen in Figure 3.8 upon induction of control cells with lactogenic hormones Stat5 translocated into the nucleus. There were no differences observed between rapamycin and non-treated cells. After 15 minutes the amount of protein in the nuclear fraction clearly increased. As seen for Stat5 phosphorylation (Figure 3.7) this translocation last 30 minutes and then slowly returns to level before DIP stimulation. The small amount of Stat5 detected at time point 0 in the nuclear fraction may be due to a contamination of cytoplasmic proteins in the nuclear fraction. These results indicate that mTOR has no influence on the phosphorylation or nuclear translocation of Stat5.

3.1.6.3 Influence on transcriptional activation of Stat5

Since mTOR has no effect on the phosphorylation nor on the dimerization and nuclear translocation of Stat5, the mTOR could affect the transcriptional activity of Stat5. The promoter of β-casein contains GAS elements, a sequence region required for the binding of Stat5 dimers. The activation of this promoter by Stat5 leads to transcription of the β-casein gene. We surmised that the mTOR signaling pathway could influence the binding of Stat5 on the β-casein promoter. This would explain the reduced expression of β-casein after rapamycin treatment. Therefore the binding and transactivator activity of Stat5 was studied using a reporter construct. In the previously established HC11-β-casein-luc cells a luciferase gene under control of β-casein gene promoter is stably integrated into the genome. With the help of such a reporter system it is possible to estimate the binding and transactivation activity of Stat5 by measuring the enzymatic activity of luciferase. The HC11-β-casein-luc cells were cultivated to reach the competent stage and were induced for 16 hours with lactogenic hormones (DIP) with addition of rapamycin or DMSO. Finally the cells were lysed in a special luciferase assay buffer (see Material and Methods, chapter 2.2). The luciferase activity in the cell extracts was measured in a luminometer by addition of the luciferin substrate. The obtained data were normalized against the protein concentration in each sample and plotted in a diagram (see Figure 3.9).

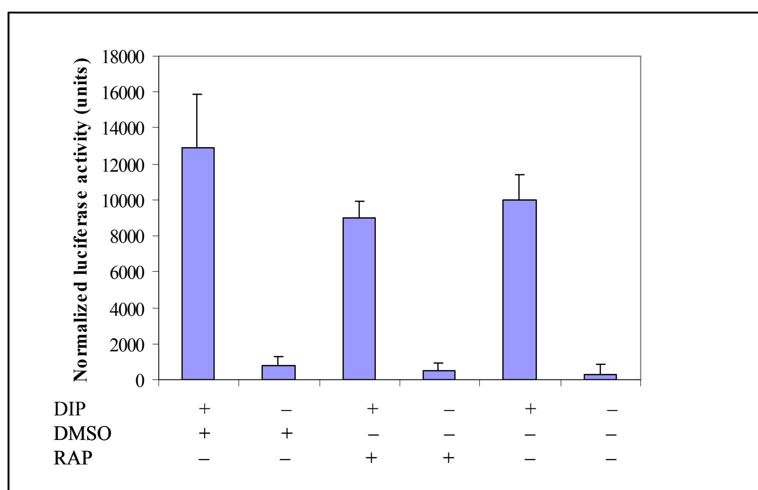

Figure 3.9 Rapamycin has no effect on the activation of the β-casein promoter by Stat5. HC11-β-casein-luc were grown to reach competency and than induced for differentiation with lactogenic hormones (DIP) with addition of rapamycin or DMSO as a control. After 16 h the level of luciferase activity was measured in luminometer. The activity was normalized against the total protein concentration in each sample.

As can be seen (Figure 3.9) the transcriptional activity of Stat5 was induced with lactogenic hormones (DIP) when the activity of luciferase between non-induced (column 6) and induced control cells (column 5) were compared. There was no significant difference between the induced cells treated with rapamycin (column 3) or DMSO (column 1). In both cases Stat5 binding was stimulated upon addition of DIP to the medium.

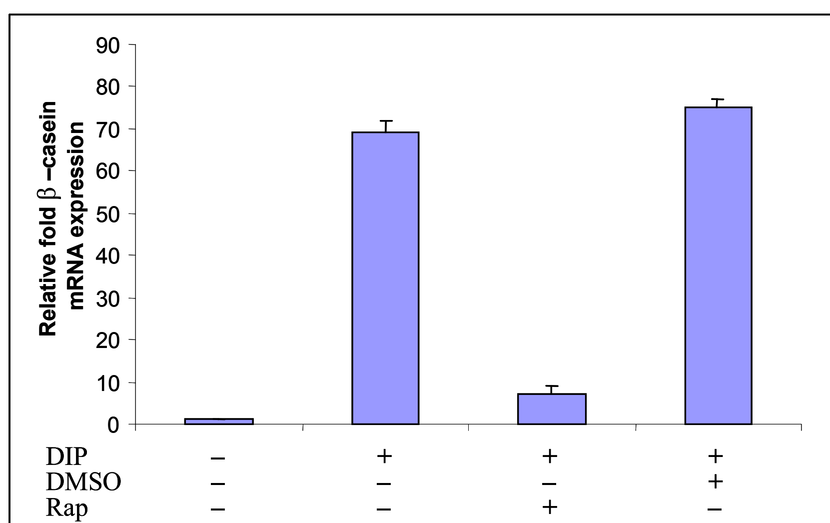

Figure 3.10 Rapamycin reduces expression of β-casein mRNA in HC11-luc cells. The HC11-luc cells were cultivated to reach competent stage and were stimulated for 16 with lactogenic hormones. The total RNA was isolated and reverse transcribed. The cDNA was used in real-time PCR reaction run with primers specific for β-casein gene. The data were normalized against the 18S ribosomal subunit mRNA that serve as internal standard.

Because there was no effect of mTOR on Stat5 signaling, it was important to investigate whether β-casein expression in the HC11-luc cells is the same as in normal HC11 cells. Therefore the amount of β-casein mRNA and protein was also analyzed in these cells. To confirm that mTOR also affects the endogenous β-casein mRNA accumulation in the HC11-luc cells, total mRNA was isolated from the HC11-luc cells after 16h of stimulation with lactogenic hormones. The mRNA was reverse-transcribed into cDNA, which was then used for a quantitative Real-Time PCR reaction with primers specific for the β-casein gene. As an internal control, also primers amplifying the 18S ribosomal unit were used. β-casein mRNA was then normalized against the amount of 18S mRNA. The PCR data (Figure 3.10) showed a clear increase of mRNA expression in cells stimulated with DIP (column 2) when compared with non-induced cells (column 1). Treatment with rapamycin (column 3) significantly reduced the accumulation of mRNA as compared with control cells treated with DMSO (column 4). This is in agreement with data shown in figure 3.2B.

To further confirm that induction of functional differentiation with lactogenic hormones on the protein level, the HC11-luc cells were stimulated with DIP for 4 days. The isolated protein extracts were analyzed in a western blot with an antibody specific for β-casein protein (Figure 3.11).

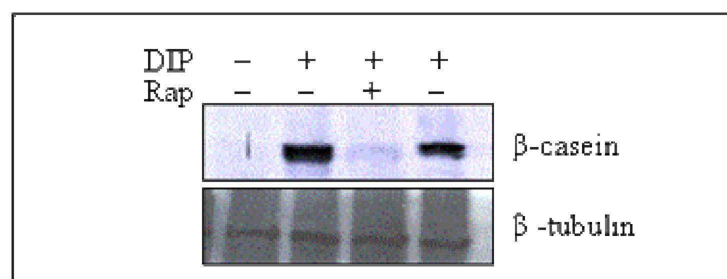

Figure 3.11 Rapamycin inhibits the expression of β-casein in HC11-luc cells. The HC11-luc cells were cultivated to reach competency and than stimulated for 4 days with lactogenic hormones (DIP) in presence of Rapamycin or DMSO (drug solvent). To compare the lactogenic stimulation one sample was left untreated and non-stimulated. Total proteins were isolated and western-immuno-blotted with an antibody specific for β-casein. To verify the equal loading of the sample the membrane was stripped and reprobed with anti β-tubulin.

The western blot analysis in Figure 3.11 confirms the induction of functional differentiation with DIP in HC11-luc cells (lane 2). The differentiation marker β-casein is expressed, as compared to non-stimulated cells (lane 1). Also in the HC11-luc cells rapamycin (lane 3) inhibits β-casein expression when compared with cells treated only with the drug solvent DMSO (lane 4).

The results showed that mTOR has no influence on the prolactin pathway. Treatment of mammary epithelial cells with rapamycin, inhibiting mTOR, had no effect on the Stat5 phosphorylation status, nor on its nuclear translocation or binding ability to the β-casein promoter. Moreover, the results showed that although Stat5 binds to the β-casein promoter upon rapamycin treatment there was no full activation of the β-casein gene transcription. The level of β-casein mRNA was clearly reduced in treated HC11-luc cells. As a result the β-casein protein expression was inhibited, indicating that cells were not functionally differentiated. This was also not caused by mRNA instability of β-casein as shown in Figure 3.3. Therefore, other factors important for the expression of β-casein must be influenced by mTOR.

3.1.7 The Influence of mTOR on the expression of Idb proteins

The mTOR protein is one of the down stream targets of the insulin signaling pathway (Wang et al., 2006). Several studies have shown that activation of the insulin pathway is required for the expression of milk proteins (Rosen et al., 1999). Insulin signaling was also shown to regulate the activity of Idb proteins (Belletti el al., 2002; Navarro et al., 2001). These observations suggest, that there might be a correlation: possibly mTOR is a mediator of insulin by influencing the action of Idb proteins. Another observation supports this idea: if mTOR is inhibited by treatment with rapamycin the HC11 cells proliferate slower. This is in agreement with the observation that the down-regulation of Id1 also reduces cell proliferation. In the following chapter the possible link between mTOR and Idb proteins was studied in more detail.

3.1.7.1 Regulation of Id1 by mTOR

If insulin regulates the activity of mTOR and if mTOR affects Idb proteins several experiments can be performed to test this hypothesis. To test the influence of mTOR on Id1 expression the HC11 cells were seeded at low density to allow proliferation and were treated for 24 hours with rapamycin (+) or as negative control with an equivalent dilution of DMSO (-) or left untreated (0). The proteins and total mRNA were than isolated and the mRNA was reverse transcribed. The obtained cDNA samples were used as templates for Real-Time PCR reactions, which were performed with primers specific for Id1. The PCR data were normalized to 18S ribosomal subunit mRNA.

In parallel, the isolated protein extracts were analyzed on a western blot using an antibody specific for Id1. To control equal loading of the samples on the gel, the membrane was re-probed with anti β-tubulin antibody.

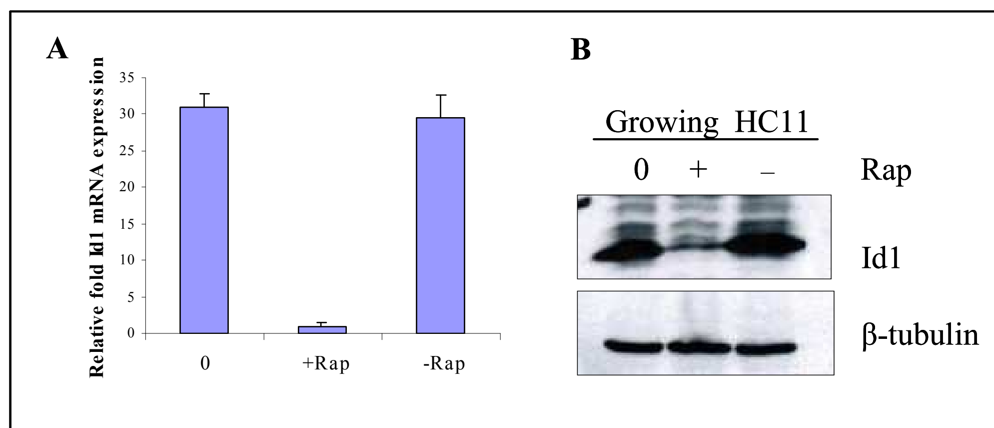

Figure 3.12 mTOR influences the expression level of Id1. The HC11 cells were proliferating in presence of rapamycin (+) or DMSO as negative control (-) or were left untreated (0). The total RNA and proteins were isolated after 24h of treatment. (**A**) The isolated mRNA was reverse transcribed to cDNA. The Real-Time PCR reaction was performed with primers specific for Id1 and for normalization with primers specific for 18S ribosomal subunit mRNA. The data were normalized and plotted in a diagram. (**B**) Protein extracts were western blotted with an antibody specific for Id1. To control the equal loading of the samples, the membrane was re-probed with anti β-tubulin antibody.

The Real-Time PCR analysis (Figure 3.12A) showed that rapamycin treatment clearly down-regulates the expression of Id1 (the relative expression level is reduced 30 times) when compared with control or untreated cells. This result indicated that mTOR controls the expression of Id1 on the transcriptional level. The western blot (Figure 3.12B) showed also the down-regulation of Id1 on the protein level upon treatment with rapamycin and confirmed the Real-Time PCR data. These experiments indicated that mTOR might control the proliferation of HC11 cells by influencing the expression level of Id1. Since Id1 has also been shown to be involved in the regulation of cell invasiveness it is well possible that inhibition of its expression with rapamycin not only reduces the cellular proliferation rate, but is also responsible for reduced ability of the rapamycin treated cells to migrate in the surrounding matrix during morphological differentiation in 3D cultures (Figure 3.5).

3.1.7.2. Regulation of Id2 expression by mTOR.

The HC11 cells induced for differentiation with lactogenic hormones were not able to express β-casein in presence of rapamycin (Figure 3.2 and 3.11).

Studies with an epithelial cell line showed that Id2 is required for functional differentiation and that inhibition of Id2 was followed by a decrease in β-casein expression (Parrinello et al., 2001). It is therefore possible that mTOR regulates differentiation by controlling the expression of Id2. To study the effect of mTOR inhibition on Id2 expression, HC11 cells were grown to the competent stage and than induced with lactogenic hormones in the presence of rapamycin (+DIP+Rap) or DMSO (+DIP-Rap) or were left unstimulated (-DIP). After lactogenic stimulation for 16 hours the total RNA was isolated and reverse transcribed. The obtained cDNA samples were used as a template in Real-Time PCR reactions, which were run with primers specific for β-casein or Id2. Again a primer pair for amplification of ribosomal subunit 18S mRNA was used as internal standard for the normalization of obtained Id2 PCR data.

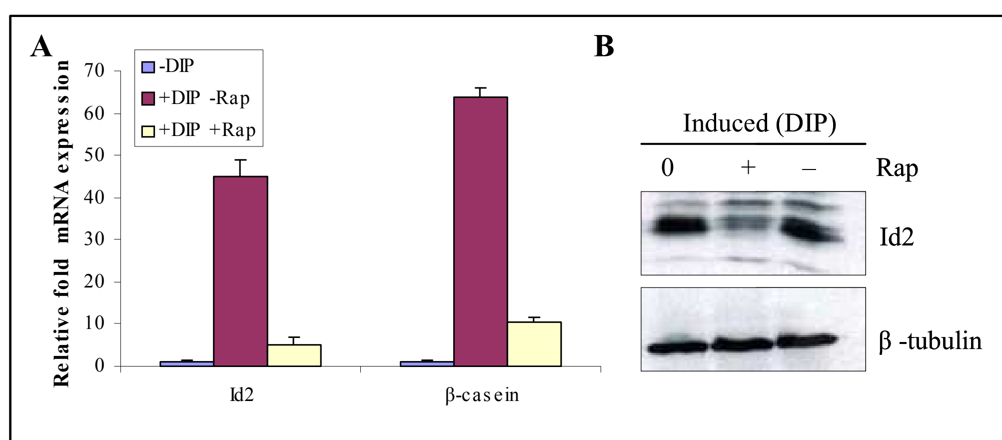

Figure 3.13 mTOR regulates the expression of Id2. The HC11 cells were cultivated to the competent stage and than induced for terminal differentiation with lactogenic hormones (+DIP) in presence of rapamycin (+Rap) or DMSO (-Rap) or were left unstimulated (-DIP) or were induced but not treated (0). (**A**) The isolated total RNA was reverse-transcribed and the obtained cDNA used as template in a Real-Time PCR reaction with primers specific for β-casein or Id2. The results were normalized against 18S mRNA. (**B**) The protein extracts were western blotted with an antibody specific for Id2. To control equal loading of the samples the membrane was reprobed with anti β-tubulin antibody.

As can be seen in Figure 3.13A treatment with DIP induced the expression of Id2 and as expected also of β-casein. In HC11 cells treated with rapamycin the expression of Id2 was significantly reduced and reflects the decrease in mRNA observed for β-casein.

Also, proteins were isolated after 16 h of lactogenic stimulation from induced and untreated cells (0) as well as cells induced in presence or absence of rapamycin.

The protein extracts were western blotted with an antibody specific for Id2. To verify equal loading of the samples the membrane was reprobed with an anti β-tubulin antibody. The western blot analysis (Figure 3.13B) showed that the protein level of Id2 in cells induced with DIP in presence of rapamycin is strongly reduced in comparison to control cells. These western blot data correlate with the obtained Real-Time PCR data (Figure 3.13A). These results confirm the hypothesis that mTOR is involved in the regulation of functional differentiation of mammary epithelial cells by controlling the expression of Id2 on the transcriptional level.

3.1.8 The influence of rapamycin on primary mammary epithelial cells

HC11 treated with rapamycin were not able to proliferate and to form branch-like structures when cultured in a 3D matrix. Inhibition of mTOR signaling also had a negative effect on the functional differentiation of these mammary epithelial cells. Furthermore, HC11 cells induced for differentiation with lactogenic hormones (DIP) in the presence of rapamycin were not able to express β-casein, which additionally indicates a lack of terminal differentiation. To confirm that the obtained result from these studies with HC11 cells are not only true for this *in vitro* established epithelial cell line, the influence of rapamycin on the morphological and functional differentiation was also investigated in primary mammary epithelial cells, since the behavior of primary cells *in vitro* resembles the response of cell in an *in vivo* situation more closely. Primary epithelial cells were freshly isolated from mammary glands of virgin mice and transferred into culture dishes. To investigate the effect of rapamycin on the morphological differentiation of the primary cells the isolated primary mammary epithelial cells were seeded in collagen gels and induced for morphological differentiation in the presence of rapamycin or DMSO (negative control). After 4 days of incubation the cells grown in the gels were analyzed under the microscope (Figure 3.14A). The control primary cells formed well developed tree-like structures. In contrast, the primary epithelial cells treated with rapamycin were not able to form branch-like structures. Their morphology resembled that of rapamycin treated HC11 cells. These results indicate that like for the HC11 cells the ability of murine primary mammary epithelial cells to differentiate in 3D cultures depends on an intact mTOR signaling pathway. It also shows that the HC11 cells responses are still comparable to those of primary cells and confirms that HC11 cells are a usefull model system for these studies.

To study the role of mTOR on functional differentiation of primary mammary epithelial cells the isolated cells were induced for functional differentiation with lactogenic hormones in the presence of rapamycin. The control cells were stimulated in the presence of DMSO. After 4 days of lactogenic stimulation proteins were extracted with RIPA buffer. The obtained protein samples were analyzed in a western blot with an antibody specific for the milk protein β-casein.

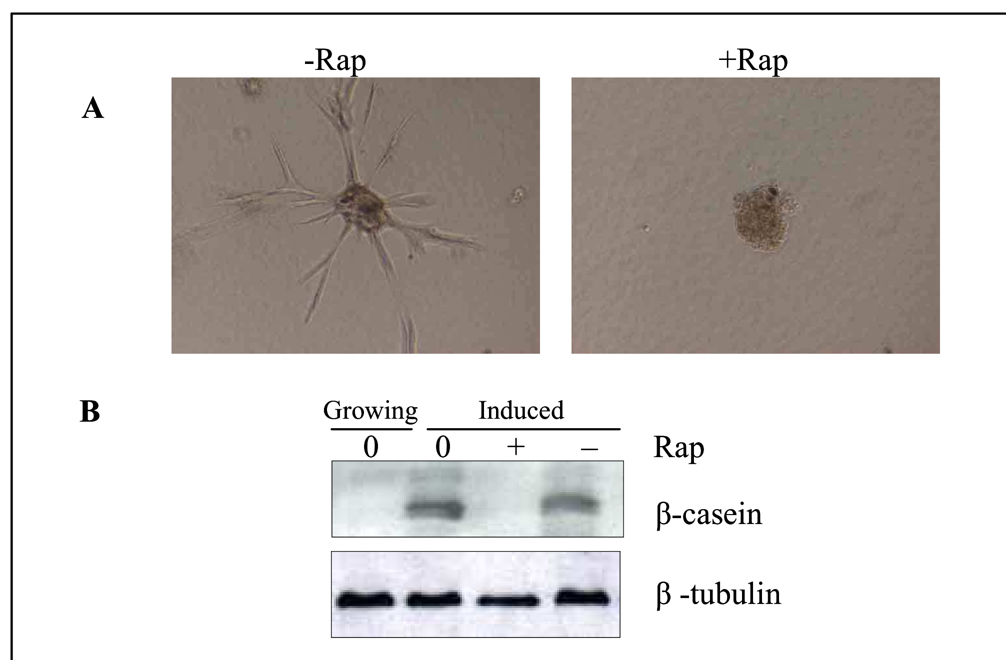

Figure 3.14 The role of mTOR in the morphological and functional differentiation of primary mammary epithelial cells. (**A**) Freshly isolated primary epithelial cells were seeded in collagen gel and induced for differentiation in presence of rapamycin (+Rap) or DMSO (-Rap). (**B**) The primary mammary epithelial cells were grown (Growing) and than induced for functional differentiation with lactogenic hormones in presence of rapamycin (+) or DMSO (-) or were left untreated (0). The total protein extracts were western blotted with an anti β-casein antibody. To verify equal loading of the samples the membrane was stripped and reprobed with an anti β-tubulin antibody.

The western blot analysis (Figure 3.14) showed that the control primary cells induced with lactogenic hormones in presence of DMSO (lane 4) as well as the induced primary cells without any treatment (lane 2) expressed the milk protein β-casein compared to non-induced cells (lane 1). This indicated successful induction of terminal differentiation of primary cells. In contrast the primary cells induced in presence of rapamycin were not able to functionally differentiate as indicated by absence of β-casein protein (lane 3). The studies indicate that mTOR signaling is important for morphological and functional differentiation of primary mammary epithelial cells *in vitro* and confirm the data obtained with our established HC11 cell line.

3.2 The role of mTOR during development of the mammary gland

The *in vitro* studies with the established mammary epithelia HC11 cell line as well as with the freshly isolated primary epithelial cells showed that mTOR signaling is essential for the formation of branch-like structures if cells are seeded in a three-dimensional culture system. More importantly, mTOR signaling is crucial for the terminal differentiation of these cells. Inhibition of mTOR by rapamycin leads to an inhibition of the milk protein β-casein expression, which is a marker used as indicator to determine the differentiation status of mammary epithelial cells. To support these *in vitro* studies, it was essential to analyze the role of mTOR signaling *in vivo*. Therefore, the effect of rapamycin treatment on mammary gland development was investigated in mice.

3.2.1 Role of mTOR during pregnancy

During pregnancy epithelial cells start to proliferate extensively (see Introduction chapter 1.1). This process leads to the extension of ducts and finally to the formation of the secretory compartment, which almost completely fills the mammary gland stroma at the end of pregnancy. *In vitro* studies showed that mTOR signaling is required for the proliferation of mammary epithelial cells and the formation of branch-like structures in gels (see Figures 3.5 and 3.6). To study the role of mTOR *in vivo* mid pregnant (10 days postcoitum) wild-type NMRI mice were treated i.p. up to the day of delivery with daily doses of 1.5 mg/kg of rapamycin (Kind gift of Dr. K. Brinck Wyeth Pharma). The group of control animals was treated with the drug solvent alone (N,N-Dimethylacetamid, Polysorbat 80, Polyethylenglycol 400 (in ratio 2:1:7)) at the same time points as the rapamycin treated mice. At the day of delivery the treated and the control animals were anesthetized and sacrificed. The mammary glands from treated and control animals were isolated and analyzed. Also the embryos of the mice were investigated. Rapamycin totally blocked the development of the embryos (Figure 3.15B). In comparison, the embryos of the control animals were fully developed (Figure 3.15A). Since abortion of pregnancy reduces the level of pregnancy hormones, which directly influence the development of the mammary gland, it was not possible to define the cause of the observed changes. They could be caused by the direct inhibition of proliferation and differentiation of mammary epithelial cells by rapamycin. Another possibility is that the reduction of mammary gland development is caused indirectly by the inhibition of embryonic development by rapamycin.

