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Differential gene expression of potential stem/progenitor cell markers in murine mammary epithelial cells (HC11)

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For my family and Marietta

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Abstract

Epithelial cells of the mammary gland have the ability to proliferate, differentiate and undergo apoptosis. A murine model system for studying mammary epithelial cell behavior is the HC11 cell line, which has some stem and/or progenitor cell characteristics. HC11 cells can be maintained, in response to special treatment conditions, as undifferentiated cells, competent cells capable of responding to lactogenic hormones, or they can be induced to differentiate. Based on data obtained from a gene array, the aim of this project is to investigate differentially expressed genes in HC11 cells, which might be stem/progenitor cell marker candidates and determine their functions in HC11 cells. Polymerase chain reaction (PCR) experiments revealed that *Lgals1* and *Ran* were upregulated in undifferentiated compared to competent and induced HC11 cells. These findings were confirmed and quantified in this project with real-time PCR experiments. Western Blot experiments confirmed the upregulation of galectin-1 (Gal-1), the *Lgals1* gene product, in undifferentiated compared to competent, induced and differentiated HC11 cells. Fluorescent activated cells sorting (FACS) analysis of undifferentiated HC11 cells revealed that 60.85% of the gated HC11 cells expressed stem cell antigen 1 (Sca1), 90.12% of the cells expressed Gal-1 and 60.77% of the gated HC11 cells expressed Gal-1 and Sca1. Galectin-1 positively influences cell proliferation and migration of certain cell types. Knockdown of *Lgals1* in HC11 cells followed by functional assays measuring proliferation or migration might give further insight into how undifferentiated cells are regulated. In this study it has been shown that galectin-1 (Gal-1), the *Lgals1* gene product, is upregulated in undifferentiated compared to competent, induced and differentiated HC11 cells, functional assays might reveal an important function of galectin-1 in HC11 cells, as well as in stem/progenitor cells of the mammary gland and FACS experiments revealed that galectin-1 might be used to enrich stem/progenitor cell populations of the mammary gland.

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1. Introduction

1.1 The mammary gland

The mammary gland in humans as well as in other organisms is an organ that goes through significant developmental changes during embryogenesis, puberty, pregnancy, lactation and involution (Liu *et al.*, 2005 and Hennighausen *et al.*, 2001). The mammary gland consists of two cellular compartments, the epithelium and the stroma (Hennighausen *et al.*, 2001). The epithelium consists of a ductal tree that develops during puberty and a lobuloalveolar compartment that develops during each pregnancy (Hennighausen *et al.*, 2001). The branching structure of ducts, which are surrounded by myoepithelial cells, end in lobules, which are composed of alveoli and these structures in turn are composed of epithelial cells (Hennighausen *et al.*, 2004). The epithelial cells, that mainly form the alveoli, are secretory cells, which undergo differentiation during each pregnancy (Hennighausen *et al.*, 2001).

In summary, two primary epithelial cell lineages (myoepithelial and luminal) form the epithelium of the mammary gland (Shackleton *et al.*, 2006). Expansion of the mammary epithelium during puberty and with each pregnancy before the subsequent involution, characterized by a rapid loss of tissue function, degeneration of alveolar structures and loss of epithelial cells, are evidence for the drastic changes the mammary gland undergoes through

all the developmental stages and that supports the theory of the existence of stem-like cells in the mammary gland (Deome *et al.*, 1959; Kordon and Smith, 1998; Smith and Chepko, 2001; Welm *et al*., 2002; Clayton *et al*., 2004; Dontu *et al*., 2004; Liu *et al*., 2005; Woodward *et al*., 2005; Shackleton *et al.*, 2006; Smith, 2006; Stingl *et al*., 2006 and Tan *et al*., 2006).