If the embryos are not able to develop, this results in a decreased level of lactogenic hormones which normally stimulate mammary gland development in treated mice. Intact mTOR signaling is required for embryonic development.

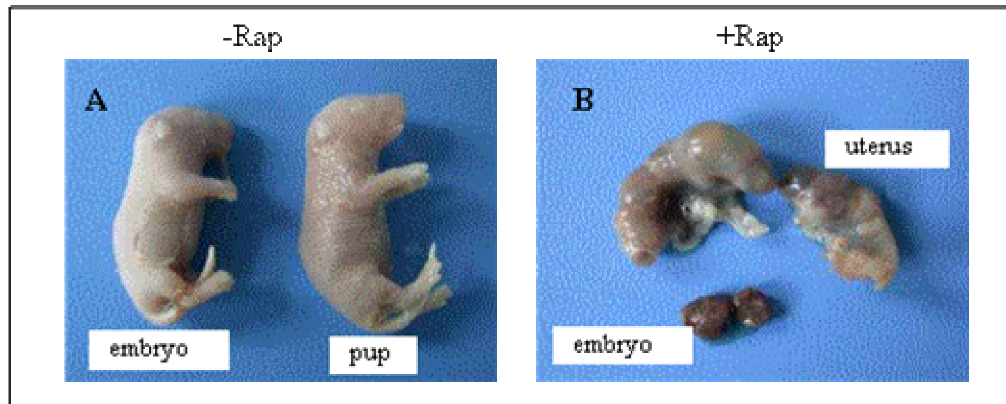

Figure 3.15 Treatment with rapamycin inhibits the development of mouse embryos. Pregnant mice were treated with rapamycin or drug solvent as a control from mid pregnancy (10 day postcoitum) up to the day of delivery. (A) Fully developed embryos of a mouse treated with placebo (left) in comparison with new born pups (right). (B) Uterus of rapamycin treated mice with undeveloped and dead embryos.

3.2.2 The role of mTOR during lactation

The experiments with HC11 cells indicate that intact mTOR signaling is required for the morphological differentiation of mammary epithelial cells. The untreated HC11 cells when seeded in collagen gel or martigel® formed three dimensional structures that resembled their glandular organization *in vivo*. This process was significantly inhibited by the addition of rapamycin in the culture medium. Furthermore, mTOR signaling was extremely important for the functional differentiation of mammary epithelial cells *in vitro*. Blocking mTOR activity with rapamycin during the induction of HC11 cells with lactogenic hormones caused a total inhibition of β-casein protein expression. Induction of the insulin signaling pathway is essential for lactation, and mTOR is one of the downstream targets of the insulin pathway. Therefore the role of the mTOR pathway during lactation of mammary epithelial cells was analyzed *in vivo*. Mammary epithelial cells reach their peak of functional differentiation during lactation where they are part of alveolar compartment secreting high amounts of milk proteins. To study the role of mTOR during this developmental stage, pregnant mice were treated with rapamycin or placebo starting from 19 days postcoitum until day 10 of lactation. During this 12 day treatment the development of the mammary gland of the mothers as well as the development of their new born pups was monitored.

3.2.2.1 The influence of rapamycin on the development of pups

New born mice are naked with closed ears and eyes. Pups start to eat solid food around 14-16 days later when their eyes become fully functional. During the first two weeks after birth they are fully dependent on the mother. The most important factor influencing growth of the pups is milk produced in the lactating mammary glands of the mother. The quality and quantity of milk has therefore a direct impact on the development of the growing pups. During the first 10 days after birth infant mice usually triple their weight. Therefore the growth curve of pups can be used as indicator for milk availability and quality. The weight of pups of mothers treated with inhibitor of mTOR and of mothers treated with drug solvent was determined daily. The measurement was continued up to 10 days after birth and the data were plotted into a graph (Figure 3.16). As can be seen, the pups from the mothers injected with rapamycin gain weight more slowly, when compared with pups of control mice treated with drug solvent. This indicates the possibility that differentiation and as a consequence milk expression is reduced in rapamycin treated glands.

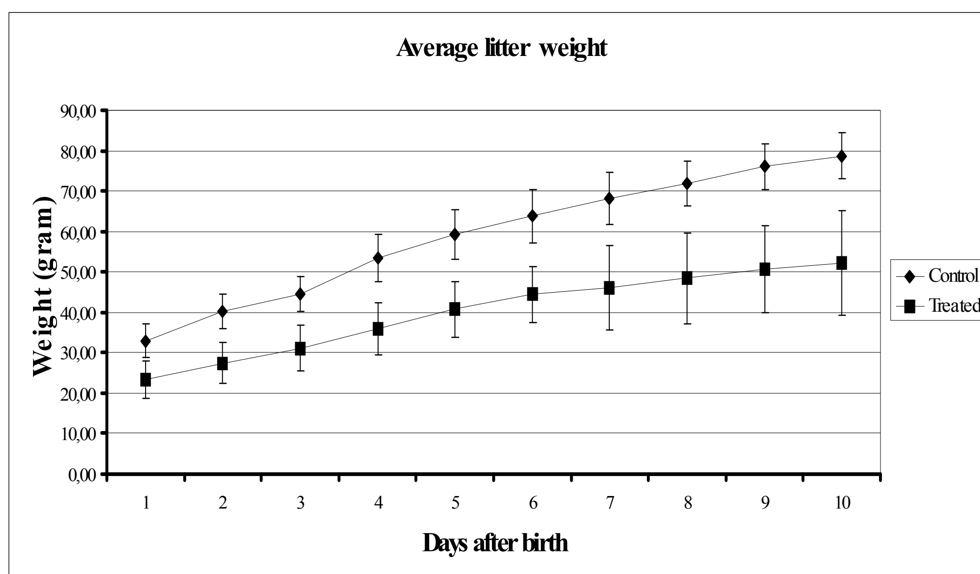

Figure 3.16 Inhibition on mTOR reduces the growth of new born mice. Mothers were injected daily with rapamycin (1.5 mg/kg i.p.) or placebo for 10 days after birth. The weight of the pups (12 pups/mother) of treated and control mothers was measured daily. The average litter weight is plotted in the graph.

3.2.2.2 Effect of mTOR inhibition on gland weight and milk production

During the first days of life the newborn pups are fully dependent on their mothers milk. The milk proteins and fatty acids are the only source of nutrition and are required for proper growth. Small changes in milk quality or quantity have a direct impact on development of the pups. The observed reduced growth of the pups of mothers treated with rapamycin might be caused by the drug inhibiting the terminal development of the mammary gland and milk production. Another possibility is that rapamycin is secreted with the milk influencing growth of the pups directly. To investigate this question the lactating mice were sacrificed after 10 days of lactation and the mammary glands were isolated. The first difference observed was the weight of the glands of control and treated mice. Mice treated with rapamycin had significantly flatter glands in comparison to non-treated mice indicating that there is either less milk production or less developed gland tissue when mTOR is inhibited. Their average weight was reduced from 1.5 gram to 0.6 gram upon rapamycin treatment (Figure 3.17).

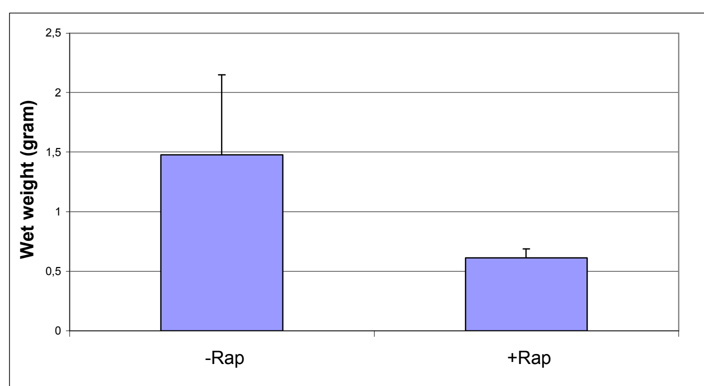

Figure 3.17 Rapamycin treatment reduced the wet-weight of the mammary gland *in vivo*. Mice were treated with rapamycin intra p. (1.5 mg/kg) or placebo up 10 days of lactation. Than the animals were anesthetized and sacrificed. The mammary glands number 4 were carefully isolated. The wet weight of treated and control glands was measured immediately after dissection. The obtained data are averaged in the diagram.

To investigate the idea that there is a reduction in milk protein synthesis influencing the body weight of the pups, the mammary glands of treated and non-treated animals were used for isolation of the milk and the concentration was measured. As can be seen in Figure 3.18 mice treated with the drug have significantly less milk proteins. The expression of milk proteins is reduced around 60% (Figure 3.18) and is comparable to the average decrease in mass of the treated pups (Figure 3.16).

These results indicate that the inhibited growth of the pups was at least partly due to a reduction in the amount of produced milk.

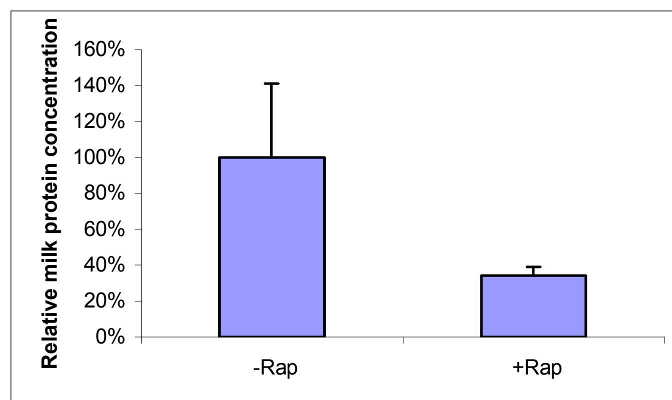

Figure 3.18 Milk protein concentration is lower in mice treated with Rapamycin Mice were injected with rapamycin or as a control placebo. Glands number 4 has been removed by surgery from mice and store in 60% EtOH for 48h. The obtained milk protein pellets were resuspended in RIPA buffer and the suspensions were spin again and protein concentrations were determined using Bradford solution for measurement.

3.2.2.3 Morphology of the mammary gland of rapamycin treated mice

During lactation, the mammary epithelial cells that contribute to alveoli, are terminally differentiated and secrete milk. The decrease in milk protein expression in treated animals could be due to a reduced development of the alveolar compartment since rapamycin clearly inhibited terminal differentiation of mammary epithelial cells *in vitro*. To test if rapamycin reduced the differentiation of alveolar cells *in vivo,* the histology of the control and rapamycin treated glands was studied. Initially, the isolated glands were investigated as whole mounts spread on a glass slide. As can be seen in Figure 3.19A the control glands are fully developed. They are bigger and the well-developed secretory units are visible. In comparison, glands isolated from rapamycin treated animals are visibly thinner and the alveolar compartment is clearly less developed. Between the alveolar units there was still a lot of stroma tissue compared with the control glands, where no stroma was observed in the alveolar compartment. To analyze the secretory compartment in more detail glands isolated from treated and control mice were cut into thin slices and stained with a combination of two dyes: hematoxylin and eosin. The gland slices were analyzed under the microscope. As can be seen in Figure 3.19B the alveolar compartment of the control was filled with milk. The alveoli are big and open and the epithelial cells form a thin layer around the alveolar space.

In comparison the alveolar compartment of the treated gland is clearly affected by rapamycin. The single alveoli were smaller and less extended indicating that the epithelial cells secreted less milk and do not completely fill the alveoli lumen.

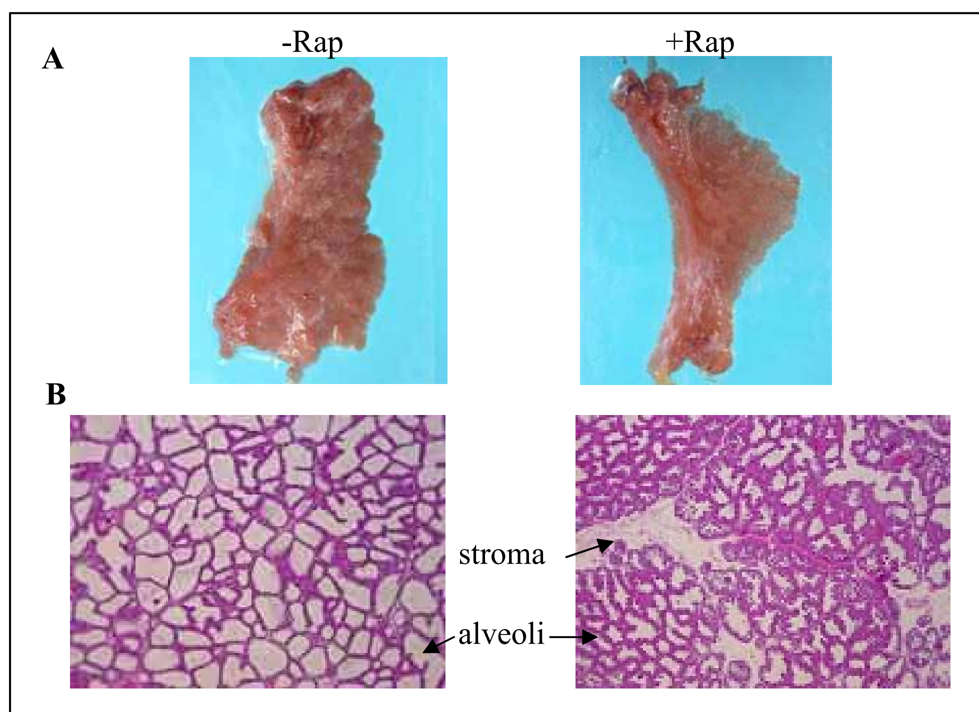

Figure 3.19 The mammary gland of mice treated with rapamycin are filled with less milk in comparison to the controls. Mice were treated with rapamycin or drug solvent up to the 10 days of lactation. (A) The whole mounts of isolated glands were photographed. (B) The isolated glands were fixed and cut into thin slices and stained with hematoxylin-eosin.

3.2.2.4 The effect of rapamycin on expression of Id2 and β-casein.

Inhibition of mTOR with rapamycin during differentiation of mammary epithelial cells *in vitro* leads to a block of β-casein expression and simultaneously reduces the level of Id2 protein which is involved in regulation of mammary gland development. Id2 has been shown to be expressed in parallel to β-casein *in vivo*. Both proteins accumulate during lactation (Parrinello et al., 2001). The reduced growth of pups was probably due to a reduction in milk synthesis. In rapamycin treated mothers the drug evidently affected proper differentiation of the alveolar compartment. To confirm that rapamycin inhibits the terminal differentiation of mammary epithelial cells in *vivo,* the β-casein expression (marker of functional differentiation) was investigated. The expression of β-casein *in vitro* was shown to be regulated by mTOR through Id2. It can be expected that the same mechanism plays a role *in vivo*. Thus, a reduction in expression of the β-casein should be accompanied by a reduction in the expression of Id2.

To confirm this hypothesis total RNA and proteins were extracted from isolated glands of rapamycin treated and control mice at 10 day of lactation. The RNA was reverse transcribed to cDNA, which was used as template in a Real-Time PCR reaction run with primers specific for β-casein or Id2. The obtained results were normalized against data from reactions run with primers for 18S ribosomal subunit and plotted into a diagram (Figure 3.20A). As expected the β-casein as well as Id2 mRNA levels were reduced by 3.5 fold and 4.2 fold, respectively, in the rapamycin treated glands. The western blot analysis (Figure 3.20B) confirmed the decrease in β-casein in treated animals which is accompanied by a reduced in expression of Id2.

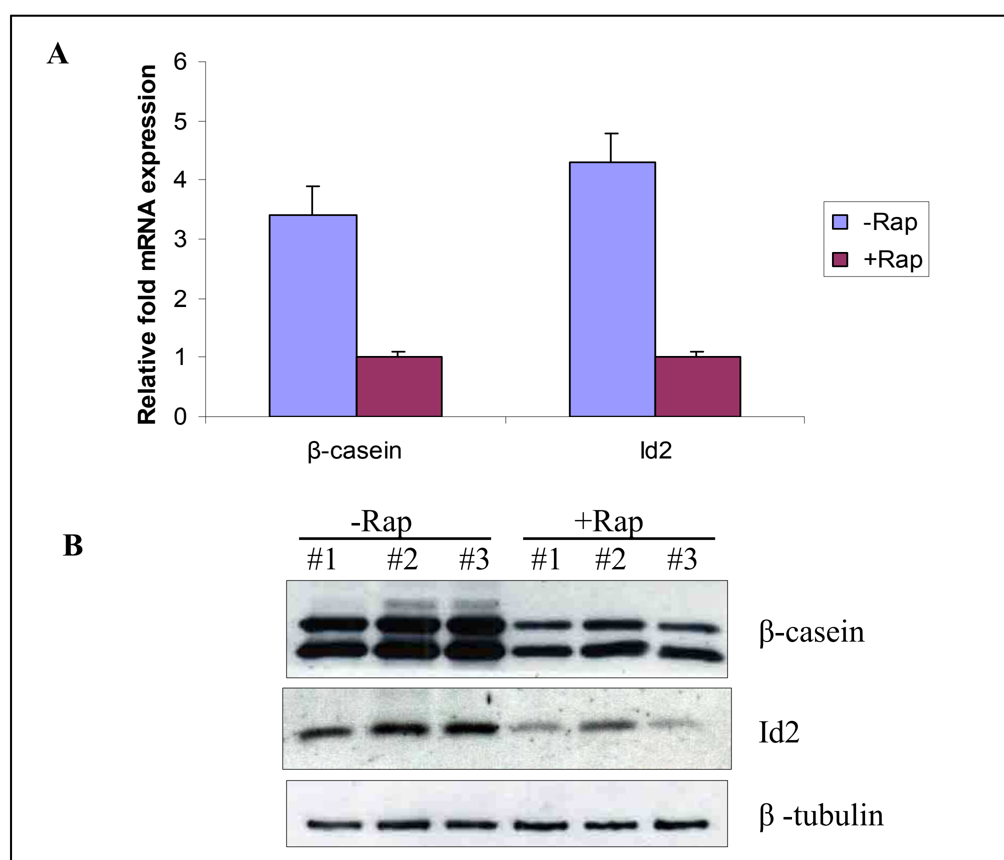

Figure 3.20 Rapamycin reduces expression of β-casein and Idb2 in mammary glands of mice during lactation (**A**) Total RNA was extracted from three glands at 10 day of lactation and reverse transcribed into cDNA. The PCR reactions were run with primers specific for β-casein or Idb2. Data were normalized against 18S ribosomal unit mRNA. (**B**) Total proteins isolated from 3 glands of treated (+Rap) and non-treated (-Rap) mice were western blotted with anti β-casein or Idb2. To control equal sample loading, the membrane was stripped and reprobed with an anti β-tubulin antibody.

Treatment of induced HC11 cells with rapamycin down-regulated the expression of Id2 on the mRNA and the protein level. This correlated with a decrease in milk protein β-casein expression (Figure 3.20). The in *vitro* experiments are thus in agreement with the results obtained *in vivo*.

3.3 The effect of rapamycin on mammary epithelial cells over-expressing Id proteins.

The *in vitro* and *in vivo* experiments indicate that treatment of mammary epithelial cells with rapamycin reduces the level of Id1 and Id2 proteins. The Ids inhibit DNA binding of bHLH transcription factors and were shown to be important regulators of proliferation and differentiation of mammary epithelial cells *in vitro*. Previously, it was proposed that mTOR regulates the growth and functional differentiation of the mammary epithelial cells through Idb proteins. To confirm this, the effect of rapamycin on HC11 cells over-expressing Id1 or Id2 was investigated. If there is a direct connection, over-expression of Id1 or Id2 would bypass the negative effect of rapamycin. To over-express the genes in HC11 cells, transient transfection can be used. However, several methods of transient transfection were tested and usually only low transfection efficiencies (10-15% positive cells) were obtained (see Figure 3.4.3). Therefore, a lentiviral vector system was used for the stable transduction of HC11 cells. The coding sequences of Id1 or Id2 were cloned in a lentiviral vector pSiEW, which is schematically shown in Figure 3.21. The long terminal repeat sequences (LTRs) are required for integration in the host genome. The SFFV promoter allows stable over-expression of the transgene and of EGFP. The internal ribosome entry sequence (IRES) allows that a single Id-EGFP-mRNA is translated into two proteins. EGFP, serves as marker for the identification of positively transduced cells and for a fast estimation of the transduction efficiency under a fluorescence microscope or by FACS analysis. The HC11 cells stably over-expressing Id1 or Id2 protein were treated with rapamycin and the effect on proliferation and differentiation was studied.

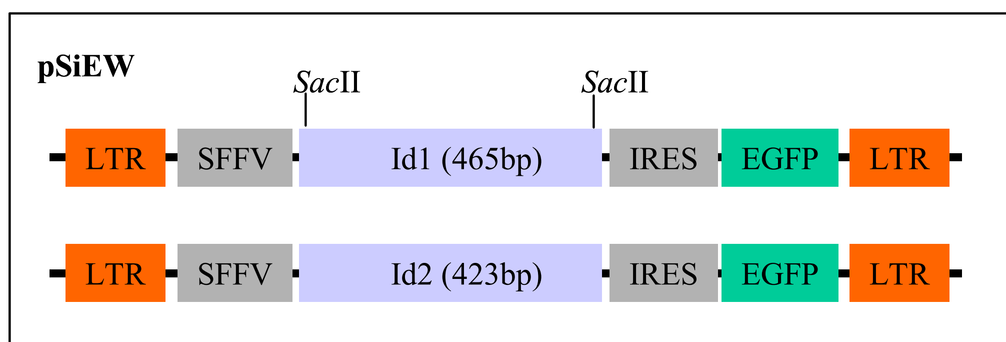

Figure 3.21 Schematic representation of the pSiEW vector. LTR (long-terminal repeat) sequences are required for target cell integration. SFFV (spleen focus forming virus) promoter allow for amplification of Idb proteins. IRES (internal ribosome entry sequence) allows for co-expression of Id1 (or Id2) and GFP from one mRNA. The EGFP (enhanced green fluorescence protein) serves as marker of transduced cells.

3.3.1 Preparation of plasmids for the production of lentiviruses

The plasmids pCMV-Sport6-Id1 or pCMV-Sport6-Id2 (obtained from RZPD, Germany), which contained the cDNA sequence of Id1 or Id2 respectively, were used as template in PCR reactions run with specially designed primer pairs. The primers contain restriction sites for *Sac* II and only allow the amplification of the protein coding region of Id1 or Id2 (Figure 3.22A). The amplified DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen). To obtain sticky ends the amplified fragments were cut with *Sac* II. The digestion products were again purified and cloned into pSiEW, which was also linearized with *Sac* II. After ligation the plasmids were transfected to TOP10 cells and to select the insert positive clones the isolated plasmid DNA samples were digested with *Sac* II. The positive clones showed a band of 465 bp (in cease of Id1 insert) or 423 bp (in cease of Id2 insert) in addition to 9.6 kbp vector band (Figure 3.22B).

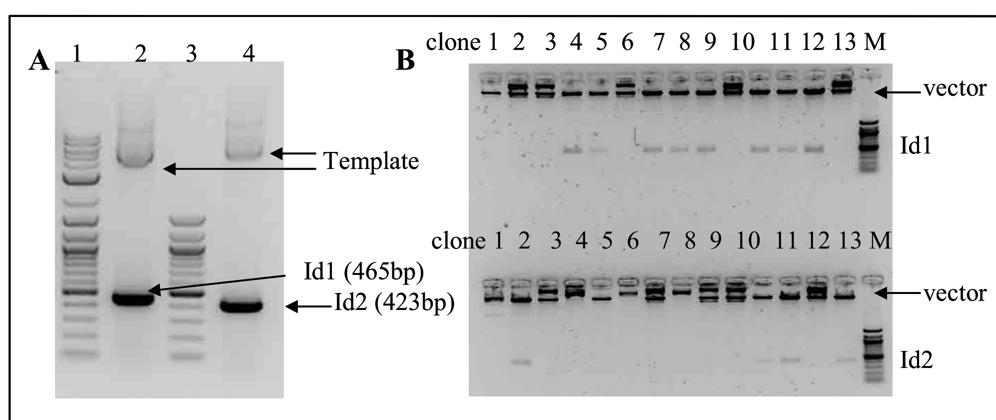

Figure 3.22 Cloning of Id1 and Id2 in the pSiEW lentiviral vector. (**A**) The Id1 and Id2 coding sequence was amplified in a PCR reaction. 1 kb marker (lane 1), Id1 PCR product (lane2), 100bp marker (lane 3) Id2 PCR product (lane 4). Thin bands show pCMVSport6 template. (**B**) Plasmids from several bacterial clones were isolated (mini prep) and digested with *Sac* II. Products of digestion were separated on a 1.2% agarose gel. The pSiEW clones containing Id1 or Id2 insert could be identified on the basis of presence of 465 bp (in cease of Id1) or 423 bp (in cease of Id2) digestion products. M–100 bp DNA ladder.

The pSiEW-Id1 and pSiEW-Id2 plasmids were purified in large scale (maxi preps). The obtained plasmid DNA was sequenced and the presence of the sequence coding of Id1 or Id2 was confirmed. The plasmids were also digested with *Xba* I and *Xho* I enzymes to verify the presence of the LTRs required for integration of the viral genome into the host genome. Both pSiEW-Id1 and pSiEW-Id2 vectors contained the LTRs. The prepared lentiviral plasmids were used for the transfections of 293T packaging cells to obtain virus particles.

3.3.2 Transfection of 293T cells for the generation of the lentiviral particles

To produce lentivirial particles, a cell line is used which is easy to cultivate (fast proliferation) and to transfect with high efficiency. The human cell line 293T fulfills these requirements and is commonly used for packaging of viral constructs. The 293T cells were transfected using the calcium phosphate method with the prepared lentiviral plasmids pSiEW-Id1 or pSiEW-Id2, as a control the empty pSiEW-plasmid was used. Additionally, the two packaging plasmids pMD2.VSVG and pCMVΔR8.91 were co-transfected. One day after transfection the 293T cells were analyzed under the fluorescence microscope. A high presentage of EGFP positive 293T cells was observed indicating efficient transfection of these cells (Figure 3.23). This is important to obtain a high virus titer in the medium.

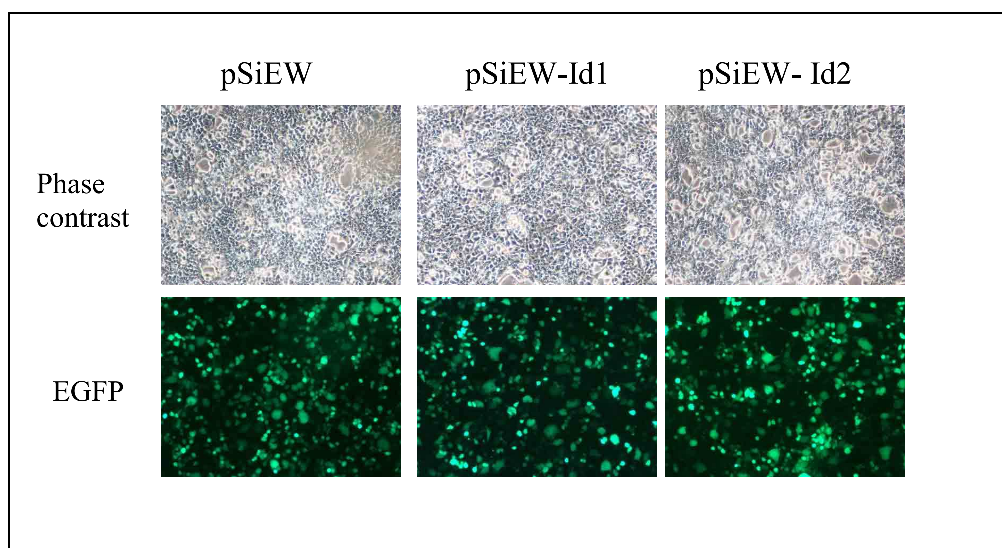

Figure 3.23 High efficiency of transfection of 293T cells with plasmids. The 293 cells were transfected with plasmids for production of lentiviruses. Cells were transfected with pSiEW-Id1 (middle) pSiEW-Id2 (right) or empty pSiEW plasmid (left) in combination with packaging plasmids. Green cells are the transfected cells expressing EGFP encoded on the pSiEW vector. If the packaging plasmids were co-transfected the cells will produce viral particles within 48h after transfection.

The culture medium of the transfected 293T containing the lentiviruses was collected every 24h for the next following 2 days. The viral titer in the medium was determined as described in Material and Methods (see 2.2.2.2). The lentiviruses were subsequently used for stable transduction of the mammary epithelial cell line HC11.

3.3.3 Viral transduction of HC11 cells and over-expression of Idb proteins

It has been shown that the HC11 cells are hard to transfect using many different transfection methods (see chapter 3.4.3). To improve this, the HC11 cells were transduced with the prepared lentiviral particles. Generally, MOI 5 or 10 was used for this experiments. The efficiency of infection was checked the next day under the fluorescence microscope. EGFP served as a marker for positively transduced mammary epithelial cells (Figure 3.24). The transduction with lentiviral particles resulted in a high percentage (95-97%) of positive cells.

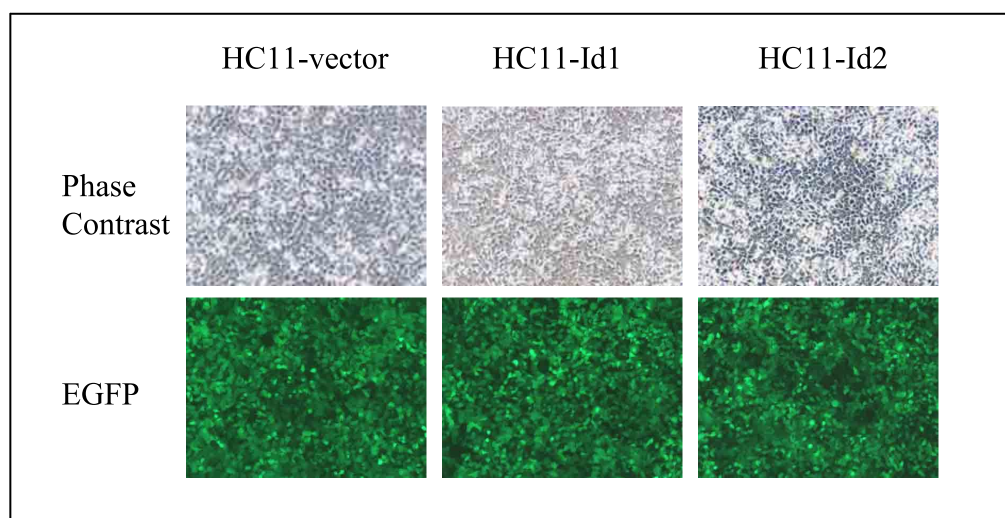

Figure 3.24 Transduction of HC11 with lentiviral particles. HC11 cells were seeded in a 6-well plate and transduced with lentiviral vectors using an MOI 5 or 10. The efficiency of infection with virus was determined under the fluorescence microscope by estimating the presentage of EGFP. Green staining of cells correlate with over-expression of Id1 or Id2.

To confirm that the lentiviral genome was inserted into the host genome leading to the over-expression of Id1 or Id2 and not only to the expression of EGFP a western blot analysis was performed. The HC11 cells transduced with lentiviral particles SiEW-Id1 (HC11-Id1), or SiEW-Id2 (HC11-Id2), or empty SiEW (HC11-vector) were seeded in 10 cm dishes and cultivated in growth medium for 2 days. Also non-transduced HC11 cells were used as a control. Total proteins extracts were isolated and a western blot with antibodies specific for Id1 or Id2 was performed.

As shown, (Figure 3.25) the HC11 cells transduced with lentiviral particles SiEW-Id1 (lane 1) or SiEW-Id2 (lane 2) over-expressed either Id1 or Id2 protein when compared with the level of Id proteins in the controls (lanes 3 and 4).

The HC11 cells transduced with empty lentiviral vector over-expressing only EGFP (lane 3) and the non-transduced HC11 cells (lane 4) had the same level of Id1 and Id2 expression indicating that insertion of the lentiviral construct or EGFP-expression did not influence the expression of the Id endogenous-proteins. The HC11 cells stably over-expressing Id1 or Id2 proteins were used in functional assays described below.

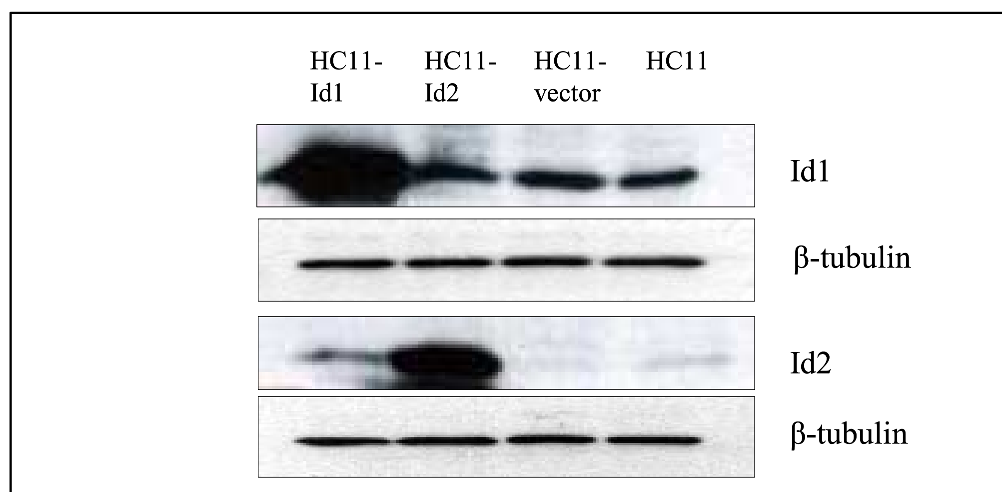

Figure 3.25 The HC11 cells infected with lentiviral vectors stably express Idb proteins. HC11 cells positive for EGFP were seeded in 10 cm dishes and cultivated in growth medium for 48h. Total proteins extracts were isolated and western immuno-blotted with an antibody against Id1 or Id2 protein. To verify the equal loading of the samples the membranes were reprobed with an anti β-tubulin antibody.

3.3.4 The effect of Id1 over-expression on proliferation and differentiation of HC11 cells

As discussed in the introduction, Id1 plays a key role in the proliferation of epithelial cells in culture as well as in *vivo* (Chapter 1.4). The Id1 protein level is high during puberty when cells extensively divide to build ducts as well as during early pregnancy when cells proliferate to form the secretory compartment. Inhibition of the mTOR kinase by treatment with rapamycin was shown in the previous chapter (3.1) to inhibit the proliferation and also to decrease the expression of Id1. Therefore, it was interesting to investigate if the effect of rapamycin on proliferation depends on the down-regulation of Id1.

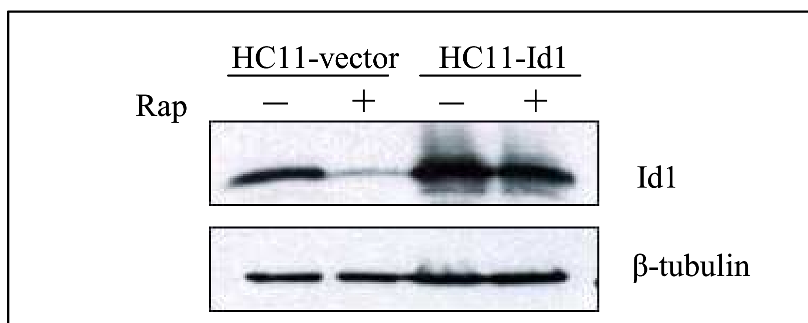

Figure 3.26 Lentiviral over-expression of Id1 in HC11-Id1 cells is insensitive to the down-regulating effect of rapamycin. The HC11 cells transduced with lentiviral particles SiEW-Id1 (HC11-Id1) or with empty lentiviral SiEW vector (HC11-vector) were grown in presence or absence of rapamycin (10 nM). After 24 h incubation total proteins were isolated and the obtained extracts were western blotted with an anti Idb1 antibody. To confirm equal loading of the samples the membrane was reprobed with an anti β-tubulin antibody.

The initial experiment where growing HC11-vector and HC11-Id1 cells were treated with rapamycin or DMSO showed (Figure 3.26) that the HC11-Id1 cells expressed high levels of Id1 protein and were resistant against the down-regulating effect of rapamycin on Id1 protein expression. To test the effect of this rapamycin insensivity on proliferation the HC11-Id1 cells stably over-expressing Id1 protein were seeded in a 96-well titration plate and were treated with rapamycin or DMSO for 96 hours. As a control, HC11-vector cells transduced with empty vector and expressing only EGFP were grown under the same conditions. The number of living cells was estimated every 24 h by using the XTT proliferation assay (Roche Molecular Biochemicals). The obtained data were averaged and are presented as growth curves (Figure 3.27).

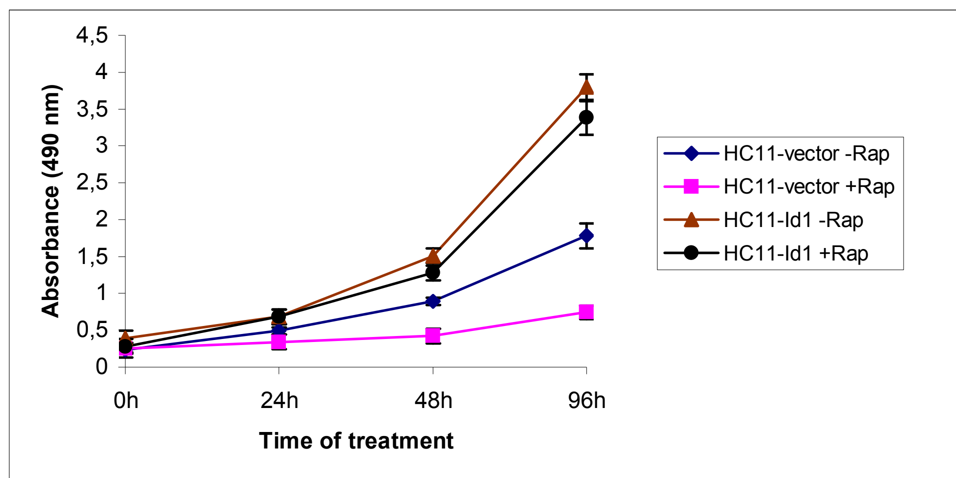

Figure 3.27 Rapamycin does not inhibit the proliferation of HC11 cells over-expressing Idb1. The HC11-Id1 cells were seeded per well of 96 well micro titration plate and cultivated in 100 µl of growing medium in presence or absence of rapamycin (10nM) for 96 hours. The control cells HC11-vector transduced with empty lentiviral vectors were incubated under the same condition. The number of living cells was counted every 24h at indicated time points of rapamycin treatment with help of XTT proliferation assay.

As shown (Figure 3.27) the growth of the HC11 cells treated with rapamycin was clearly affected compared with the DMSO (-Rap) treated cells. The HC11 cells over-expressing Id1 were proliferating much faster compared to the control cells. The growth of HC11-Id1 was not influenced by the inhibitory effect of rapamycin. These data confirm that Id1 controls the proliferation potential of HC11 cells and indicate that mTOR regulates the proliferation of mammary epithelial cells by influencing the expression level of Id1.

It was also interesting to investigate how over expression of Id1 influences the differentiation of the HC11 cells. Normally Id1 protein is expressed at high level in proliferating cells and is down-regulated during confluency. That is a prerequisite for differentiation. Interestingly, western blot analysis (Figure 3.28) of HC11 cells induced for differentiation with DIP showed that over-expression of Id1 prevents the expression of Id2 as well as the induction of β-casein by lactogenic hormones. This result indicate that Id1 is responsible for holding the cells in a proliferation stage and prevents pre-mature differentiation by blocking the proteins involved in activation of differentiation including Id2.

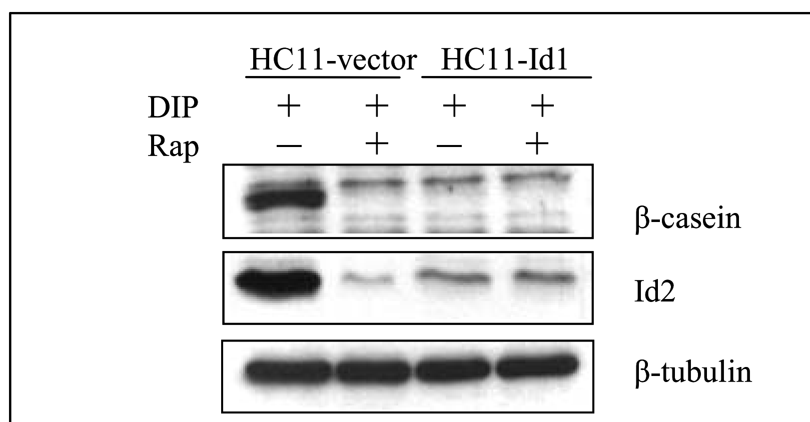

Figure 3.28 Over-expression of Id1 in HC11 cells prevents the expression of Id2 and β-casein after stimulation with DIP. The HC11-Id1 and HC11-vector cells were induced for differentiation with DIP in presence or absence of rapamycin (10 nM). After 4 days of incubation the proteins were isolated and the obtained protein extracts were western blotted with an anti Id2 antibody as well as β-casein antibody. To confirm the equal loading of the samples the membrane was stripped and reprobed with an anti β-tubulin antibody.

3.3.5 Branching of HC11 cells over-expressing Id1

HC11 cells seeded in a three-dimensional environment are able to form structures that resemble ducts of the mammary gland. Treatment with rapamycin inhibits the development of duct-like structure in collagen and matrigel (Figure 3.5). A similar effect was observed in the case of HC11 cells expressing siRNA silencing the Id1-expression (Figure 3.38). Rapamycin was shown to down-regulate the Id1 protein level and as a consequence cell proliferation. It was interesting to test, if the HC11 cells over-expressing Id1, in which growth is insensitive to the inhibitory effect of rapamycin, are also able to differentiate morphologically in 3D culture when treated with the drug.

The HC11-Id1 over-expressing Id1 protein and the HC11-vector cells transduced with empty lentiviral vector were seeded in collagen gel and cultured in the presence of rapamycin or DMSO. The expression of EGFP by HC11-Id1 and HC11-vector allowed the detection of the duct like structures in the gels analyzed under the fluorescence microscope (Figure 3.29).

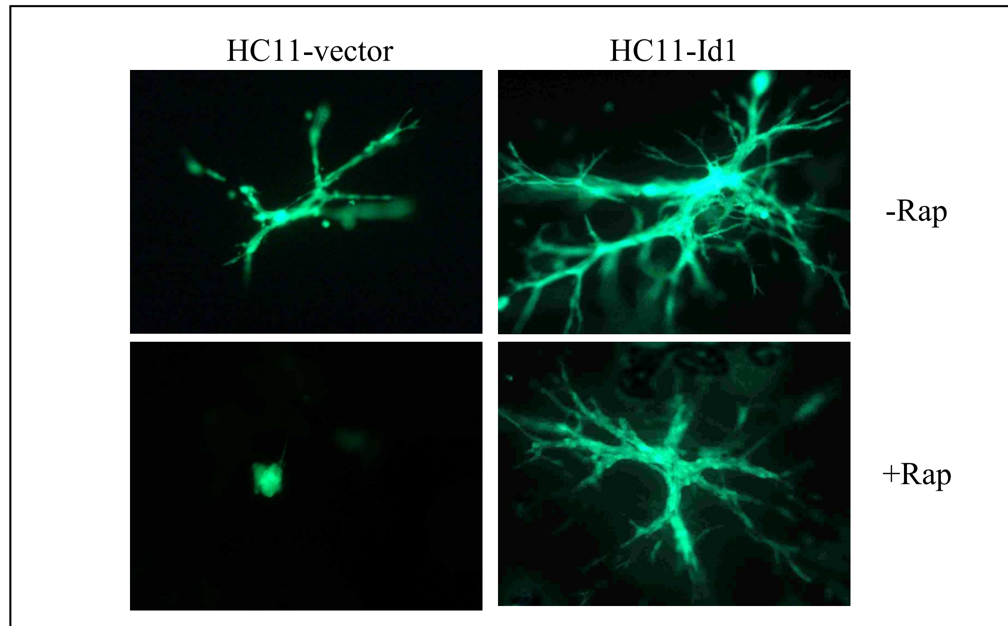

Figure 3.29 Branching morphogenesis is not blocked by rapamycin in HC11 over-expressing Id1. Around 2000 HC11-Id1 or HC11-vector cells were seeded within 100 µl of collagen gel and cultivated in presence of rapamycin (10 nM) or DMSO as a control. The medium was replaced every day. After 4 days the cells were analyzed under the fluorescence microscope. Because the cells also expressed GFP due to the lentiviral transduction the green branch-like structures can be observed.

Without addition of rapamycin the HC11-Id1 over-expressing Id1 protein formed tree-like structures already after 4 days of stimulation. Furthermore, the branches were far more developed in comparison to control HC11-vector transduced only with empty vector. Normal HC11 cells needed around 8-10 days to form such structures. This accelerated growth was not surprising, since we already observed that the HC11-Id1 cells proliferate faster than the control cells (Figure 3.27). More importantly, the treatment with rapamycin did not block the morphological differentiation of HC11 cells over-expressing Id1. This was in clear contrast to the control cells, which proliferate only weakly and could not invade the surrounding gels. These results strongly indicate that mTOR controls morphological differentiation of mammary epithelial cells in the 3D cultures through the regulation of Id1. This is in agreement with previously published data showing that Id1 directly regulates proliferation and the invasive potential of mammary epithelial cells.

3.3.6 Effect of Rapamycin on the functional differentiation of HC11 over-expressing Id2

Not only Id1, but also Id2 was shown to play an important role in the development of the mammary gland. Knock-down of Id2 caused a block of functional differentiation of epithelial cells and a reduction of milk protein expression (Mori et al., 2000). HC11-Id2 cells over-expressing Id2 and HC11-vector cells were seeded in 10 cm plates and cultivated in growth medium for 2 days. During the next 2 days the cells were brought into the competent stage in starvation medium and finally induced for differentiation with lactogenic hormones (DIP) in the presence of rapamycin or DMSO. After 4 days of stimulation the total proteins were extracted with RIPA buffer and western immuno-blotted with an antibody specific for β-casein. Detection of the milk protein serves as a marker for functionally differentiated mammary epithelial cells. To verify equal loading of the samples the membrane was stripped and reprobed with an anti β-tubulin antibody.

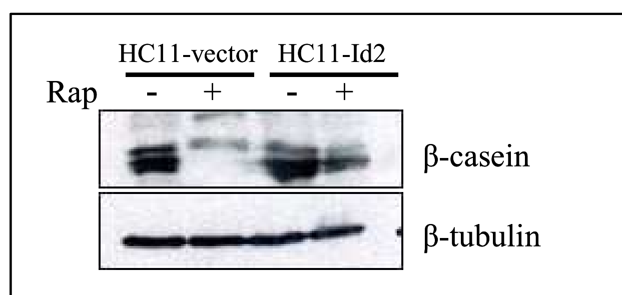

Figure 3.30 Rapamycin has no effect on the terminal differentiation of HC11 over-expressing Id2. HC11-Id2 and HC11-vector cells were seeded in 10 cm dishes grown and induced for differentiation with DIP in presence of rapamycin or DMSO as control. After 4 days of stimulation with lactogenic hormones total proteins were isolated with RIPA buffer and western blotted with an antibody against β-casein. To confirm the even loading of the samples the membrane was stripped and reprobed with anti β-casein antibody.