1.2 Stem cells and the "cancer stem cell" theory in the mammary gland

1.2.1 Stem cells of the mammary gland

Stem Cells (SCs) are undifferentiated cells that have the unique capacity of self renewal, i.e.: the ability to regenerate new SCs, as well as the potential to generate all of the cell types of the tissue in which they exist (Chepko and Smith, 1996; Reya *et al.*, 2001; Dontu and Wicha, 2005 and Morrison and Kimble, 2006). To ensure self-renewal, SCs undergo two types of cell division: symmetric, that gives rise to two identical cells with SC properties and asymmetric, that gives rise to one cell with SC properties and one progenitor cell, which is more committed to differentiation (Morrison and Kimble, 2006). In the mammary gland stem/progenitor cells have the capacity of self-renewal as well as the ability to generate myoepithelial cells, ductal epithelial cells and alveolar epithelial cells, the three lineages that comprise the lobulo-alveolar structures of the gland (Dontu *et al.*, 2004). SC populations in general differ from progenitor cell populations: progenitor cells proliferate actively in response to special signals (Dontu *et al.*, 2004) and they have limited self-renewal potential in comparision to SCs (Smith, 2006).

So far, due to the lack of specific markers it is not possible to isolate SCs from progenitor cells in the mammary gland (Stingl *et al.*, 2006), although they can be distinguished morphologically (Gudjonsson *et al.*, 2002).

1.2.2 The breast "cancer stem cell" theory

Breast cancer is the most diagnosed form of cancer and has the second highest mortality rate of all forms of cancer in western women (Jemal *et al.*, 2006). Over the past decades a lot of progress has been made in modeling human breast cancer in mice and using these models to improve cancer prevention and cancer drug discovery, but there is still no cure for the deadly disease of metastatic breast cancer (Jemal *et al*., 2006).

Cancer is currently viewed as a disease that depends on multiple genetic mutations and undifferentiated, division-competent cells are seen as prime targets for mutations (Al-Hajj *et. al.*, 2004). Researchers now believe that tumours might originate from transformed normal adult SCs which gives rise to the idea that similar signaling pathways may regulate the cell fate of normal adult SCs and cancer cells, whereby cancer cells may include a small population of "cancer stem cells" (CSCs) (Reya *et al.*, 2001). Therefore, the attention of many cancer biologists has turned to the SC compartments of all tissues. There are several facts that support the theory of a CSC population within the tumour (Liu *et al.*, 2005). The most important implication of this theory is that normal adult SCs and CSCs might share a wide variety of features such as the capacity for self-renewal, the ability to differentiate, anti-apoptotic pathways, membrane transporter activity, anchorage independence and the potential to migrate and form metastasis (Liu *et al.*, 2005).

Due to the fact that adult SCs are slowly dividing and long-lived cells they are exposed to damaging agents over long periods of time which might result in an accumulation of mutations and in the worst case, in transformation (Liu *et al.*, 2005). Mammary carcinogenesis might result from the deregulation of normal SC pathways and therefore those pathways can be seen as new targets for new approaches of breast cancer treatment (Liu *et al.*, 2005).

A solid tumour is a complex structure consisting of heterogenous cancer cells (Reya *et al.*, 2001). There are two models who explain this heterogenity (Figure 1a and 1b). Figure 1a illustrates a situation where most of the heterogenous tumour cells can proliferate without restriction and form new tumours (Reya *et al.*, 2001). Figure 1b suggests the presence of CSCs from which the heterogenous cancer cells originate (Reya *et al.*, 2001).

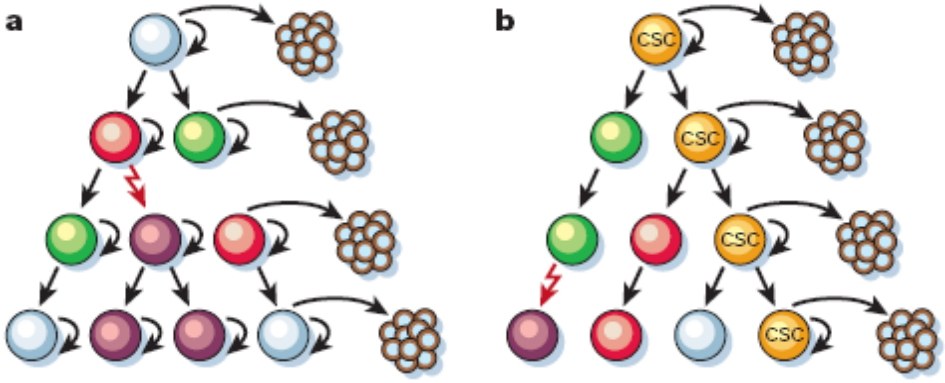


Figure 1a and 1b (Reya *et al.*, 2001): The "cancer stem cell" theory. **Figure 1a** illustrates a situation where most cancer cells in a tumour have the potential to proliferate extensively and form new tumours. **Figure 1b** suggests instead, that within a tumour only a subset of CSCs have the potential for extensive proliferation and the ability to form new tumours (Reya *et al.*, 2001).