The western blot analysis (Figure 3.30) showed that HC11-vector cells transduced with empty lentiviral vector and treated with rapamycin are not expressing milk protein β-casein (lane 2) indicating that they are not terminally differentiated in contrast to the control cells treated with DMSO (lane 1). The HC11-Id2 cells over-expressing Id2 were still able to differentiate under treatment of rapamycin as indicated by the presence of milk protein β-casein (lane 4). These experiments together with the previous results provide evidence that mTOR influences terminal differentiation of mammary epithelial cells through the regulation of Id2 expression.

3.4 Identification of genes involved in proliferation and differentiation of mouse mammary epithelial cells

During mammary gland development several hormones are able to activate signaling pathways, which in turn activate transcription of hundreds of genes involved in the formation of a functional gland. Although some important signaling pathways and their activators have been studied in detail, little is known about the regulation of the various target genes during mammary epithelial cell proliferation and differentiation. Development of the micro-array technology has provided a novel tool to study the transcription profile of thousands of genes in one experiment. In this work this method was used to identify genes differently expressed during proliferation and differentiation of HC11 cells. Another aim of this work was to establish a fast and easy *in vitro* system that allows testing the role of the identified candidate genes in proliferation and differentiation of the mammary gland.

3.4.1 Micro-array analysis of gene expression during differentiation of mammary epithelial cells

To identify genes that are regulated during proliferation and differentiation of mammary epithelial cells, the HC11 cell line was used as a model. The HC11 were originally isolated from mid pregnant mice. This cell line is able to proliferate well *in vitro* and can be induced for differentiation. The addition of the lactogenic hormones dexamethasone, insulin and prolactin (DIP) in the medium leads to the expression of the milk protein β-casein which is used as indicator for functional differentiation of the mammary epithelial cells.

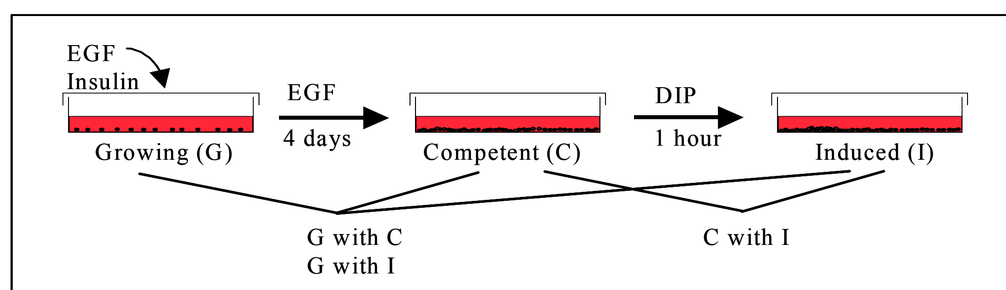

Figure 3.31 The **gene expression pattern in three different stages of HC11 cells was compared.** The HC11 cells can be grown in plastic dishes. They proliferate until they reach confluency and can be brought to competency and than induced with lactogenic hormone mix (DIP) for terminal differentiation. The gene expression patterns of the three stages of culture were compared to each other: growing (proliferating) cells were compared to competent cells (G to C), growing cells were compared with cells induced for 1h with lactogenic hormones (G to C), competent cells were compared with cells induced for 1h with lactogenic hormones. This allowed for identification of genes involved in proliferation and/or early stages of differentiation.

To compare the gene expression pattern between the differentiated and non-differentiated (proliferating) stage the HC11 cells were grown until they reached the competent stage and were than induced for differentiation (Figure 3.31). Total RNA was isolated from three different stages of cultures of growing cells which proliferate extensively, from competent cells, which are not able to divide because the plates are confluent, and from cells stimulated for 1h with lactogenic hormones (DIP). This allowed the identification of genes that are expressed at high level in proliferating cells as well genes specifically activated during early stages of mammary epithelial cell differentiation. The isolated RNA was reverse transcribed to cDNA. Micro-array analyse was performed in cooperation with the lab of Dr. Peter Angel (German Cancer Research Center, Heidelberg). They hybridized the obtained cDNAs with the NIA-Chip, which is a PCR-based cDNA micro-array containing 15,000 unique genes (78% are novel, 22% are known). The selected genes are known to be expressed during mouse embryonic development (Tanaka et al., 2000). We could initially detect expression of around 3000 genes in our HC11 samples**.** We have identified around 1800 replicates after the color switch (60% of the total detected genes). We identified false positives (7%**)** which showed an inconsistent regulation pattern after the color switch. Around 397 proliferation associated and 639 differentiation associated genes were initially selected as candidates involved in these processes. Several genes, found to be regulated during proliferation and differentiation of HC11 cells, were picked and analyzed in this work in more detail.

3.4.2 Confirmation of micro-array data by Real-Time-PCR analysis.

The expression patterns of target genes obtained from the micro-array analysis were verified by PCR. By reverse transcription of mRNA of HC11 cells in combination with Real-Time PCR it is possible to verify regulation of the mRNA level for particular gene in the investigated samples. Therefore the HC11 cells were grown to reach competency and were induced with lactogenic hormones (DIP) for one hour. The total RNA was isolated from growing (proliferating), competent (confluent plate) and induced HC11 cells. The extracted RNA samples were reveres-transcribed ito cDNA. The obtained cDNAs were use as templates for in Real-Time PCR reactions run with primers specific for the selected candidate genes. An example of the data obtained in a Real-Time PCR reaction performed with primers specific for Id2 gene is shown in chapter 2.2.1.15.

All data obtained form Real-Time PCR reactions was analyzed as in the Id2 example. The fold difference in gene expression levels, between growing, competent and induced cells were calculated as ∆Ct values and were compared with micro-array results (Table 3.1). Only genes, which expression pattern was confirmed were considered for further analysis in functional assays.

Gene Name	Type of analysis	Growing vs. competent (ΔCt value)	Competent vs. Induced (ΔCt value)	Growing vs. Induced (ΔCt value)
Idb1	M-A-Data	2.6	-3.9	-3.1
	Real-Time-PCR	3,1	1.1	4.2
Idb2	M-A-Data	-2.6	-0.4	-3.2
	PCR-PCR	-2.5	-1.6	-4.1
Cited4	M-A-Data	-2.8	-1.5	-
	RealTime-PCR	-1.7	-0.4	-2.3
Nedd8	M-A-Data	-0.9	-0.4	-0.5
	RealTime-PCR	0.3	-0.1	0.2
Ltf	M-A-Data	-2.1	-1.0	-1.0
	RealTime-PCR	-6.6	-1	-7.6
Atp5l	M-A-Data	-1.9	-1.2	-1.6
	RealTime-PCR	-0.1	-0.2	-0.3
Nkd2	M-A-Data	-2.2	0.6	-1.1
	RealTime-PCR	-2.6	-1.1	-3.7
Hdac2	M-A-Data	-	1.6	-
	RealTime-PCR	1.9	-0.5	1.6
Sap30	M-A-Data	-	1.4	-
	RealTime-PCR	-0.8	-1.7	-2.5
Esg1	M-A-Data	-	2.1	-
	RealTime-PCR	-0.8	1.6	0.8
Rae1	M-A-Data	1.8	2.2	1.5
	RealTime-PCR	-0.6	-0.4	-1
Nap1l1	M-A-Data	1.5	2.1	1.5
	RealTime-PCR	1.5	-0.3	1.2
Hsp105	M-A-Data	2.8	-	1.9
	RealTime-PCR	1.8	0.3	2.1
Pdlim1	M-A-Data	2.8	-	1.8
	RealTime-PCR	-2.2	0.1	-2.1
Nol5	M-A-Data	1.5	0.8	2.5
	RealTime-PCR	3.3	-0.4	2.9
Biklk	M-A-Data	1.2	0.5	2.3
	RealTime-PCR	-4.8	-1.1	-5.9
Kpna2	M-A-Data	1.4	0.9	1.6
	RealTime-PCR	3.6	0.3	3.4

Table 3.1 Comparison of results obtained from micro-array and Real Time-PCR analysis. The data obtained from micro array and PCR were plotted as delta Ct values of compared stages of HC11 cell differentiation (Growing, Competent, Induced). The Relative fold induction of target gene can be calculated according to formula $2^{\Delta Ct}$ where delta Ct is difference in the Ct (threshold amplification cycle) between two compared stages ($\Delta Ct = Ct_1 - Ct_2$).

3.4.3 Silencing of candidate genes by RNA interference

Over-expression or down-regulation of candidate genes can be used to investigate the role of these genes during differentiation of mammary epithelial cells. The siRNA technology has become a potent tool in functional genomics to silence the expression of genes of interest. There are several methods to introduce vectors leading to expression of siRNA. Transient transfection and stable transduction are widely used. Before the effect of siRNA on the proliferation and differentiation of HC11 can be analyzed, the newly designed siRNA has to be tested for its silencing potential. Therefore transient tansfection was used, since this is an easy and fast method for validation of the siRNA silencing power. To select the most efficient transfection method for mammary epithelial cells, several different reagents were tested for transfection of HC11 cells with pSuper vectors containing siRNAs. The results are shown in Figure 3.32.

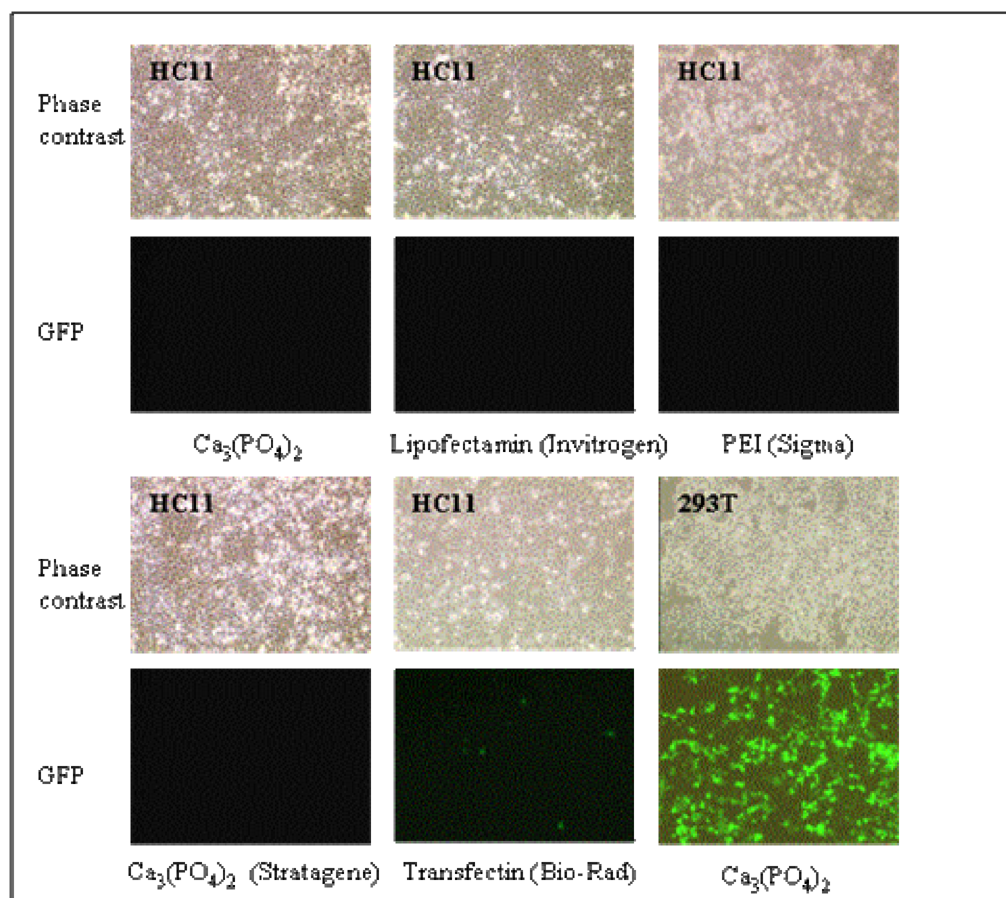

Figure 3.32 Mammary epithelial cell HC11 are difficult to transfect. Mammary epithelial cells HC11 were transfected using different methods and kits for transient transfection. As control the 293T cells were transfected using calcium phosphate. GFP-Green Fluorescence Protein is used as marker for transfected cells.

All transient transfection methods tested resulted in a very low transfection efficiency (5-15%) indicated by the low fraction of cells expressing GFP. The experiment showed that HC11 cells are not efficiently transfected compared to 293T cells although different reagents and protocols were used. Therefore we decided to use the lentiviral vector system, which requires more time and work, but gives high transduction efficiencies (95-100%). Moreover, lentiviral transduction results in stable expression of siRNA, which is required for subsequent functional analysis.

3.4.3.1 Preparation of lentiviral vectors with siRNA targeting Id1 and Id2 mRNA

To optimize the siRNA silencing system, it was important to analyze siRNA knock down on a gene, whose function is known. In this way it is possible to compare the obtained phenotype with previous results. As shown in this work the Id1 and Id2 proteins are essential for proper development of mammary gland as well as for proliferation and differentiation of mammary epithelial cells *in vitro*. They were also detected in our micro-array analysis and their differential expression pattern during proliferation and differentiation of HC11 was confirmed by Real Time PCR analysis. Therefore, the Id proteins were chosen as a model for the establishment of the siRNA knock-down technique. The siRNA sequences for silencing the expression of Ids were delivered in pSuper vectors downstream of a H1 promoter. The H1 promoter and siRNA were than cloned into lentiviral vector pSEW. The final vector is schematically presented in Figure 3.33.

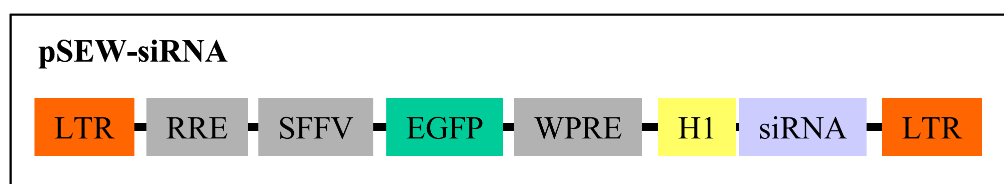

Figure 3.33 Schematic presentation of pSEW vector containing the cloned H1 promoter and siRNA sequences. The siRNA sequence with H1 promoter was subcloned from a pSuper plasmid and was inserted between LTR (long terminal repeat)of lentiviral plasmid pSEW. RRE (Rev-responsive element) as nuclear export and encapsidation permissive element. SFFV (spleen focus forming virus) promoter for stable expression of GFP. WPRE (woodchuck hepatitis virus posttranscription response element) enhancer for transgene expression in target cells. EGFP (enhanced green fluorescence protein). H1 (inducible Pol III) promoter, allowing expression of si (short interfering) RNA.

The LTR allows the stable insertion of the construct into the host genome. The siRNA sequence is under the control of the H1 promoter required for stable expression.

The expression of GFP is controlled separately by the SFFV promoter, and is used for identification of positively transduced cells and fast estimation of transduction efficiency using fluorescence microscopy or FACS analysis.
For silencing the Id1 gene, three siRNA sequences were selected: A115, B131, C152. For silencing Id2 gene, two siRNA sequences were selected 7 and 9. The pSuper vectors containing siRNA were used to transform TOP10 cells. On the next day, several colonies were picked up from plates and used to perform Mini Prep isolation of plasmid DNA (NucleoSpin® Macherey-Nagel). To select the siRNA insert positive clones, the isolated pSuper plasmid samples were digested with *Bgl* II and *EcoRI* enzymes. The *Bgl* II restriction site is eliminated after insertion of siRNA into pSuper. The digestion of positive clones gives only one band while digestion of negative clones yields two bands after digestion and resolution on the 1.2% agarose gel. The selected pSuper siRNA insert positive vectors were sequenced. The bacterial clones containing pSuper vectors with the proper siRNA sequences were used for the preparation of Maxi Preps of plasmid DNA (Nucleobond® Macherey-Nagel). The fragments containing the H1 promoter and the siRNA sequence were cut from the pSuper plasmids with *Sma* I and *Hinc* II (blunt ends). Products of digestion were solved on 1.2% agarose gels. The expected 300 bp fragment containing H1 promoter and siRNA sequence could be recognized and was cut out from the gel with a sterile scalpel and than purified. (QIAquick, Qiagen). The pSEW vector was digested with *Sna BI.* To increase the insertion efficiency of ligated fragment and to prevent the circularisation of open pSEW plazmid, the blunt ends of the vector obtained after digestion reaction were dephosphorylated. After purification the open pSEW vector were ligated with isolated 300 bp fragment (H1 promoter+siRNA). The ligation mix was used for transformation of TOP10 cells. After overnight incubation, several bacterial clones were used for mini plazmid isolation. The positive clones were selected in digestion step with *EcoRI* enzyme. The insert positive vectors gave 2 kb band after digestion reaction products resolution on the 1.2% agarose gel. The positive clones with the proper orientated insert H1 promoter -siRNA were used for Maxi Prep preparation. The obtained vectors were than sequenced and the correct insertion of siRNA sequences in pSEW was verified. The prepared vector pSEW-A115, pSEW-B131, pSEW-C152, pSEW-7, pSEW-9 were used for transfection of 293T packaging cells together with packaging vectors pMD2.G and pCMVRΔ8.91.

3.4.3.2 Transfection of packaging cells with lentiviral vectors

For expression of lentiviral particles, human 293T cells are commonly used. cell. The 293T cells were seeded in 10 cm plate and transfected using calcium phosphate as described in material and methods, with the lentiviral plasmids pSEW-siRNA-Id1 or pSEW-siRNA-Id2 or as a control with an empty lentiviral plasmid (pSEW without the H1-promoter-siRNA insert). Two additional plasmids pMD2.G and pCMVRΔ8.91 were co-transfected for expression of viral proteins required for virus replication and packaging. The next day the transfected cells were examined under the fluorescence microscope. A high amount of GFP expressing 293T cells were detected (Figure 3.34). The transfection efficiency was estimated to be 60-80%.

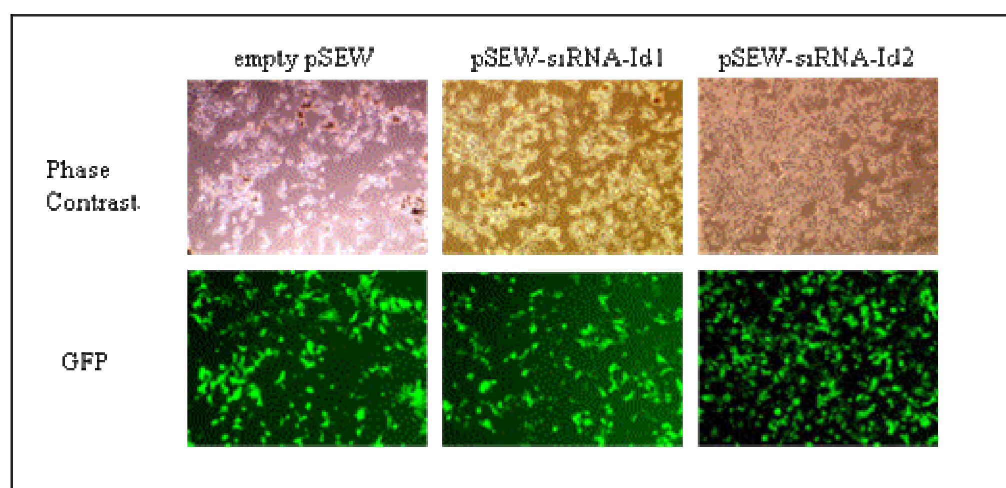

Figure 3.34 Transfection of 293T cells with vectors for production of lentiviral particles. The 293T were seeded in 10 cm plates and transfected with lentiviral plasmids using the calcium phosphate method. The GFP indicates cells expressing viral proteins. 293T cells transfected with pSEW-siRNA-Id1-A115 construct (middle). 293T cells transfected with pSEW-siRNA-Id2-7 construct (right). 293T cells transfected with empty pSEW plasmid (left). The cells were also co-transfected with evenlope plasmid pMD2.G and packaging plasmid pCMVRΔ8.9.

The transfection medium was changed to fresh medium and after 16 h the medium was aspirated and stored after filtration. The medium containing lentiviral particles was used for stable transduction of the mammary epithelial cells in the following experiments.

3.4.3.3 Stable transduction of HC11 cells with lentiviral vectors

To investigate the role Id proteins during proliferation and differentiation of the mammary epithelium, the HC11 cells were transduced with lentiviral particles to obtain stable expression of different siRNA sequences targeting Id1 and Id2 proteins. As a control the cells were transduced with empty vector to monitor the influence of transfection procedures and expression of the green fluorescent protein on normal HC11 proliferation, morphology and differentiation. The efficiency of transduction was investigated 24 hours after transduction under the fluorescence microscope. As can be seen, almost all cells expressed GFP indicating high transduction efficiency (Figure 3.35). The high efficiency of viral transduction allowed to perform functional analyses using almost a homogenous HC11 population stably expressing siRNA targeting Id1 or Id2.

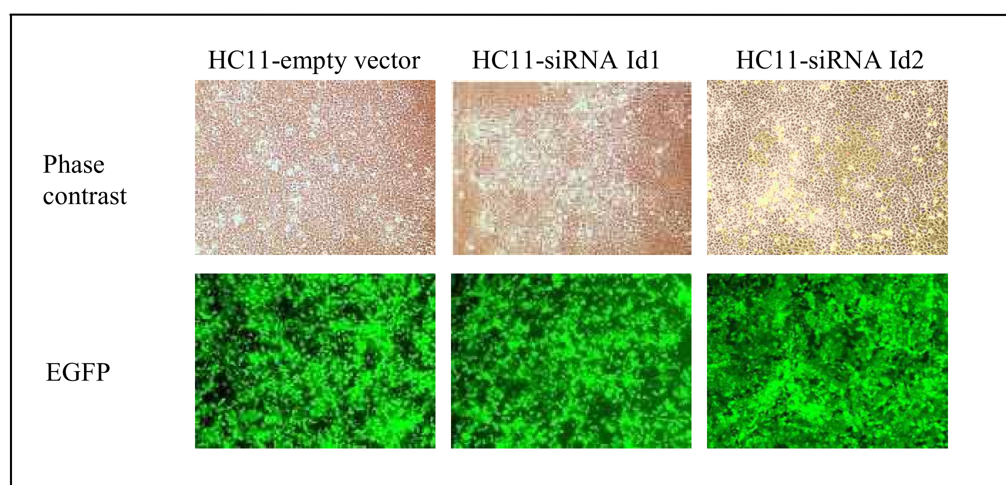

Figure 3.35 High lentiviral transduction efficiency of mammary epithelial cells. The HC11 cells were transduced with lentiviral particles containing the siRNA targeting Id1 or Id2. The control cells were transduced with empty lentiviral vectors. GFP-green fluorescence protein was used as a marker for viral uptake. The viral genome integrates in the host genome ensuring stable expression of siRNA.

The silencing potency of the cloned siRNA sequences was investigated before functional experiments were performed. The total RNA and proteins were isolated form HC11 cells transduced with lentiviral vectors, stably expressing siRNA. The extracted RNA was reverse transcribed into cDNA. The obtained cDNA samples were used as templates in Real-Time PCR reactions, run with primers amplifying a specific sequence of Id1 or Id2. To verify equal loading of the samples in the reaction the obtained data were normalized using primers amplifying the 18S ribosomal subunit.

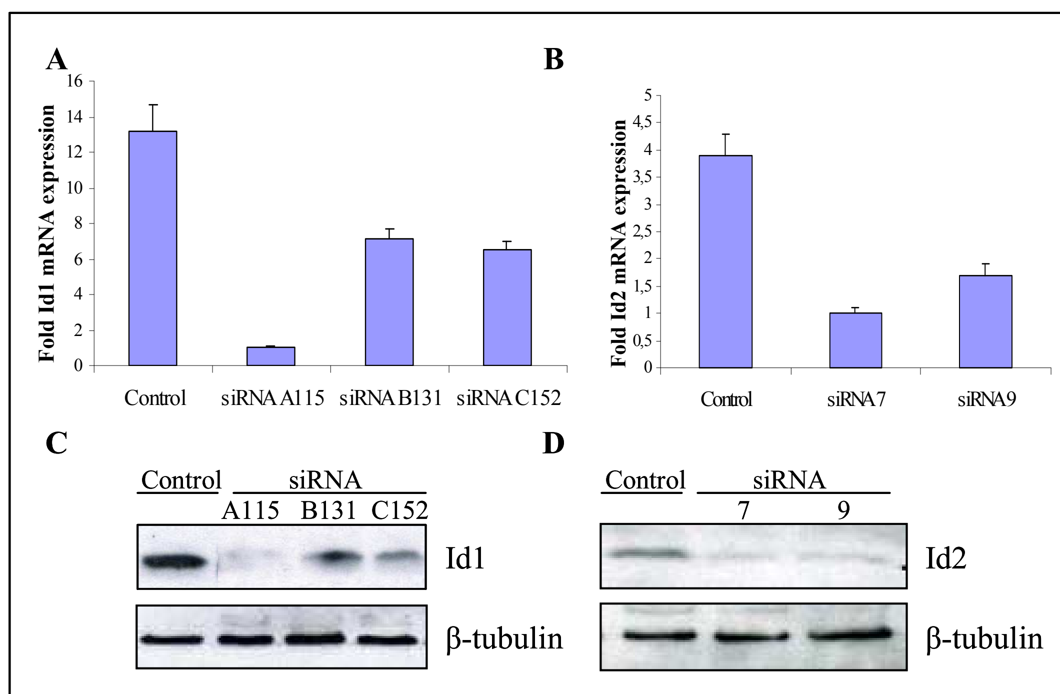

Figure 3.36 The expressed siRNA down-regulates the expression of targeted mRNA. HC11 cells stably expressing siRNA against Id1 or Id2 were used for the isolation of mRNAs and proteins. The mRNAs were reversed transcribed to cDNA and used as template in a Real-Time PCR reaction run with primers specific for Id1 (A) or Id2 (B). The extracted proteins were western-immuno-blotted with an antibody specific for Id1 (C) or Id2 protein (D). To confirm equal loading of the samples the blots were stripped and reprobed with anti β-tubulin antibody.

The analysis of Id1 or Id2 mRNA expression by Real-Time PCR (Figure 3.36 A and B) showed that the selected siRNAs effectively down-regulate the expression of Ids mRNA in stably transduced HC11 cells in comparison to control cells (transduced with empty lentiviral vector). The isolated total protein extracts were western immuno-blotted with an antibody against Id1 or an antibody against Id2 protein. To verify the equal samples loading the membranes were stripped and reprobed with antibody specific for β-tubulin. The western blot analysis (Figure 3.36 C and D) further confirmed the Real-Time PCR results. The expression of Id1 and Id2 in siRNA expressing HC11 cells was clearly down regulated on the protein level. The HC11 population transfected with the most effective siRNA (for Id1 the sequence A115 and for Id2 the sequence 7 and 9) were used for further functional experiments.

3.4.4 Differentiation of HC11 cells expressing siRNA against Id2

Id2 plays an essential role during late stages of mammary gland development. Id2 knock-out mice have a lactation defect with a strong inhibition of β-casein expression (Mori et al., 2000; Miyoshi et al., 2002). To test the effect of Id2 silencing on the functional differentiation of mammary epithelial cells, the HC11 cells transfected with siRNA 7 and 9 effectively down regulating the Id2 expression level were grown to the competent stage and then induced with lactogenic hormones (DIP) for functional differentiation. Total RNA was isolated after 24 h (β-casein mRNA accumulation is high at this point) of incubation with DIP and reverse transcribed into cDNA that served as a template in Real-Time PCR reactions. Primers specific for β-casein (differentiation marker) or the 18S ribosomal subunit were used. Total proteins were isolated after 4 days of stimulation with DIP (β-casein protein expression is high at this time point) and western immuno blotted with an anti β-casein antibody. To control the equal loading the membrane was stripped and reprobed with an anti β-tubulin antibody.

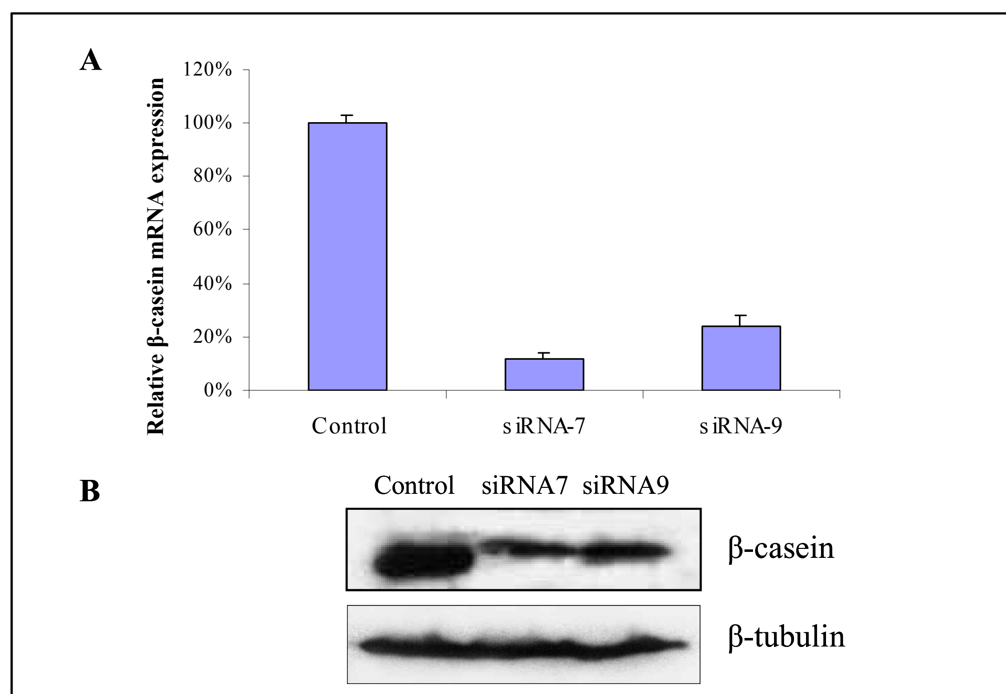

Figure 3.37 Down-regulation of Id2 by siRNA inhibits expression of milk protein β-casein. HC11 cells stably expressing siRNA against Id2 were induced with DIP. (**A**) After 24h lactogenic stimulation the mRNA was isolated and reversed transcribed into cDNA which was used as template in Real Time PCR reactions run with primers specific for β-casein and 18S ribosomal subunit which serve as internal loading control. (**B**). After 96 h of lactogenic stimulation total proteins were extracted and western-immuno-blotted with antibody specific for β-casein. To control the equal loading of the samples the membrane was reprobed with anti β-tubulin antibody.

As can be seen in Figure 3.37A the silencing of Id2 expression by siRNA 7 or 9 leads to a clear reduction of β-casein mRNA. The obtained Real-Time PCR data are in correlation with results from western blot analysis showed in Figure 3.37B. The expression of β-casein protein is clearly reduced in comparison to control cells transduced with empty vector. These results clearly show that Id2 is involved in transcriptional regulation of β-casein and indicate that Id2 is one of the major regulators of the terminal differentiation of HC11 cells *in vitro*.

3.4.5 Morphological differentiation of HC11 cells expressing siRNA targeting Id1

If mammary epithelial cells are seeded in a three dimensional context they are able to form branch-like structures. It order to study the role of Id1 in the morphological differentiation of mammary epithelial cells, the HC11 cells stably expressing siRNA-A115, which silence Id1 expression (Figure 3.36) and control HC11 cells transduced with empty vector, were seeded in a collagen gel, and induced for differentiation. After 8 days of cultivation the formation of branch-like structures was analyzed under the fluorescence microscope. The presence of GFP positive branch-like structures confirmed that they are exclusively formed by HC11 cells transduced with lentiviral particles and not by non-transduced HC11 cells.

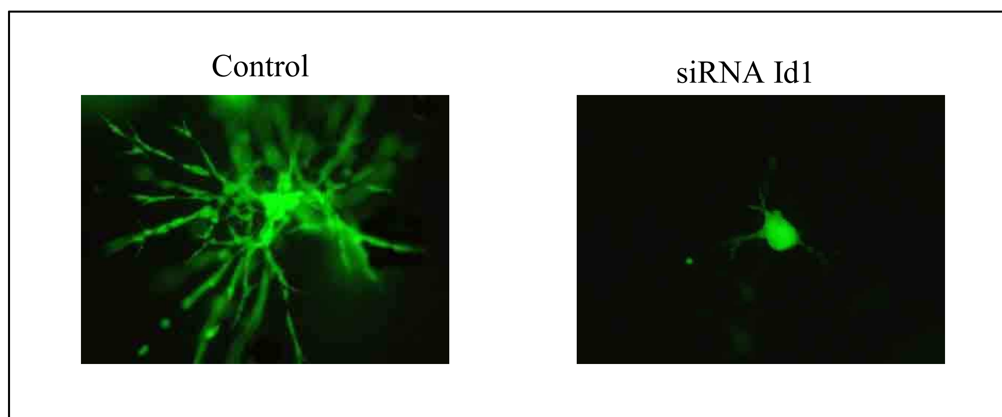

Figure 3.38 Inhibition of branching due to silencing of Id1 protein expression. Around 2000 HC11 cells stably expressing siRNA against Id1 were seeded in 100 µl of collagen gel and were induced for differentiation. After 8 days the picture was taken using the fluorescence microscope associated camera. GFP protein is a marker for transgene positive HC11 cells expressing siRNA targeting Id1.

As can be seen in Figure 3.38, HC11 cells stably expressing siRNA silencing Id1 were not able to drive the morphological differentiation. No or only small branches were observed. The cell growth is not totally impaired indicating that the cells were not able to invade the stroma. Most of cells formed only globular colonies. The control HC11 cells transduced with empty vector formed normal branch-like structures indicating that expression of GFP did not influence the morphological differentiation process. The obtained results are due to silencing of Id1 expression. The experiment indicates that Id1 controls the ability of HC11 cells to invade the stroma ultimately influencing the formation of branch-like structures. In addition, it seemed that also proliferation was reduced, which could have contributed to the reduced outgrowth. Therefore in next experiment the effect of silencing of Id1 on HC11 cell proliferation was measured using the XTT proliferation assay.

3.4.6 Proliferation of HC11 expressing siRNA silencing Id1

The experiment using 3D cultures indicated that Id1 regulates the morphological differentiation of mammary epithelial cells. Since Id1 has been shown to be involved in the regulation of epithelial cell proliferation it was necessary to investigate if the inhibition of branching is due to a reduction in mammary epithelial cell proliferation. Therefore, proliferation rate of HC11 cells stably expressing siRNA-A115 targeting Id1 mRNA was compared with control cells transduced with empty vector. The number of living cells was measured every 24 h using the XTT proliferation assay.

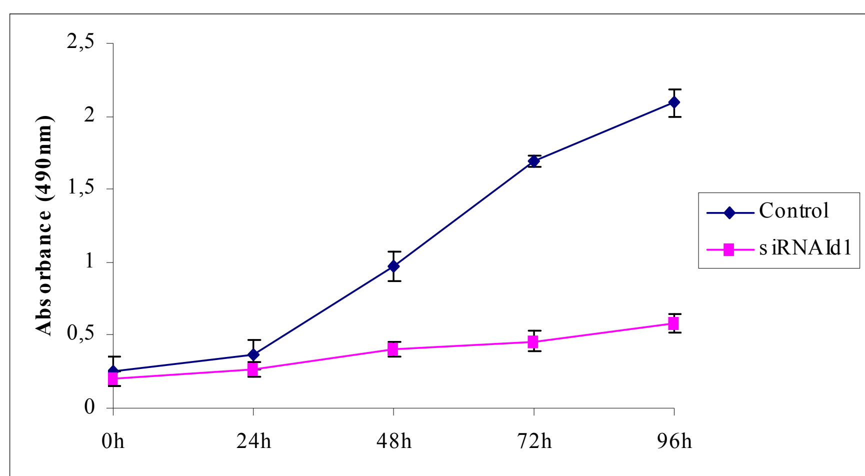

Figure 3.39 The siRNA targeting Id1 inhibits proliferation of HC11 cells *in vitro*. The HC11 cells were stably transduced with empty lentiviral particle or lentiviral vector expressing siRNA-A115 targeting Id1. $5x10^3$ of transduced HC11 cells were seeded in a 96-well plate in growing medium and cultured for the next 96 h at 37°C. The amount of living cells was quantified by using the XTT assay at indicated time points.

As shown in Figure 3.39 the silencing of Id1 expression by siRNA clearly inhibited the proliferation of HC11 cells in comparison to control cells. The experiment indicated that Id1 is one of the regulators of proliferation of mammary epithelial HC11 cells. Reducing its expression inhibited cell growth possibly blocking the morphological differentiation in a collagen gel.

The obtained results parallel the studies performed with rapamycin, presented in first part of this work. Treatment with rapamycin negatively influenced branching and proliferation. The results obtain with HC11 silencing Id1 in 3D culture was similar to the action of rapamycin on the morphological differentiation of HC11 cells in the gels. Rapamycin inhibits also expression of β-casein, whose expression was dependent on Id2. The siRNA and the rapamycin experiments show that mTOR regulates proliferation and differentiation of mammary epithelial cells by controlling the expression of Id1 and Id2.

4. DISCUSSION

The development of organs requires a perfect coordination of multiple extracellular signals to assure a perfect balance between growth, proliferation, and differentiation. This is essential to achieve and maintain the specialized functions of tissue structures. Many signaling pathways and their activated targets genes involved in regulation of organogenesis are still unknown. This work contributes to a better understanding of the intracellular pathways and the transcriptional events, which regulate the process of mammary gland development. The importance of the obtained data presented in this work will be discussed in more detail below.

4.1 The mTOR pathway plays a role in proliferation and differentiation of the mammary gland

The mTOR signaling pathway was implicated in controlling many cellular functions (Schmelzle and Hall, 2000). However, the role of the mTOR pathway in the development of mammary gland had not been studied in detail. Therefore, the activity of mTOR was inhibited with rapamycin, and the consequences on proliferation and differentiation of mammary epithelial cells were studied in this work.

4.1.1 HC11 cells as a model system to study the mTOR pathway

The development of the mammary gland occurs mainly after birth and unlike for most other organs, the experiments on developmental regulation can be performed in adult organisms. During each pregnancy a cycle of proliferation, functional differentiation and massive apoptosis occurs in the mammary gland. The mammary gland reaches the peak of its development during pregnancy when mammary epithelial cells start to proliferate extensively, to extend the ductal system and to form alveolar units. The epithelial cells contributing to the alveolar compartment terminally differentiate, become functional and start to secrete milk, which is necessary for proper development of newborns. Therefore, the mammary gland is a perfect system for studying the pathways and genes regulating the process of organogenesis (Medina, 1996). However, molecular analysis of signaling pathways in mammary epithelial cells *in vivo* is complicated by the presence of other cell types in the gland like adipocytes and fibroblasts, contributing to the mammary gland stroma. Therefore, the initial studies were performed using an *in vitro* model.

The isolated mammary epithelial cell line HC11 shows similar characteristics as mammary epithelial cells *in vivo*. The HC11 cell line is an established mammary epithelial cell line selected as a cell clone from the COMMA-1D cell line, which was initially isolated from a mid-pregnant Balb/c mouse (Ball et al., 1988). Stimulation with hormones leads to proliferation and functional differentiation of these cells (Wartman et al. 1996). Terminal differentiation can be monitored by studying the expression level of β-casein mRNA and protein, which is a component of milk and serves as a marker for functionally differentiated mammary epithelial cells. The HC11 cell system has been used in this work to analyze the role of the mTOR pathway on proliferation and differentiation of mammary epithelial cells. The results were compared to results obtained with primary mammary epithelial cells and showed that HC11 cells are able to respond in similar way to freshly isolated cells (Figure 3.1 and 3.14). These experiments clearly show that HC11 cells can be used as a model system to study the role of signaling pathways essential for mammary gland development.

4.1.2 Morphological differentiation of mammary epithelial cells can be studied in 3D culture

The inhibition of mTOR with rapamycin influenced the morphological differentiation of mammary epithelial cells. It is not possible to investigate the effect of the drug on cell morphogenesis in petri-dish, since the cells can only grow in two dimensions resulting in a single layer of cells. The use of a 3D culture system allows epithelial cells to proliferate and to form structures that resemble the glandular organization of the cells *in vivo*. The 3D cultures have been used in the past also to investigate pathways required for branching and alveolar morphogenesis (Niemann et al., 1998). For example, to study oncogene induced changes in the morphology of mammary epithelial cells (Shaw et al., 2004) and to analyze epithelial plasticity and metastasis (Jeschlinger et al., 2002) as well as to investigate mechanisms governing apoptosis in normal and malignant mammary epithelial cells (Weaver and Bissell, 1999).

Therefore, in this study the 3D culture system was chosen to study the role of mTOR in mammary epithelial morphogenesis. If the HC11 cells are seeded in a collagen gel or matrigel they form branch-like structures (Figure 3.4). The studies in this work using the 3D culture system showed that the morphological differentiation was inhibited by rapamycin treatment (Figure 3.5). The same effect on morphogenesis was observed when Id1 was silenced with siRNA (Figure 3.38).

This result corresponds to other studies showing that Id1 is an important factor regulating proliferation, migration and invasiveness of normal (Lin et al., 1999) as well as of cancer cells (Norton, 2000; Sikder et al., 2003). Moreover, over-expression of the Id1 protein in HC11 cells prevented the inhibition of morphological differentiation with rapamycin indicating that mTOR may regulate the proliferation and migration by influencing Id1. The Id1 protein has been shown to be expressed at high levels during puberty and early pregnancy, when cells proliferate and migrate into the stroma to form the ductal system (Parrinello et al., 2001; Richter et al., 2000). Also insulin signaling has previously been implicated in regulation of ductal morphogenesis (Hadsell and Bonnette, 2000). In agreement with this observation, both mTOR (Rosen et al., 1999) and Id1 (Navarro et al., 2001; Belletti et al., 20002) are downstream components of insulin signaling. From the 3D culture study presented here it can be concluded that mTOR mediates signals from insulin to Id1, which in turn regulate proliferation and migration of mammary epithelial cells leading to the formation of the ductal system during mammary gland development.

4.1.3 The mTOR pathway controls the proliferation of mammary epithelial cells

The analysis of branch-like growth of HC11 cells in 3D cultures indicates that mTOR plays an important role in the regulation of morphological differentiation. It seemed that proliferation was reduced if the drug was added to HC11 cells, which is not surprising since rapamycin has been shown in other studies to reduce proliferation of normal as well as of malignant cells (Metcalfe et al., 1997; Hidalgo and Rowinsky, 2000). The performed proliferation assay confirmed that rapamycin reduced the proliferation rate of HC11 cells (Figure 3.6), indicating that the inhibition of branch-like structure formation by rapamycin was in part due to a reduction of proliferation. Like in the case of rapamycin treated cells, the growth rate of HC11 cells in which the expression of Id1 was silenced with siRNA resulted in a similar inhibition of proliferation (Figure 3.39). From these results it can be concluded that rapamycin reduced the proliferation of mammary epithelial cells by inhibiting mTOR, which caused the down-regulation of the expression of Id1 (Figure 3.12).

4.1.4 The mTOR pathway is essential for the functional differentiation of mammary epithelial cells

4.1.4.1 Rapamycin inhibits β-casein expression *in vitro*

The expression of the milk protein β-casein serves as a marker for terminally differentiated secretory cells. This protein can be detected in the mammary gland of late pregnant mice and is highly expressed during lactation. The HC11 cells can be induced for functional differentiation by adding a lactogenic hormone mix (DIP) to the medium (Figure 3.1). It was shown that expression of β-casein is dependent on an intact mTOR signaling pathway. By adding rapamycin into the medium HC11 cells stop to express β-casein on the protein as well as on the mRNA level (Figure 3.2). This indicates that mTOR controls the expression of β-casein not (only) on the translation level, although the control of translation is a well-known major function of mTOR (Schmelzle and Hall, 2000). It also regulates the expression of β-casein on the transcriptional or post-transcriptional level. To confirm that the results obtained with HC11 cells are not only true for this *in vitro* established epithelial cell line the influence of rapamycin on functional differentiation was also investigated on primary mammary epithelial cells. Therefore, the freshly isolated primary mammary epithelial cells were induced for differentiation while mTOR was blocked. Like in HC11 cells, rapamycin also blocked the terminal differentiation of primary cells as shown by the lack of β-casein protein in the treated cells (Figure 3.14).

The fact, that mTOR plays a role in differentiation is consistent with recent data showing a role for mTOR in the regulation of differentiation of many other types of cells. It was shown to be involved in differentiation of *Drosophila melanogaster* cells (Bateman et al., 2004). Furthermore, the differentiation of megakaryocytes (Raslova et al., 2006), myoblast (Sarker et al., 2004) and vascular smooth muscle cells (Martin et al., 2003), the differentiation of female granulosa cells (Alam et al., 2004), the control of adipogenesis in murine (Yeh et al., 1995) and human adipocytes (Bell et al., 2000) were all found to be regulated by the mTOR signaling pathway.

4.1.4.2 Intact mTOR signaling is essential during mammary gland development

To study the role of mTOR *in vivo* mid-pregnant female mice were injected with rapamycin. However, the used drug concentration had an acute teratogenic activity, and completely inhibited the development of embryos (Figure 3.15), making it impossible to study the effects of rapamycin on the mammary gland during pregnancy. This inhibitory effect of rapamycin on embryonic development is in correlation with other studies showing that functional mTOR is required during early embryonic development to support growth and proliferation of embryonic stem cells (Gangloff et al., 2004; Murakami et al., 2004). To overcome this limitation it is possible to study the effect of rapamycin on ductal and alveolar formation during pregnancy *in vitro* using whole organ cultures of the mammary gland. In such a culture the development of the mammary gland can be stimulated by the addition of lactogenic hormones and is in this way independent of pregnancy (Ginsburg and Vonderhaar, 2000).

It was possible to investigate the role of mTOR during proliferation and differentiation of epithelial cells in the lactating mammary gland *in vivo*. Mice were treated starting at day 19 of pregnancy and up to 10 days of lactation before the mammary glands were isolated. Lactating glands from rapamycin-treated mice appeared smaller; the volume of secretory compartment was less developed in comparison with the control mice (Figure 3.19). This was accompanied by a decreased wet weight (Figure 3.17) and a decreased milk production, explaining the decreased gland mass and the reduced gain of weight of the pups (Figure 3.16). The histological analysis of glands of the treated mothers showed that the lumina of the alveoli were less extended than the controls, confirming the reduction in milk production. The alveolar cells still had a cuboidial shape similar to the epithelial cells at the beginning of functional alveoli formation indicating that the cells were not fully differentiated. As expected, a reduction in the amount of β-casein was detected in protein extracts of rapamycin-treated glands (Figure 3.20), which correlated with the decrease in volume of functionally differentiated secretory compartment in mammary glands of treated mice (Figure 3.19). Taken together, these *in vitro* and *in vivo* results showed that mTOR is required to establish the secretory function of mammary epithelial cells.

4.1.4.3 The mTOR-dependent expression of β-casein is not mediated by Stat5 signaling.

The inhibition of mTOR with rapamycin had no influence on the stability of β-casein mRNA as indicated by experiments using actinomycin D (Figure 3.3). Therefore inhibition of mTOR must influence other important signaling pathways controlling the functional differentiation and transcription of the milk protein β-casein gene.

To induce terminal differentiation and expression of β-casein a mixture of lactogenic hormones (DIP) was used. These lactogenic hormones (dexamethasone, insulin and prolactin) in turn activate three major signaling pathways regulating the transcription of target genes involved in differentiation, including the milk genes (Hennighausen, 1997). The prolactin pathway is a very important pathway in this respect and regulates the phosphorylation and activation of Stat5 (Gouilleux et al., 1994). Stat5 is the major player in prolactin-activated signaling governing proliferation and functional differentiation of alveolar epithelial cells (Miyoshi et al., 2001), and is also required for the maintenance of their secretory ability during lactation (Cui et al., 2004). From these data it can be surmised that rapamycin prevents β-casein expression by inhibiting the Stat5 signaling. However, western blot analysis showed that rapamycin has no influence either on phosphorylation of Stat5 (Figure 3.7) or on the nuclear translocation of Stat5 (Figure 3.8). Both experiments showed that there was no significant difference between treated and control cells. Finally, luciferase assays revealed that the transcriptional activation of the target gene by Stat5 was comparable in treated and control cells (Figure 3.9). From these results it can be concluded that mTOR mediates its effect on the differentiation of mammary epithelial cells independently of the Prolactin/Stat5 signaling.

The mTOR protein is a down-stream target of the insulin signaling pathway (Wang et al., 2006). Many studies have shown that activation of the insulin pathway is required for the expression of milk proteins (Rosen et al., 1999). Maximal activation of β-casein expression *in vitro* requires insulin in addition to prolactin (Henninghausen, 1997). Insulin signaling regulates the activity of Idb proteins (Belletti at al., 2002; Navarro et al., 2001), which are crucial for milk protein expression (Parrinello et al., 2001). Therefore, in this work it was tested if mTOR influences the functional differentiation and the level of β-casein through regulation of Idb protein expression. The results are discussed in the following chapter.

4.1.5 mTOR controls morphological and functional differentiation of mammary epithelial cells through Idb proteins.

The important question remaining to be answered was how mTOR actually controls the fate of mammary epithelial cells. Various experiments performed in this work have shown that proliferation and differentiation of mammary epithelial cells can be blocked. First, inhibition of mTOR with rapamycin blocks both proliferation and morphological differentiation of mammary epithelial cells *in vitro*. Similar effects could be observed when HC11 cells were stably transduced with siRNA silencing the expression of Id1. Intact mTOR signaling was also essential for proper functional differentiation of mammary epithelial cells in culture. Treatment of female mice with rapamycin during lactation impaired milk protein expression. Reducing the expression of Id2 by siRNA decreased the β-casein level, indicating a block in functional differentiation.

These observations raised the question if mTOR regulates the proliferation and differentiation by influencing the expression of Idb proteins. Indeed, cells treated with rapamycin showed a reduced level of Id1 during proliferation. More importantly, over-expression of Id1 in HC11 cells completely rescued the mammary cells from the inhibitory effect of rapamycin on proliferation confirming the essential role of Id1 in proliferation. Interestingly, the cells over-expressing Id1 were not able to functionally differentiate and have a reduced level of Id2 (Figure 3.28). The results indicate that Id1 in addition to promote proliferation inhibits the expression of Id2, blocks differentiation, and confirm a previously postulated model of Idb protein action in the mammary gland (Desprez et al., 2003). In protein extracts isolated from rapamycin treated cultures and from the lactating gland, a reduction in the level of milk protein β-casein was accompanied by a decrease in the Id2 level (Figure 3.20). The cells over-expressing Id2 could bypass the requirement of functional mTOR for β-casein expression (Figure 3.30). The results demonstrate for the first time a central role for mTOR in the precise regulation of pathways governing proliferation and differentiation of mammary epithelial cells in the developing mammary gland and identify the Id1 and Id2 as major downstream mediators of mTOR action.

4.1.5.1 mTOR regulates Idb expression on the mRNA level

Rapamycin treatment during proliferation down-regulates the mRNA of Id1 (Figure 3.12) whereas rapamycin addition to induced cells reduced the Id2 mRNA level (Figure 3.13). Regulation of Id1 and Id2 by mTOR on the mRNA level was surprising, since the mTOR signaling pathway is known to affect the expression of genes involved in the protein translation machinery. mTOR regulates the initiation of protein translation by controlling the activity of its down-stream targets eIF4E/4E-BP1 and S6Kinase (Manteuffel et al., 1996; Brown et al., 1995**).** Therefore, the regulation of Id1 and Id2 on the mRNA level is probably only indirectly affected by mTOR possibly via the enhanced or decreased translation of certain transcription factors important for Id1 and Id2 expression.

4.1.5.2 Potential transcriptional regulation of Idb proteins by C/EBPβ

Since Id1 and Id2 are regulated on the mRNA level, a specific transcription factor is involved in their transcriptional regulation, which also plays a role in the mTOR pathway. The C/EBPβ transcription factor could be an interesting candidate, since it is activated by the insulin pathway.

The insulin/PI3K pathway induces the activation of mTOR and has been shown to dramatically increase the phosphorylation of the mTOR down-stream targets S6Kinase and 4E-BP1 involved in the regulation of protein expression on the translational level (Chung et al., 1994; Brunn et al., 1996; Brunn et al., 1997). An intact insulin/IGF pathway is essential for terminal end bud formation, for ductal morphogenesis (Kleinberg et al., 2000) as well as for proper lactation during functional differentiation of the mammary gland (Lau et al., 1993). Furthermore, insulin has been shown to regulate the transcription of C/EBPβ in adipocytes, which plays an essential role in proper formation of the mammary gland (MacDougald et al., 1995). The C/EBPβ protein (CCAAT/enhancer binding protein β) is a member of a highly conserved family of transcription factors, which belongs to the leucine zipper class of DNA binding proteins (Lekstrom et al., 1998). C/EBPs contain an amino terminal transactivation domain followed by a highly basic DNA-binding region, and a carboxy terminal leucine-rich dimerization domain (Landschulz et al., 1989).

They can form dimers with other C/EBP family members, or with different groups of leucine zipper proteins. Dimerization is essential for their transcriptional activity (Patel et al., 1994).

The C/EBPs transcription factors are involved in the regulation of proliferation and differentiation of a number of cells and tissue types, including the mammary gland (Grimm et al., 2003). Mice deficient for C/EBPβ were generated (Screpanti et al., 1995) and development of the mammary gland was found to be impaired. In particular the ductal morphogenesis in the knock-out virgin mouse was found to be affected, showing distended ducts and decrease branching. In addition, the lobuloalveolar proliferation and functional differentiation of mouse epithelial cells during mammary gland development was affected. Furthermore, the expression of the functional differentiation marker β-casein was dramatically inhibited in C/EBPβ null glands (Seagroves et al., 1998). These observations resemble the phenotype seen in rapamycin treated cells or mice.

Transcription of the C/EBPβ gene results in a unique intronless mRNA that can be translated via alternative start sites into three isoforms: full length liver-enriched transcriptional activator protein LAP1, a shorter isoforms LAP2, and a transcriptional inhibitory protein LIP. The LIP protein lacks more than half of the transactivation domain and as a consequence is not able to activate gene transcription. LIP can thus compete with LAP for the same DNA binding site as homodimer or by forming heterodimers with LAP, blocking the transcription of LAP target genes. Since the LIP isoform has a high affinity for DNA the inhibitory effect occurs even at substoichiometric ratios of LIP: LAP (Descombes et al., 1991). LAP expression is not changing dramatically during mammary gland development, the protein level increases 2-3 fold at onset of pregnancy and decreases at parturition. It is readily detectable again during lactation and involution. The LIP isoform is expressed at low level in the virgin gland and is strongly upregulated (about 100 fold) during pregnancy. At parturition the LIP level decreases to almost undetectable and stays low during lactation (Raught et al., 1995 Seagroves et al., 1998). Therefore the LIP: LAP ratio is a very important determinant of the overall C/EBPβ function during mammary gland development.

The translational regulation of the C/EBP isoforms (Calkhoven et al., 2000) by mTOR during mammary gland development might be the mechanism by which the mTOR pathway affects proliferation and differentiation of mammary epithelial cells (Jundt et al., 2005; Kim et al., 2004). Similar to the inhibition of mTOR by rapamycin, the lack of C/EBPβ in mammary cells severely inhibits the expression of β-casein. Moreover, the phenotype of mammary glands treated with mTOR inhibitor was close to that of C/EBPβ null gland, which displayed only limited lobuloalveolar development of the mammary gland in response to pregnancy hormones (Robinson et al., 1998; Seagroves et al., 1998). Interestingly, the C/EBPβ null mammary gland phenotype resembles that of the gland of Id2-null mammary epithelium, where the formation of the functional secretory alveolar compartment is impaired and the expression of β-casein is clearly reduced (Mori et al., 2000). More importantly, preliminary evidence linking C/EBPβ to Id2 was recently provided by a study, which demonstrated that Id2 is a direct target of C/EBPβ since the expression of Id2 is reduced during pregnancy in C/EBPβ deficient mammary glands (Karaya et al., 2005). Additionally, it has previously been shown that Id1 is regulated by C/EBPβ in pro-B cells (Saisanit et al., 1997). However, if mTOR controls Id1 and/or Id2 protein expression through the regulation of C/EBPβ protein has to be investigated in more detail.

4.1.5.3 Potential regulation of Idb proteins by HIF

The downstream targets of mTOR influencing the regulation of Idb protein expression might vary depending on the developmental stage of the mammary gland, and as discussed above, other transcription factors regulated by mTOR pathway might play a role. In this respect it is of interest to mention hypoxia inducible factor 1 (HIF1). Treatment of mice with the mTOR inhibitor rapamycin during lactation resulted in a phenotype similar to that of mice deficient for the transcription factor HIF1 (Seagroves et al., 2003). Although the mammary glands of these mice displayed normal development of the alveolar compartment during pregnancy, loss of HIF1 leads to a defect in terminal differentiation. The lactation was impaired and striking changes in the milk composition were observed (Seagroves et al., 2003). The insulin/PI3K/mTOR pathway regulates HIF1 protein expression through a translation-dependent pathway and is required for transcriptional activity of HIF1 (Dekanty et al., 2005, Treins et al., 2002).

It can thus be surmised that rapamycin inhibiting insulin/PI3K/mTOR signaling affects the function of the lactating mammary gland at least partly due to a reduction of HIF1 protein translation. Moreover, recent studies have provided evidence that Id2 is a novel target of HIF1. Id2 expression is regulated by HIF1, since it induces the transcription of the Id2 gene in response to hypoxia in neuroblastoma cell lines (Lofstedt et al., 2004). Interestingly, the activation of HIF1 by the PI3K/Rheb/mTOR pathway is necessary for induction of several protein markers of follicular differentiation (Alam et al., 2004).

Thus, a model can be postulated in which mTOR signaling during development of the mammary gland controls the translation of the HIF1 protein. HIF1 in turn activates the transcription of the Id2 gene, promoting functional differentiation and milk secretion in mammary alveolar cells during lactation. Further studies will be necessary to investigate this model.

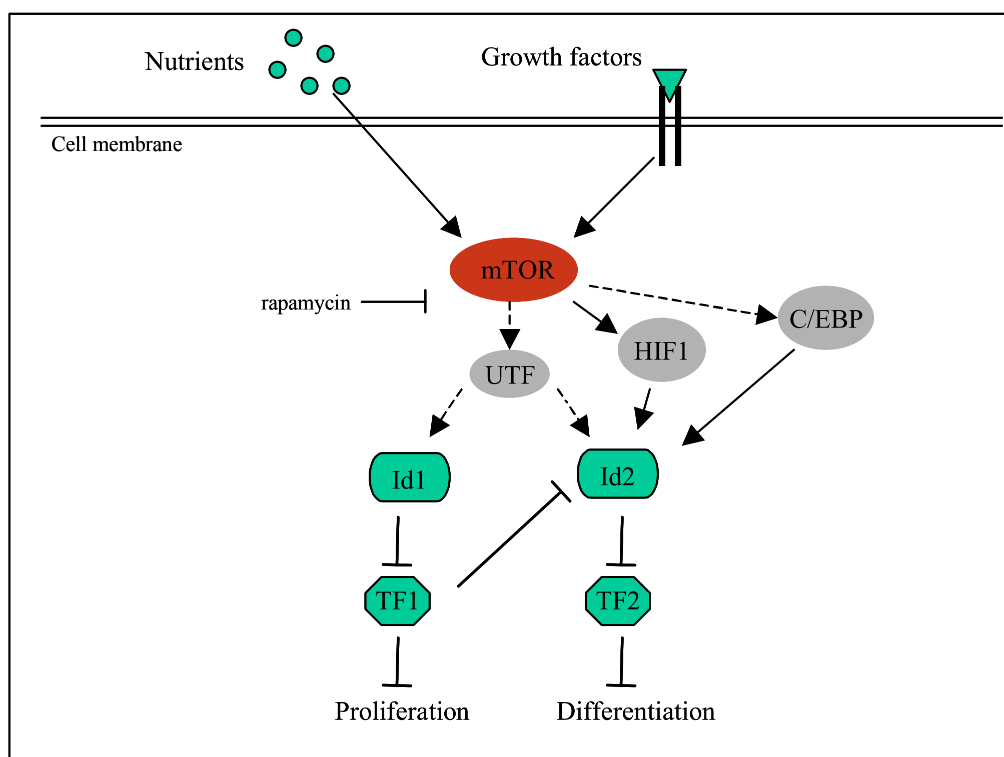

Figure 4.1 Model of regulation of the Id1 and Id2 protein level by the mTOR signaling pathway during proliferation and differentiation of mammary epithelial cells. The mTOR pathway, activated by nutrients and growth factors may stimulate translation of HIF1 and/or C/EBPβ or unknown transcription factor (UTF). These transcription factors in turn are potentially involved in the activation of the transcription of Id1 or Id2. Id1 promotes proliferation by counteracting the anti-proliferative action of bHLH protein (TF1). Id2 promotes differentiation by inhibiting the action of TF2 which is an inhibitor of differentiation. Dotted lines indicate a possible link.

4.1.6 Role of mTOR and Idb proteins in cancer

Apart from its role during the development of the mammary gland, the mTOR pathway has been implicated in development of cancer. Several studies clearly showed that various components of the mTOR signaling pathway, including PI3K, Akt, Rheb, Pten, TSC upstream of mTOR as well as the mTOR downstream targets 4EBP, eIF4E and S6 Kinase which are involved in regulation of translation, are frequently mutated, over-expressed or deregulated during tumor development. Inhibitors of the mTOR pathway are currently used in several clinical studies to treat various types of tumors, including breast cancer. The investigations performed in patients revealed, however differences in patient responses and drug efficacy (Janus et al., 2005: Hynes and Boulay, 2006). To develop a more tolerable but efficient anti-cancer treatment targeting the mTOR signaling pathway it is crucial to identify more downstream targets (genes and proteins) regulated by mTOR.

To gain a better understanding of the function of mTOR in mammary tumorigenesis the role of mTOR during normal mammary gland development was investigated in this work. Obviously, mTOR controls the proliferation and differentiation of mammary epithelial cells through Id1 and Id2, respectively. Interestingly, overexpression of Id1 correlates with an aggressive form of breast tumors (Lin et al., 2000) whereas Id2 maintains a differentiated and non-invasive phenotype of breast cancer cells (Itahana et al., 2003). Deregulation of the expression of Id1 and Id2 proteins also altered proliferation and differentiation of many other cell types resulting in uncontrolled growth and increase invasiveness (Perk et al., 2005).

As discussed above, the Idb proteins were shown to be regulated by C/EBPβ (Saisanit et al., 1997; Karaya et al., 2005). Interestingly, an increased level of one of the downstream targets of mTOR (eIF4E) favors LIP translation (Calkoven et al., 2000) and eIF4E over-expression correlates with high level of LIP translation found in tumors (DeBenedetti et al., 1999). However, if mTOR controls Id1 and/or Id2 protein expression through the regulation of translation of C/EBPβ isoforms in normal mammary gland and the role of mTOR/C/EBPβ/Idb axis in cancer development has to be investigated in more detail. The presented findings broadening our understanding of the events that contribute to breast cancer development and should help to design better anticancer therapies targeting the components of the mTOR pathway including its downstream targets contributing to cancer.

4.1.7 Conclusions and Outlook

The analysis of the effect of inhibiting the mTOR signaling pathway by using rapamycin on mammary epithelial cells for the first time demonstrate that mTOR plays a central role in the coordination of pathways governing the proliferation and differentiation of epithelial cells during mammary gland development. More detailed analysis led to the identification of Id1 and Id2 as two major down stream effectors of the mTOR signaling pathway regulating proliferation and differentiation respectively. These findings clearly contribute to our understanding of the signals regulating the expression of the genes involved in the development of mammary gland.

Future studies will be performed to investigate the effect of mTOR inhibition during puberty and pregnancy, using for example whole organ gland cultures. Studying the components of the mTOR pathway especially downstream of mTOR will help to identify their role in tumor development and/or progression.

4.2 Identification of genes involved in mammary gland development may help to identify factors essential for breast cancer

Breast cancer is the leading cause of cancer related deaths among women around the world. The factors that contribute to breast cancer development can be divided into genetic and non-genetic components. Genetically inherited breast cancer accounts for around 10% of all breast cancer cases (Nathanson et al., 2001). However, the development of the majority of diagnosed breast cancers appears to be primarily induced by non-genetic factors like diet, obesity, alcohol consumption, age, race, geographic location, parity, early menarche, late menopause, use of postmenopausal hormones, ect. (Ketcham and Sindelar, 1975; Russo and Russo, 1999; Lichtenstein et al., 2000). These non-genetic factors cause genetic alterations leading to the deregulation of the expression profile of genes governing proliferation and differentiation (Perou et al., 1999). Knowing the gene expression profile during normal mammary gland development will be important to understand the molecular basis of breast cancer induction. Identification of potent genes involved in the regulation of growth and differentiation will help to design more effective anti-cancer drugs, since deregulation of such genes can possibly be observed in breast cancer samples (Perou et al 2000; Desai et al., 2002). Therefore, in this study the gene expression profile of mammary epithelial cells was analyzed in detail and HC11 cells were used for this purpose. This immortal cell line was established as a cell clone from COMMA-1D cells initially isolated from a mid pregnant Balb/c mouse (Ball et al., 1988). The HC11 cells are able to grow and functionally differentiate when induced with lactogenic hormones *in vitro* (Wartman et al. 1996). The terminal differentiation is accompanied by an increase in the level of total mRNA expression, which at later stages of differentiation correlates with the appearance of β-casein protein (Rosen et al., 1999). To characterize the genes that are involved in proliferation and differentiation of mammary epithelial cells the expression patterns of HC11 cells during growing, competent and induced stage, were compared to each other using the microarray technology. The important findings will be discussed in the following chapters.

4.2.1 The use of microarrays allowed the identification of differently expressed genes.

The first arrays were performed with radioactively labeled cDNA from one sample which was hybridized to cDNA clones of a second sample spotted on nylon membranes (Drmanac and Drmanac, 1994). Arrays on glass were first used to study the expression pattern of 45 selected genes of *Arabidopsis thaliana* (Schena et al., 1995). Nowadays, thousands of cDNA clones are robotically spotted on chemically treated glass slides giving the “printed” microarray containing a collection of thousands of different genes (Eisen and Brown, 1999).

The microarray technology was also used in this study for the identification of hundreds of genes differently regulated during proliferation and differentiation of mammary epithelial cells. The obtained data provided additional insight into the molecular basis of mammary gland development (Rudolph et al., 2003). The RNA isolated from growing, competent and induced cells were directly labeled during Reverse Transcription PCR with one of the two (Cy-3 or Cy-5) fluorescent dye-conjugated deoxyuridine triphosphates (dUTPs)(Manduchi et al., 2002).

The use of Cy-3 and Cy-5-labelled cDNA is important as a control. This color switch is included to eliminate false positive cDNAs which can appear as results of differences in incorporation of the dye-conjugated nucleotide during labeling. It is known that Cy3-dUTP is more efficiently used than Cy5-dUTP during cDNA synthesis. Furthermore, it is possible that the dye-conjugated nucleotide is not incorporated with the same efficacy into cDNA transcribed from different RNA sequences. The cDNA sequence during reverse transcription could have more or less dye incorporation than expected from the sequence, so the gene could appear in the microarray analysis to be down-regulated although it is *defacto* up-regulated in the tested sample and reversibly the gene that is normally activated appears to be silenced.

The labeled cDNA samples were mixed in equal quantities in following pairs G/I and G/C and C/I and then hybridized to cDNA spotted on a microarray chip. The NIA chip used in this study was a PCR-based cDNA library and contained 15,264 genes expressed during mouse embryonic development (Tanaka et al., 2000). In parallel, a second microarray was hybridized with the same RNA but the cDNA samples in each compared pair G/C, C/I or G/I had opposite dye labeling (Cy-3/Cy-5 color switch) in comparison to the initial array.

With the microarray technique initially the differential expression of around 3000 genes was detected in the HC11 samples. Furthermore, around 1800 replicates in all hybridization experiments were identified (60% of the total detected genes). The color switch allowed identification of the genes which expression pattern was inconsistent. In total, 7% of the detected genes showed an inconsistent regulation pattern after the color switch. With this step false positive genes were eliminated from further analysis.

4.2.2 Selection of candidate genes

Around 1800 genes were identified in the micro-array analysis, which were differently regulated during different stages of mammary epithelial cell culture. The genes that were expressed in growing cultures and showed a 2-fold up-regulation compared to the induced stage were chosen for further analysis. Using this selection criteria around 397 candidates were selected as proliferation and growth associated genes. The genes, which were 2-fold up-regulated in induced stages in comparison to growing and competent cultures were selected as differentiation associated genes. Around 639 candidate genes matched these criteria. To obtain detailed information about the selected genes the UniGene-Databank as well as the Locus Link and the HomoGene databases were used. In this way it was possible to find out more about the function of the selected genes and if they are known to play a role during mammary gland development, during organogenesis or other important cellular processes.

4.2.3 Characterization and functional analysis of genes identified in the microarray

The differential expression of multiple genes was verified using Real-Time PCR. The Real-Time PCR analysis has confirmed the regulation for many genes whereas others showed an inconsistent regulation pattern and were not used for further analysis. The Idb genes were the first candidate genes analyzed in more detail, since their crucial role during proliferation and differentiation of mammary epithelial cells as well as mammary gland development was shown in previous studies (Mori et al., 2000; Desprez et al., 2003). The analysis was performed with the HC11 cell model system and the expression pattern of the Idb genes in these cells served as a first control to verify microarray and PCR data.

Apart from Idb genes various other genes were found to be of interest and can be analyzed in a similar way in future studies. A selection of these genes will be described in the following chapters. They are possibly important players in proliferation and/or differentiation of mammary epithelial cells as well as in the process of tumorigenesis.

4.2.3.1 Nkd2

The Nkd2 gene (nacked cuticle 2, a homolog of *Drosophila melanogaster* Nkd1) showed in micro-array the expression pattern indicating its involvement in the process of regulation of the functional differentiation of mammary epithelial cells. The same regulation pattern of Nkd2 was found in Real-Time PCR experiment, showing a 12.9 fold up-regulation in DIP-induced cells.

The Nkd2 protein has been shown to play a role in the regulation of the Wnt signalling pathway. The intracellular signaling events activated by Wnt proteins can be divided into two categories: canonical and noncanonical. One of the targets of the canonical Wnt pathway is β-catenin (Peifer et al., 1992). In the absence of Wnt signaling, the β-catenin undergoes phosphorylation-dependent ubiquitination by Dishevelled which induces rapid degradation of β-catenin by the proteasome (Aberle et al., 1997). The activation of the Wnt signaling stabilizes β-catenin by antagonzing its phosphorylation. This leads to β-catenin accumulation (Peifer et al., 1994) and its nuclear translocation (Schneider et al., 1996). To stabilize β-catenin the Wnt proteins interact with the cytoplasmic protein Dishevelled. Normally, Dishevelled inhibits the activities of a multiprotein complex, which function is to phosphorylate β-catenin. In the nucleus, β-catenin binds TCF/Lef family DNA binding proteins to directly regulate many Wnt responsive target genes (Riese et al., 1997).

There are at least two noncanonical pathways, which do not involve β-catenin stabilization and accumulation in the cytosol. The Wnt/PCP (Planar Cell Polarity) pathway shares the Dishevelled protein with the canonical pathway (Strutt, 2003). This pathway is thought to regulate the expression of cytoskeletal elements and possibly of JNK (Yamanaka et al., 2002). The Wnt/Ca^{2+} signaling also involves the Dishevelled protein (Sheldahl et al., 2003). The Wnt/Ca^{2+} pathway activates the release of intracellular calcium that affects the function of calcium-modulated kinases (Kuhl et al., 2000).

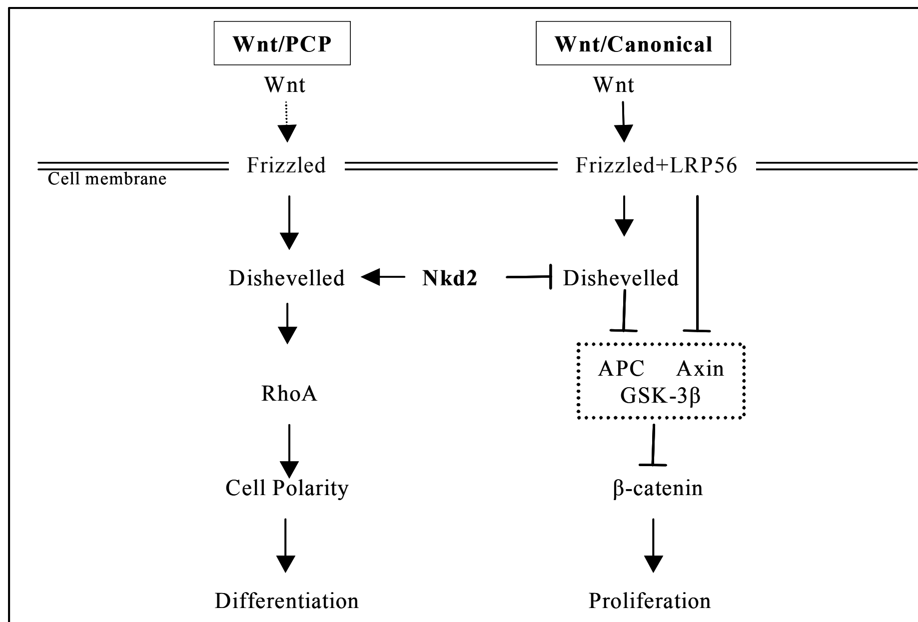

Figure 4.2 Model of regulation of Wnt signalling by Nkd2 during proliferation and differentiation. Activation of Wnt/β-catenin signalin leads to extensive proliferation. Nkd may interact with Dishevelled to inhibit the canonical Wnt pathway. The Nkd2 may act then as a switch to direct the Dishevelled activity towards the PCP (Planar Cell Polarity) pathway. Proper cell polarity is required for differentiation and polarized milk secretion.

Excessive Wnt signaling can lead to an up-regulated cell proliferation and therefore the activity of the Wnt pathway controlling development is auto-regulated via a positive and negative feedback loop (Warton et al., 2001). Nkd2 is a cytoplasmic Wingless (Wg, Wnt protein)-inducible protein that can bind the Dishevelled protein and thereby antagonize Wnt signaling (Rousset et al., 2001, Yan et al., 2001). The Nkd2 antagonist acts then in a negative feedback loop with Wg. The Nkd2 protein accumulates and inhibits Wnt signaling in proportion to the strength and duration of the Wg signal. (Zeng et al., 2000). The Wnt pathway has been also implicated in the regulation of mammary gland development.

The Wnt proteins stimulate growth of mammary rudiments during puberty and regulate ductal branching and alveolar compartment morphogenesis during pregnancy (Brennan and Brown, 2004). Analysis of the expression pattern of Nkd2 in mammary epithelial cells predicts that the level of Nkd2 protein during mammary gland development is important to control the proper switch from extensive growth to pre-differentiation. The Nkd2 protein can also be necessary for the establishment of mammary epithelial cells in the induced stage by blocking the Wnt activated proliferation. Another important function of Nkd2 could involve the maintenance of the normal epithelial cell phenotype.

In this context it is of interest to mention that in 50% of human breast cancers the Wnt signalling pathway is hyper-active as measured by the accumulation of β-catenin (Brennan and Brown 2004). The role of Nkd2 in mammary gland development and tumorigenesis will be of high interest in the future.

4.2.3.2 Nol5

The Nucleolar protein 5 (Nol5) showed in the microarray analysis a higher expression level in growing cells and down-regulation in induced cells. Real-Time PCR analysis confirmed this expression pattern (7.5 fold up-regulation in growing cells comparing to induced cells) indicating its possible contribution to regulate mammary epithelial cell proliferation. Nol5 (known also as SIK similar protein) belongs to the Nop5/Sik protein family that consists of highly conserved nucleolar proteins (Vorburggen et al., 2000). The family members bind to the C/D box of small nucleolar RNA (Newman et al., 2000). The obtained complexes, known as small nucleolar ribonucleoproteins play an essential role in rRNA processing required for ribosome assembly (Maxwell and Fournier 1995). Importantly, their functional analysis indicated that Nol5 increases rRNA synthesis (Nakamoto et al., 2001). As shown in this study elevated proliferation leads to the expression of proteins of the biogenesis machinery required during proliferation. The elevated synthesis of new cellular components is necessary to enable cell division. The function of the Nol5 protein in stimulating ribosome biogenesis correlates well with the high Nol5 gene expression level observed in growing cells. Nol5 could be a mediator of hormone induced pathways regulating the level of protein biogenesis machinery components during the mammary gland development especially during puberty and pregnancy, when the mammary epithelial cells extensively proliferate and need to constantly express the cellular building elements.

Apart from its role during mammary gland development, Nol5 might play a role during cancer progression. Hypertrophy of the nucleolus is a characteristic cytological feature of cancer cells (Busch and Smetana 1970). The increased nucleolar size is thought to reflect the increase in activity of nuclear and nucleolar processes during the malignant progression of mammalian cells (Nakamoto et al., 2001). Nol5 might be essential for proliferation of cancer cells. Future detailed analysis of the function of Nol5 and other Nop5/Sik family members will show if these proteins can be considered as possible targets for the development of novel anticancer drugs.

4.2.3.3 Kpna2

The Kpna2 gene (karyopherin (importin) alpha 2) on microarray and Real-Time PCR analysis was found to show a higher expression level (12 fold up-regulation) in growing cells as compared to induced cells. Such an expression pattern indicates that Kpna2, like Nol5 might be involved in the proliferation process of mammary epithelial cells.

The karyopherins play a key role in the nuclear import of proteins containing a classical nuclear localization signal (NLS). Proteins smaller than 60 kDa can diffuse non-selectively through the nuclear pore complex. The transport of larger proteins is a carrier mediated process and depends on cellular signaling (Gorlich and Kutay, 1999).

The karyopherin (importin) alpha 2 protein is a part of the karyopherin alpha-beta heterodimer and serves as the NLS binding site for transported proteins. The substrate in the cytoplasm binds to karyopherin alpha-beta heterodimer via the alpha subunit. The resulting protein-karyopherin alpha-beta heterodimer complex docks to the nuclear pore complex (NPC) via the beta subunit and is subsequently translocated through the nuclear pore, which is an energy consuming process. In the nucleus the karyopherin alpha-beta heterodimer releases its cargo upon binding to the nuclear Ran-GTP protein (Gorlich and Kutay, 1999).

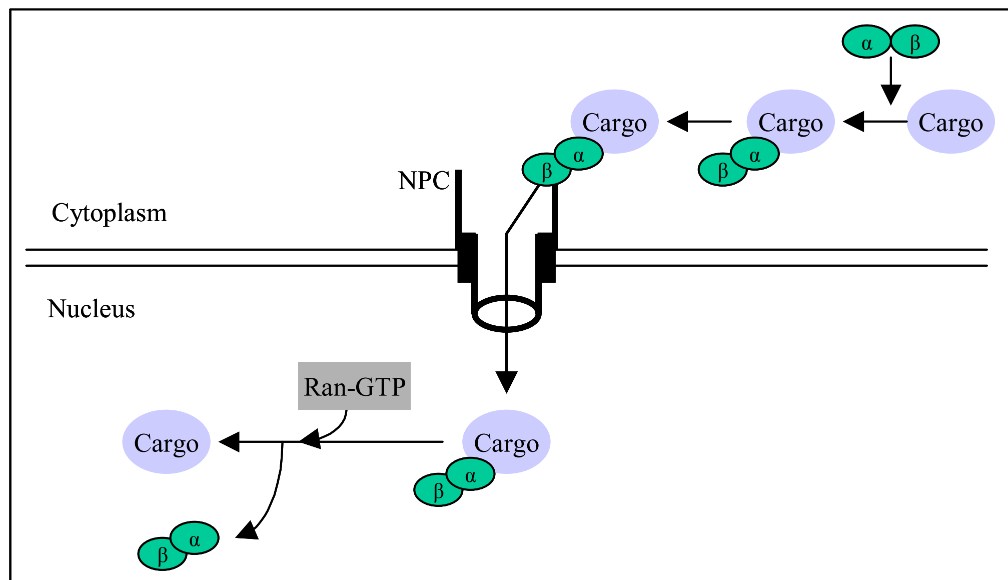

Figure 4.3 The role of Kpna2 in nuclear import. Proteins larger than 60 kDa are imported by nuclear import factors belonging to the karyopherin protein family. In the cytoplasm the karyopherin alpha beta heterodimer binds its protein cargo. The resulting protein-karyopherin complex docks to the nuclear pore complex (NPC) via the beta subunit and is subsequently translocated through the nuclear pore. In the nucleus the karyopherins release their cargo upon binding to nuclear Ran-GTP.

Interestingly, the diverse karyopherin alpha subunits have different NLS binding specificities (Kohler et al., 1999) and the members of this family show in various tissues a differential expression pattern on both the RNA and protein level (Nachury et al., 1998). Thus the nuclear import system is regulated by the expression of the different karyopherin family members. Additionally regulation occurs through competition of different proteins for the same karyopherin or different karyopherins for one protein (Jans et al., 2000). Kpna2 may play a role in the regulation of mammary epithelial cell growth, since many proteins involved in growth regulation have to be transported into the nucleus to fulfill their function.

Recent results suggested that a mutated form of human Kpna2 is involved in tumor formation and/or progression of breast cancer by preventing sufficient nuclear import of p53 and/or other tumor suppressor proteins. In the studied breast cell line Kpna2 was truncated and p53 was found in the cytoplasm but not in the nucleus (Kim et al., 2000). The cytoplasmic localization of wild type p53 was also detected in tissue samples from breast cancer patient cases (Moll et al., 1992). Additionally, BRCA1 was shown to interact with Kpna2 (Chen at al., 1996) and the wild-type form of BRCA1 was found aberrantly located in the cytoplasm of advanced breast cancer cells (Chen et al., 1995). The mislocation of nuclear tumor suppressor proteins leads to their inactivation.

In conclusion, Kpna2 is expressed in growing cells and mutations and/or its deregulation may contribute to cancer formation and progression. Therefore it would be interesting to uncover the signaling pathway that regulates the function of Kpna2 during normal mammary gland development and to determine in more detail how mutations and/or deregulation of Kpna2 contribute to breast cancer.

4.2.3.4 Cited4

The regulation pattern of the Cited4 gene (CBP/p300 interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 4) obtained with the microarray analysis and Real-Time PCR experiments indicates that Cited4 could be required for proper differentiation since it was found 4.9 fold up-regulated in induced cells. Cited4 was shown to have strong milk cycle dependent induction in pregnant and lactating mammary epithelial cells. Stronger expression of Cited4 was also noticed *in vitro* in the murine mammary cell line SCp2 during induction of differentiation with prolactin (Yahata et al., 2000).

These results are in correlation with our data and suggest possible role of Cited4 in regulation of differentiation associated gene expression in HC11 mammary epithelial cells.

The Cited protein family bind directly to p300/CBP through their conserved C-terminal acidic domain (Yahata et al., 2000). The p300/CBP (histone acetyltransferases p300/CREB-binding protein) are transcriptional integrators and play a crucial role in regulation of gene expression involved in cell growth, transformation and development. The interaction with Cited4 proteins is required for full transcriptional activation of target genes (Goodman and Smolnik, 2000).

Hypoxia-inducible factor 1 alpha (HIF1α) was shown to be a critical regulator of secretory differentiation during mammary gland development (Seagroves et al., 2003). The transactivation of target genes by HIF complexes requires the recruitment of p300/CBP by HIF1α and HIF2α (Sang et al., 2003). Since Cited4 binds to p300/CBP it is possible that Cited4, expressed at high level during pregnancy and lactation, may act as a co-activator linking the transcriptional regulator HIF to p300/CBP. Silencing the expression of Cited4 using siRNA in mammary epithelial cells can elucidate its possible role as a transcriptional co-activator during differentiation of mammary epithelial cells and mammary gland development.

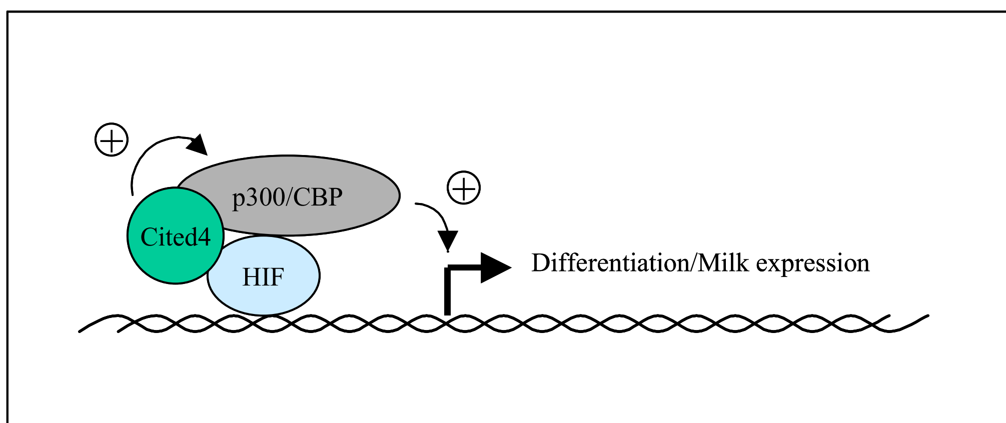

Figure 4.4 Model of Cited4 action during activation of genes involved in differentiation. Cited4 can bind to p300/CBP and may positively influence transcription of genes required for functional differentiation. Cited4 may also act as co-activator linking the transcription regulator HIF to p300/CBP and in this way co-activating the transcription of HIF targets genes involved in terminal differentiation of mammary epithelial cells.

4.2.4 Conclusions and Outlook

The data presented in this study describe an initial step to analyze genes found differentially regulated in mammary epithelial cells during their proliferation and differentiation. Various genes identified in microarrays are of high interest to analyze in more detail in order to determine their role in mammary gland development. Among the genes mentioned in the discussion two contribute to the process of proliferation Nol5, Kpna2, whereas two other genes are possibly required for proper functional differentiation Nkd2, Cited4. Apart from their normal function it was found that the selected genes can possibly contribute to breast cancer formation. To study the role of these selected candidate genes in proliferation and functional differentiation of mammary epithelial cells the established mammary epithelial cells HC11 line as well as freshly isolated primary mammary epithelial cells can be used, as shown in this study for the Idb proteins. Depending on their regulation pattern the chosen genes can be down-regulated using siRNA interference or stably over-expressed employing the lentiviral vector system for delivery and stable expression of the transgene. The effect of silencing or up-regulation of candidate genes on morphological and functional differentiation can be analyzed in a 3D culture system as described for Idb proteins.

If these genes show a clear phenotype if down-regulated or over-expressed, the role of these genes can be further studied *in vivo*. Transplantation of stably transduced mammary epithelial cells into cleared fat pads of the mammary gland will provide interesting data about the role of the candidate genes during the mammary gland development, *in vivo*. Importantly, the candidates are also interesting regarding the cancer drug development since it is possible that deregulation of their expression may contribute to tumor development. The described functional study of these genes will reveal if they can be consider as novel potential targets for anti-cancer therapies.

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ABBREVIATIONS

4EBP1	Eukaryotic initiation factor 4E binding protein 1
aa	Amino acid
ATP	Adenosine three phosphoran
APC	Adenomatous polyposis coli
APS	Ammoniumpersulfat
bp	Base pair
BSA	Bovine serum albumine
BRCA1	Breast Cancer Gene 1
BSA	Bovine Serum Albumine
CBP	CREB-binding protein
C/EBPβ	CCAAT enhancer binding protein beta
CIP	Calf intestinal phosphatase
Cm	Centi meter
CREB	Cre-element binding protein
DAPI	4´,6-diamidin-2´-phenylidol
DIP	Dexamethasone, insulin, prolactin
DMEM	Dulbeccos modified eangle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extra cellular matrix
E.Coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
eIF4A	Eukaryotic initiation factor 4A
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic initiation factor 4E
Elk-1	Ets-like protein-1
ERKs	Extracellular signal-regulated kinases
FBS	Foetal bovine serum
FCS	Foetal calf serum
FKBP12	FK506-binding protein 12 kDa
FRAP	Rapamycin associated protein
GβL	G protein β- subunit-like protein
GAS	Gamma interferon activation site
GAP	GTPase-activating protein
Grb2	Growth factor receptor bund protein 2
GSG-3β	Glycogen synthase kinase 3β
h	Hour
HBSS	Hanks bufferd salt solution
HRP	Horse raddish peroxidase
HIF	Hypoxia-inducible factor
Ig	Immunoglobulin
IGFs	Insulin-like growth factors
IR	Insulin receptor
IRES	Internal ribosome binding site
IRS	Insulin receptor substrate

JAK2	Janus-kinase 2
kDa	kilo Dalton
LAP	Liver enriched activator protein
LB	Luria-Beatani-Medium
LEF	Lymphoid enhancer factor
LIP	Liver enriched inhibitory protein
LZ	Leucine zipper domain
M	Molar
MAPK	Mitogen-activated protein kinase
MEK	MAPK/Erk kinase
MGF	Mammary gland factor
mM	mili Molar
mm	mili meter
MMP	Matrix metalloproteinase
MOI	Multiplicity Of Infection
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
NLS	nuclear localization signal
nm	nano meter
NPC	nuclear pore complex
OD_{600}	Optical wave lengh 600 nm
p	Plasmid
PAGE	Polyacrylamidgelelectrophorese
PAS	Per-arnt-sim homology
PBS	Phosphat Buffered Saline
PCR	Polymerase Chain Reaction
PDCD4	Programmed cell death protein 4
PDK1	Phosphoinositide-dependent kinase 1
PEI	Poliethylenimin
PI3K	Phosphoinositide 3-kinases
PKB	Protein Kinase B
PKCα	Protein kinase C alpha
Prl	Prolactin
PTEN	Phosphatase and tensin homolog
RAPT1	Rapamycin target 1
Rheb	Ras homolog enriched in brain)
RISC	RNA-inducing silencing complex
RNA	Ribonucleic acid
rpm	Rotation per minute
RT	Room temperature
S6K	S6 Protein kinase
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate- Polyacrylamidgelelectrophorese
SEP	Sirolimus effector protein
Shc	SRC-homology-2-domain transforming protein
siRNA	small interfering RNA
SOS	Son-of sevenless
Stat5	Signal transducer and activator of transcription
TBS	Tris Buffered Saline
TCF	T-cell factor

TSC	Tuberous sclerosis protein
TEB	Terminal end buds
TEMED	N,N,N´,N´-Tetramethylendiamin
TOR	Target of rapamycin
U	Unit
V/v	Volume to volume
WB	Western blot
WT	Wild type
W/v	Mass to volume
XTT	Sodium 3´-(1(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)bezene sulfonic acid

ZUSAMMENFASSUNG

In der vorliegenden Promotionsarbeit wurde die Funktion des Proteins mTOR (mammalian target of rapamycin) in der Organentwicklung am Modellsystem der murinen Brustdrüse untersucht. Der mTOR-Signalweg ist in die Regulation vieler zellulärer Prozesse involviert. Die Rolle des Proteins in der Organogenese scheint bis heute jedoch noch unklar zu sein. Hierbei wurde ein einzigartiges Organ (die murine Brustdrüse) verwendet, das im Laufe der Individualentwicklung verschiedene Stadien mit unterschiedlichen Proliferations- und Differenzierungseigenschaften durchläuft. Dadurch war es möglich, die Rolle von mTOR am adulten Tier unabhängig von der Embryonalentwicklung zu studieren. Um die Funktion des mTOR-Signalweges während des Wachstums und der Differenzierung zu untersuchen, wurden diverse Studien mit der Brustepithelzelllinie HC11 durchgeführt. Die Vorläufer dieser Zellen wurden ursprünglich aus einer schwangeren Maus in der Mitte der Schwangerschaft isoliert und für eine *in vitro* Kultivierung optimiert. Die HC11 Zellen proliferierten als adhärente Zellen in einer Plastikkulturschale. Durch eine laktogene Hormon-Mischung aus Dexamethason, Insulin und Prolaktin (DIP) konnte in diesen Zellen die Expression von β-Casein induziert werden. Aus der Expression dieses Milchproteins ließ sich ableiten, dass sich die Zellen im Stadium der vollen funktionellen Differenzierung befanden.

Die Zusammenfassung der Promotionsarbeit gibt einen Überblick über die erhaltenen Ergebnisse und die möglichen Schlussfolgerungen. Zu Beginn wurde die Aktivität von mTOR und vom Differenzierungsmarkerprotein β-Casein mit und ohne Inhibitor-Behandlung (Rapamycin) untersucht. Anschließend wurden Versuche durchgeführt, die zeigen, wie mTOR die Proliferation und die funktionelle bzw. morphologische Differenzierung der Brustepithelzellen durch Regulation sogenannter Idb-Proteine kontrolliert. Um die *in vitro* Daten in einem *in vivo* ähnlichen System zu bestätigen, wurden primäre, murine Brustepithelzellen einer Rapamycin-Behandlung unterzogen und die Auswirkungen auf ihre Differenzierung bestimmt. In einem weiteren Schritt wurden die Effekte einer Rapamycin-Behandlung auf die Entwicklung der murinen Brustdrüse und auf die Neugeborenen in einem Mausmodell *in vivo* analysiert. Ferner wurde die Rolle von mTOR in der Laktation von Brustepithelzellen *in vivo* untersucht.

Darüber hinaus wurden Funktionsstudien unter Verwendung zweier lentiviraler Systeme zur stabilen Überexpression bzw. zur Ausschaltung (RNA-Interferenz) der Idb-Proteine durchgeführt. Im letzten Teil der Arbeit wurden mittels Micro-Array-Technologie weitere Gene identifiziert, die in die Proliferation und die Differenzierung der Brustepithelzellline HC11 involviert sind.

In ersten Experimenten konnte gezeigt werden, dass mTOR in proliferierenden HC11 Zellen aktiviert ist. Seine für die Funktion benötigte Phosphorylierung war nach der Induktion mit laktogenen Hormonen stark erhöht, was darauf hindeutet, dass die mTOR-Aktivität in den späten Stadien der Differenzierung eine wichtige Rolle spielt. Um dies genauer zu untersuchen, wurden HC11 Zellen mit Rapamycin behandelt, einem spezifischen Inhibitor für mTOR, der die Aktivität von mTOR gegenüber seinen nachgeschalteten Zielproteinen hemmt. Wie erwartet, verloren die Rapamycin-behandelten Zellen ihre Fähigkeit funktionell zu differentieren. Die Expression der Milchproteine und die Menge an β-Casein mRNA wurden reduziert. Weitere Experimente mit Actinomycin D (Inhibitor der Replikation und Transkription) zeigten, dass Rapamycin die mRNA Stabilität nicht beeinflusst. Dies bedeutete, dass mTOR die Transkription von β-Casein regulieren konnte. Stat5 sind nachweislich essentiell für die Aktivierung der Transkription der Milchproteine. Es wäre daher möglich, dass mTOR die β-Casein Transkription durch Stat5 kontrolliert. Allerdings konnten entsprechende Experimente zeigen, dass mTOR die transkriptionelle Aktivierung des β-Casein Gens nicht durch Stat5 beeinflußt. Trotz Bindung von Stat5 an den β-Casein Promotor wurden andere wichtige Elemente für die vollständige Aktivierung der β-Casein Expression benötigt, die aber durch Rapamycin hemmbar waren. Aus diesem Grund wurde in der vorliegenden Arbeit untersucht, welche Transkriptionsfaktoren durch mTOR reguliert werden.

Es ist aus der Literatur bereits bekannt, dass mTOR ein Zielprotein im Insulin-Signalweg ist. Zahlreiche Studien belegen deutlich, dass die Aktivierung des Insulin-Signalweges notwendig ist für die Expression von Milchproteinen. Sogenannte Idb-Proteine (Inhibitoren der DNA-Bindung) werden ebenfalls durch diesen Signalweg reguliert. Diese Proteine sind Transkriptionsfaktoren. Ob zwischen den Proteinen mTOR und den Idb-Proteinen eine Verknüpfung auf Basis einer abhängigen Expression oder Aktivität besteht, ist noch nicht untersucht worden. Eine Vermutung ist, dass mTOR als Mediator durch Insulin stimuliert wird und die Expression bzw. Aktivität von Idb-Proteinen kontrolliert.

Es existieren zwei Idb-Proteine: Id1 und Id2. Id1 wird in hohem Maße während der Proliferation exprimiert. Eine Blockade der Expression dagegen bewirkt eine Abnahme in der Proliferationsrate. In der präsentierten Arbeit konnte klar gezeigt werden, dass eine Rapamycin-Behandlung eine Inhibition der Id1 Expression zur Folge hat. Diese Experimente lassen die Schlussfolgerung zu, dass mTOR die Proliferation der HC11 Zellen kontrolliert, indem es die Expression von Id1 steuert. Da Id1 auch in die Regulation der Zellinvasivität involviert zu sein scheint, wurde vermutet, dass eine Inhibition seiner Expression durch Rapamycin nicht nur die zelluläre Proliferationsrate reduziert. Gleichzeitig wurde die Fähigkeit der Zellen im Laufe einer morphologischen Differenzierung in einer dreidimensionalen Kultur in eine umgebende Matrix zu migrieren negativ beeinflusst. Studien mit der HC11 Zelllinie zeigten, dass Id2 für eine funktionelle Differenzierung benötigt wird. Einer Inhibition von Id2 folgte eine Abnahme in der β-Casein Expression. Es ist daher vorstellbar, dass mTOR die Differenzierung durch die Kontrolle der Id2 Expression kontrolliert. Tatsächlich war Expression von Id2 signifikant reduziert nach einer Behandlung der Zellen mit Rapamycin. Auch trotz einer Induktion der Zellen mit DIP war die Proteinexpression von Id2 stark gesenkt im Vergleich zu den Kontrollzellen. Diese Resultate weisen darauf hin, dass mTOR in die Regulation der funktionellen Differenzierung der Brustepithelzellen involviert ist, indem es die Id2 Expression auf transkriptionellem Level kontrolliert.

Um diese mittels *in vitro* Untersuchungen erhaltenen Ergebnissen der HC11 Zellen zu bestätigen, wurden die Auswirkungen einer Rapamycin-Behandlung auf die morphologische und funktionelle Differenzierung von primären, murinen Brustepithelzellen bestimmt. Diese Zellen gleichen in ihrem Verhalten einer *in vivo*-Zellantwort. Der Effekt von Rapamycin auf die morphologische Differenzierung wurde ermittelt, indem isolierte primäre Brustepithelzellen in einem Kollagen-Gel ausgesät und durch DIP eine morphologische Differenzierung induziert wurden. Die mit Rapamycin behandelten primären Zellen waren nicht mehr in der Lage, die charakteristischen, verzweigungsähnlichen Strukturen zu bilden. Morphologisch gesehen ähnelten sie den Rapamycin behandelten HC11 Zellen. Das heißt, dass die Fähigkeit von primären, murinen Brustepithelzellen in einer dreidimensionalen Kultur zu differenzieren von einem intakten mTOR-Signalweg abhing. Es zeigte außerdem, dass die *in vitro*-Daten der HC11 Zelllinie mit den Ergebnissen der Versuche der primären Zellen vergleichbar waren und die HC11 Zellen als zuverlässiges

Modellsystem für diese Art von Studien verwendet werden konnten. Die DIP-induzierten und Rapamycin behandelten Primärzellen waren nicht mehr fähig funktionell zu differenzieren, was zu dem beobachteten Fehlen des β-Casein Proteins passt. Daraus ließ sich schließen, dass der mTOR Signalweg wichtig war für die morphologische und funktionelle Differenzierung der primäre Brustepithelzellen *in vitro* und dass diese Ergebnisse im Einklang stehen mit den Studien der HC11 Zelllinie. Um die *in vitro* Studien zu bestätigen, war es nötig die Rolle vom mTOR Signalweg *in vivo* zu analysieren. Darum wurde der Effekt einer Rapamycin-Behandlung auf die Brustdrüsenentwicklung in einem Tiermodell getestet. Während der Schwangerschaft der Maus begannen die Brustepithelzellen in hohem Maße zu proliferieren. Dieser Prozess führte zu Verlängerung der Drüsengänge und letztendlich zur Formation von sekretorischen Kompartimenten, welche am Ende der Schwangerschaft fast die gesamte Brustdrüse ausfüllten. Zu Studienzwecken wurden Wildtyp-Mäuse ab der Mitte ihrer Schwangerschaft bis zum Tage der Geburt mit Rapamycin behandelt, um die Rolle von mTOR *in vivo* zu bestimmen. Die Kontrollgruppe dagegen erhielt nur das Lösungsmittel des Inhibitors ohne Rapamycin. Am Tage der Geburt wurden die Brustdrüsen isoliert und analysiert, ebenso die Embryos. Es zeigte sich, dass Rapamycin die Entwicklung der Embryos vollkommen blockiert hatte. Dies ist entweder auf eine direkte Inhibition der Proliferation bzw. Differenzierung der Brustepithelzellen zurückzuführen oder auf eine indirekte Hemmung der Embryonalentwicklung durch Rapamycin. Die Neugeborenen der Kontrollgruppe waren im Vergleich dazu normal entwickelt.

Die Aktivierung des Insulin-Signalweges ist essentiell für die korrekte Laktation der Brustdrüse (Milchabsonderung), wobei mTOR ein nachgeschaltetes Zielproteinen dieses Signalweges ist. Diese Tatsache war der Anlaß, die Rolle von mTOR in der Laktation von Brustepithelzellen *in vivo* zu analysieren. Die Brustdrüsenzellen erreichen den Zeitpunkt der stärksten funktionellen Differenzierung in der Laktation, wenn sie Teil des aveolaren Kompartiments sind, dass die Milchproteine sekretiert. Um die Funktion von mTOR während dieses Entwicklungsstadiums zu untersuchen, wurden schwangere Mäuse ab dem Tag 19 der Empfängnis bis zum Tag 10 der Laktation entweder mit Rapamycin oder mit einem Placebo behandelt. In der Zeit der 12tägigen Behandlung wurde die Entwicklung der mütterlichen Brustdrüse und der Nachkommen beobachtet. Der entscheidende Faktor, der das Wachstum der Nachkommen beeinflusste, ist die Milch, die in den mütterlichen Brustdrüsen gebildet

wurde. Die Wachstumskurve der Nachkommen wurde bis zum Tag 10 nach der Geburt erstellt. Hier zeigte sich, dass die Nachkommen der mit Rapamycin behandelten Mütter wesentlich langsamer an Gewicht zunahmen als die Nachkommen der unbehandelten Kontrollmäuse. Das verringerte Wachstum der Nachkommen der behandelten Mäuse konnte seine Ursachen in der Hemmung der terminalen Entwicklung der Brustdrüsen und damit der Milchproduktion haben. Um diese These zu bestätigen, wurden die Brustdrüsen von laktierenden Mäusen am Tag 10 der Laktation isoliert. Die Brustdrüsen der mit Rapamycin behandelten Mäuse waren signifikant kleiner als die der unbehandelten Kontrollgruppe. Die Expression der Milchproteine in den Brustdrüsen der behandelten Mäuse war um 60% verringert und ist zurückzuführen auf eine verschlechterte Entwicklung der aveolaren Kompartimente (Aveoli). Dies korrelierte mit dem durchschnittlichen Gewichtsrückstand der behandelten Nachkommen. Rapamycin hemmte somit klar die funktionelle Differenzierung der Brustepithelzellen *in vivo*. Die Aveoli der Kontrollmäuse waren größer und vollständig mit Milchproteinen gefüllt im Vergleich zu den Aveoli der behandelten Mäuse. Um zu zeigen, dass Rapamycin die terminale Differenzierung der Brustepithelzellen *in vivo* inhibiert, wurde die β-Casein Expression untersucht. Wie zu Beginn erwähnt, wurde die β-Casein Expression *in vitro* durch mTOR mittels Id2 reguliert. Es wurde daher vermutet, dass auch *in vivo* der gleiche Mechanismus zum Tragen kommt. Eine Abnahme der β-Casein Expression sollte demnach mit einer Reduktion der Id2 Expression verbunden sein. Es konnte gezeigt werden, dass in den Brustdrüsen von Rapamycin behandelten Mäusen am Tag 10 der Laktation die Expression von Id2 gegenüber den Kontrollmäusen reduziert war.

Die Experimente mit den HC11 Zellen deuteten an, dass eine Behandlung der Brustepithelzellen mit Rapamycin die Expressionslevel von Id1 und Id2 reduziert. Es wurde vermutet, dass mTOR das Wachstum und die funktionelle Differenzierung der Brustepithelzellen durch Idb-Proteine reguliert. Um diese Annahme zu bestätigen, wurden beide Idb-Proteine in HC11 Zellen überexprimiert. Ein lentivirales System wurde benutzt, um eine stabile Integration des Transgens (Id1 bzw. Id2) in das Genom von HC11 Zellen zu bewirken und um eine konstante Expression zu erzielen. Die entsprechenden Experimente zeigten, dass die HC11 Zellen das Id1 Protein in hohem Maße überexprimierten und resistent gegenüber einer Inhibition von Id1 durch Rapamycin waren.

Die Proliferation dieser modifizierten Zellen wurde durch eine Behandlung mit Rapamycin nicht beeinträchtigt. Diese Experimente deuteten stark darauf hin, dass mTOR die Proliferation von Brustepithelzellen durch die Expression von Id1 reguliert. Es war desweiteren in der vorliegenden Arbeit gelungen, zu analysieren, wie eine Überexpression von Id1 die Differenzierung von HC11 Zellen beeinflusst. Im Normalzustand wurde Id1 in proliferierenden Zellen hoch exprimiert. Die Expression nahm erst wieder ab, wenn das Stadium der Konfluenz erreicht war. Beides war eine Voraussetzung für eine *in vitro* Differenzierung. Interessanterweise unterdrückte eine Überexpression von Id1 eine Expression von Id2 und eine Induktion der β-Casein Expression durch laktogene Hormone. Dies heißt, dass Id1 verantwortlich war für ein Verharren der Zellen im Proliferationsstadium und eine Blockade der frühreifen Differenzierung durch Hemmung von Proteinen wie Id2, die in die Aktivierung der Differenzierung involviert waren. Eine Behandlung der HC11 Zellen mit Rapamycin inhibierte die Entwicklung der verzweigungsähnlichen Strukturen in der Kollagenmatrix. Der gleiche Effekt konnte im Falle der HC11 Zellen beobachtet werden, in denen mittels RNA-Interferenz die Id1 Expression stillgelegt wurde. Rapamycin bewirkte eine Runter-Regulation der Id1 Proteinexpression und damit einen Arrest der zellulären Proliferation. Die Id1 überexprimierenden HC11 Zellen waren dagegen insensitiv gegenüber dem inhibitorischen Effekt von Rapamycin und können Verzeigungsähnlichen Strukturen ausbilden. Dies stand im starken Gegensatz zu den Kontrollzellen, die nur sehr langsam proliferieren und nicht in die umgebende Matrix eindringen konnten. Diese Ergebnisse deuten darauf hin, dass mTOR die morphologische Differenzierung von Brustepithelzellen durch Id1 kontrolliert, welches direkt die Proliferation und das invasive Potential dieser Zellen reguliert. Nicht nur Id1 sondern auch Id2 scheint eine wichtige Rolle in der Entwicklung der Brustdrüsen zu spielen. Eine Ausschaltung der Id2 Expression verursachte eine Blockade der funktionellen Differenzierung der Epithelzellen durch eine Reduktion der Milchproteinexpression. Die Kontrollzellen, die mit dem leeren lentiviralen Vektor transduziert und mit Rapamycin bzw. DIP behandelt wurden, zeigten aufgrund von unvollständiger terminaler Differenzierung keine β-Casein Expression. Die Id2 überexprimierenden HC11 Zellen dagegen waren weiterhin in der Lage, trotz Rapamycin Behandlung funktionell zu differenzieren, was sich in der Expression von β-Casein zeigte.

Diese Ergebnisse zusammen mit früheren Beobachtungen beweisen, dass mTOR die terminale Differenzierung der Brustepithelzellen durch die Regulation der Id2 Expression beeinflusst.

An der Brustdrüsenentwicklung sind neben dem mTOR-Signalweg noch zahlreiche andere Signalwege beteiligt, die die Transkription von vielen Genen aktivieren, die allesamt involviert sind in der Bildung einer funktionellen Brustdrüse. Obwohl einige wichtige Signalwege und ihre Aktivatoren bereits bekannt sind, existieren wenige Kenntnisse über die Regulation ihrer nachgeschalteten Zielproteine. In der vorliegenen Arbeit wurde daher eine Methode (Micro-Array-Technologie) angewandt, um Gene zu identifizieren, die in die Proliferation und die Differenzierung der Brustepithelzellline HC11 involviert sind und um anschließend ihr Expressionsmuster bzw. ihre Funktion genauer zu studieren. Die Entwicklung der Micro-Array-Technologie ermöglicht parallele Studien von Transkriptionsprofilen tausender Gene in einem Experiment. Zu diesem Zweck wurde die Gesamt-RNA von drei verschiedenen Zellstadien der HC11 Zellen (Proliferationsphase, Wachstumsarrest aufgrund von Konfluenz, Stimulationsphase mittels DIP) isoliert. Dies erlaubte die Identifizierung von Genen, die zu den drei Zeitpunkten unterschiedlich stark exprimiert werden. Die Gesamt-DNA wurde in einem zweiten Schritt in cDNA (kodierende DNA) reverse transkribiert. Die Micro-Array Analyse wurde in Kooperation mit Dr. Peter Angel (Deutsches Krebsforschungszentrum, Heidelberg) durchgeführt. Die cDNA wurde dabei mit einem NIA-Chip hybridisiert. Dabei handelte es sich um einen PCR-basierenden cDNA Micro-Array, der 15000 verschiedene Gene abdeckt, von denen bekannt ist, dass sie während der murinen Embryonalentwicklung exprimiert werden. Anfangs konnte die Expression von ca. 3000 Genen in den HC11 Proben detektiert werden. Als nächstes wurden ca. 1800 Replikate identifiziert, d.h. Proben, bei denen durch einen Farbwechsel auf dem Micro-Array-Chip ein gleichbleibendes Regulationsmuster nachgewiesen werden konnte. Als Kandidaten für die weitere Analyse wurden 397 proliferations- bzw. 639 differenzierungs-assoziierte Gene ausgewählt. Das Expressionsmuster von einigen der identifizierten Zielgenen wurde in der vorliegenden Arbeit verifiziert. Nur Gene, deren Expressionsmuster bestätigt werden konnte, wurden für die weiteren funktionellen Tests verwendet.

Abschließend lässt sich aus der vorliegenden Arbeit zusammenfassen, dass Id1 und Id2 sowohl essentielle Transkriptionsfaktoren in der Entwicklung der Brustdrüsen sind als auch in der Proliferation und der Differenzierung der Brustepithelzellen *in vitro*.
Die Ergebnisse der RNA-Interferenz- und Rapamycin-Versuche zeigen, dass mTOR die Proliferation und die Differenzierung der Brustepithelzellen durch die Expression beider Idb-Proteine kontrolliert. Die Aktivität von mTOR wird demnach für das Anschalten der Proliferationsphase durch Id1 und für die Expression von differenzierungsspezifischen Genen durch Id2 benötigt. Das richtige Gleichgewicht zwischen der Expression der individuellen Idb-Proteine ist ein kritischer Faktor für die Entwicklung der Brustepithelzellen und ist vermutlich während der Tumorgenese gestört. Aus diesem Grund ist ein besseres Verständnis des mTOR-Signalweges und der damit verbundenen Idb-Protein Expression hilfreich, um weitere effektive Ansätze in der Krebstherapie zu etablieren. Beide Proteine wurden auch mittels der Micro-Array-Analyse identifiziert und ihr differenzielles Expressionsmuster während der Proliferation und Differenzierung der HC11 Zellen bestätigt.
Die Arbeit bietet eine Basis für weitere Studien bezüglich der Genexpressionsmuster während der Proliferation und der Differenzierung der Brustepithelzellen. Zahlreiche der mittels Micro-Array-Analyse identifizierten Gene eignen sich für eine weitere Untersuchung ihrer Rolle in der Brustdrüsenentwicklung. Einige dieser Gene sind möglicherweise an der Bildung von Brustkrebstumoren beteiligt. Die Rolle dieser Gene in der Proliferation und der funktionellen Differenzierung der Brustepithelzellen wird deshalb in dieser Arbeitsgruppe weiter untersucht. Abhängig vom Regulationsmuster werden die genannten Gene mittels RNA-Interferenz ausgeschaltet oder stabil überexprimiert mittels der lentiviralen Transduktion. Die Epithelzellen, die diese Modifikationen über einen längeren Zeitraum zeigen, können in Transplantationsexperimente mit Mäusen in das präparierte Fettpolster der Brustdrüse eingesetzt werden. Solche *in vivo*-Experimente würden interessante Daten zur Funktion der einzelnen Gene während der Brustdrüsenentwicklung liefern. Solche Gene können auch in der Wirkstoffentwicklung eine Rolle spielen, da eine Fehlregulation ihrer Proteinexpression möglicherweise mit einer Krebsentstehung assoziiert ist. Sie könnten dann als potentielle Ausgangspunkte für eine neue Strategie in der Krebstherapie verwenden werden.

PUBLICATIONS

1. Articles

Jankiewicz M, Groner B, and Desrivières S (2006) Mammalian target of rapamycin regulates the growth of mammary epithelial cells through the inhibitor of deoxyribonucleic acid binding Id1 and their functional differentiation through Id2.
Mol Endocrinol. 2006 Oct; 20 (10):2369-2381.

2. Posters

Marcin Jankiewicz, Sylvane Desrivieres and Bernd Groner (2005). Mammalian Target Of Rapamycin Signalling Is Required For Functional Differentiation Of Mammary Gland Epithelial Cells.
13th International AEK Cancer Congress, Wurzburg, Germany 2005

Marcin Jankiewicz Bernd Groner and Sylvane Desrivieres (2004). TOR target of rapamycin is a critical regulator of differentiation of the mammary gland.
EMBO molecular medicine meeting Bad Staffelstein, Germany 2004

Marcin Jankiewicz, Sylvane Desrivieres and Bernd Groner (2004). Role of mTOR in the differentiation of mammary epithelial cells.
COST B20 European Scientific Conference, Warsaw, Poland 2004

Marcin Jankiewicz, Sylvane Desrivieres and Bernd Groner (2004). Role of mTOR in the differentiation of mammary epithelial cells.
12th International AEK Cancer Congress, Wurzburg, Germany 2003

Sylvane Desrivières, Nahomi Castro-Palomino Laria, Marcin Jankiewicz, Carrie Shemanko, Boris Brill, Bernd Groner (2002). Insight into the differentiation program of mammary epithelial cells: Large-scale analysis of protein expression during mammogenesis
NGFN/DHGP Symposium, Berlin, Germany 2002

CURRICULUM VITAE

Marcin Jankiewicz
Chemotherapeutisches Forschungsinstitut
Georg-Speyer-Haus
AG Groner
Paul-Ehrlich-Str. 42-44
D-60596 Frankfurt am Main
e-mail: marcin.jankiewicz@interia.pl

EXPERIENCE

2002-2005 Georg-Speyer-Haus, Institute for Biomedical Research, GERMANY
Postgraduate Student

2001-2002 Medicine School of Poznan, POLAND
Department of Biology and Medical Parasitology
Research Assistant of head of the department

1998-2001 UAM, Institute of Molecular Biology and Biotechnology, POLAND
Department of Biochemistry and Biopolymers
Unergraduate Student

EDUCATION

2002-2005 Georg-Speyer-Haus in Frankfurt am Main, Germany
PhD thesis: Identification and characterisation of genes and signaling pathways involved in proliferation and differenatiation of mammary epithelial cells
Supervised by Prof. Dr. Bernd Groner

1996-2001 Adam Mickiewicz University in Poznan, Poland
Master of Science degree in molecular biology
Master essay theme: Mutagenesis of *bar* gene
Supervised by Prof. Dr. Jacek Augustiniak

1992-1996 Emilia Szczaniecka Secondary -School at Ostrow Wlkp, Poland
Secondary- school certificate specialization biochemistry

ADDITIONAL INFORMATION

Languages: English-fluent
Polish-native speaker,
German-communicative

Computer skills: Oligo v5.0, DNASIS, Dnastar, NSBI, ExPasy
Adobe Photoshop, Micrography Picture Publisher,
Corel Draw, Corel Photo-Paint,
Word, Power Point, Excel, Outlook Express, Internet Explorer

Awards: The best scientific poster Amber Rose Award, COST Meeting Warsaw, Poland 2004
The best student of Biology, Adam Mickiewicz University of Poznan, Poland 2001
The honourable mention for student very good results in learning Ostrow Wielkopolski, Poland 1996

PRACTICAL EXPERIENCE

- **Methods:** DNA, RNA and protein isolation and purification, RT-PCR, Real-Time PCR, RAPD-PCR, DNA agarose and polyacrylamide gel electrophoresis, DNA sequencing, DNA digestion with restriction nucleases, DNA insert ligation into vector, protein electrophoresis SDS-PAGE, Southern blot, Northern blot, Western blot, ELISA, immunofluorescence
- **Microscopy and Photography:** light and immunofluorescence microscope operation, taking professional research photos, microinjection microscope
- **Virology:** viral vectors generation, titration, use for stable transfection of mammalian cells (L2 laboratory)
- **Microbiology:** bacterial cell culture maintenance, transformation, plazmids and proteins overexpression, preparation of bacterial culture media, Total organic carbon (TOC) analysis, Gel clot (LAL) test
- **Plant tissue culture:** maintenance, micropropagation, bioreactors, culture media preparation
- **Mammalian cell culture:** whole organ culture, 3D culture, transient transfection, stable transduction of mammalian cells with viral vectors, over expression and silencing (siRNA) of target genes, primary cells isolation proliferation and adhesion assays, soft agar and invasion assays, reporter gene assay, FACS
- **Laboratory animals:** handling with mice and rats**,** organ and blood extractions, antibody production
- **Histology:** isolation, preparation and staining of samples
- **Functional assays**: drug testing in *vitro* and in *vivo*

PERSONAL DATA

Marital status: single
Age: 29
Nationality: Polish
Date of birth: 01.06.1977
Place of birth: Poznan, Poland

HOBBY

Practice aikido, photography, cycling, travelling

DECLARATION

The work described in this thesis is original and has not previously been submitted for a degree or diploma in any other University or College, and to the best of my knowledge, dose not contain material previously published or presented by any other person, except where due to reference is made in the text.

Frankfurt am Main 2006-11-21

Marcin Jankiewicz