Probably the most important difference between these two models is that in case of the CSC theory, the tumour originates from only a few CSCs, which might have major impact on the development of new treatment strategies (Reya *et al.*, 2001).

The importance of the CSC theory regarding new treatments is illustrated in Figure 2. Drugs which have been developed so far for cancer treatment have been identified by their ability to shrink tumours (Reya *et al.*, 2001). This is an important fact, since it seems that SCs from a given tissue seem to be more resistant against anti-cancer drugs than "normal" cells from the same tissue (Harrison and Lerner, 1991). If this is also the case with CSCs, it's likely that those cells also might be more resistant against chemotherapeutics than "normal" cancer cells (Reya *et al.*, 2001). In conclusion that supports the theory that targeting only the "normal" cancer cells while sparing out the small population of CSCs might result in a regrowth of the tumour (Reya *et al.*, 2001).

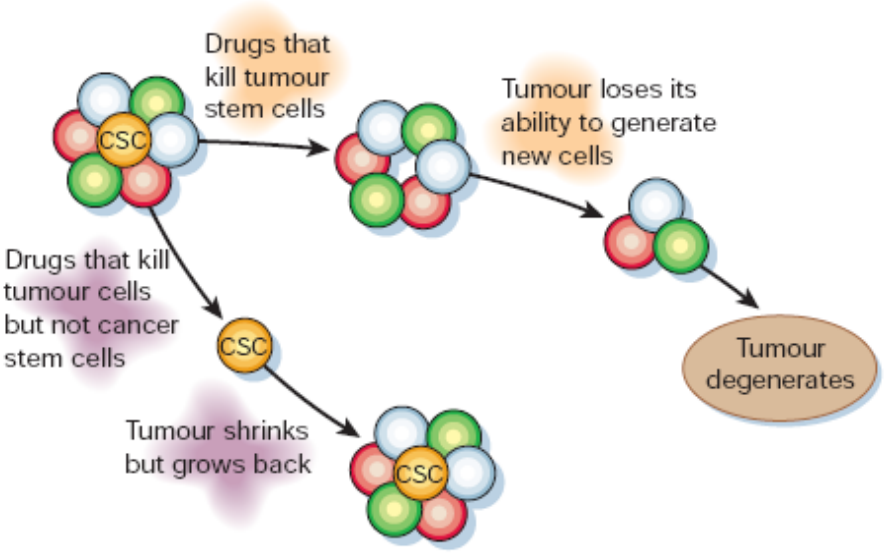


Figure 2 (Reya *et al.*, 2001): The implications of the "cancer stem cell" theory on conventional treatments and new therapeutic strategies. Current treatments might shrink the tumour, but possibly they do not terminate CSCs. By sparing out CSCs this subset of cancer cells might be able to regenerate the tumour. New cancer treatment strategies are now focusing on selectively targeting CSCs (Reya *et al.*, 2001).

A whole range of mutations are necessary for a cell to actually become cancerous (Knudson *et al.*, 1973). As mentioned above, the accumulation of many mutations requires a long time and

the life-span of progenitor and "normal" cells is limited (Al-Hajj *et al.*, 2004). CSC is a term used to describe a cancer cell that has self-renewal ability as well as the potential to differentiate (Al-Hajj *et al.*, 2004). So far it is not clear, if CSCs derive from SCs or progenitor cells (Al-Hajj *et al.*, 2004).

In order to maintain the disease, the transformed cell has to overcome the genetic restrictions for self-renewal and proliferation (Morrison *et al.*, 2002). This can only be realized either in a normal SC (Figure 3), which has lost the restriction for proliferation due to transformation, or in a progenitor cell, which gained the ability for self-renewal through mutations (Al-Hajj *et al.*, 2004).

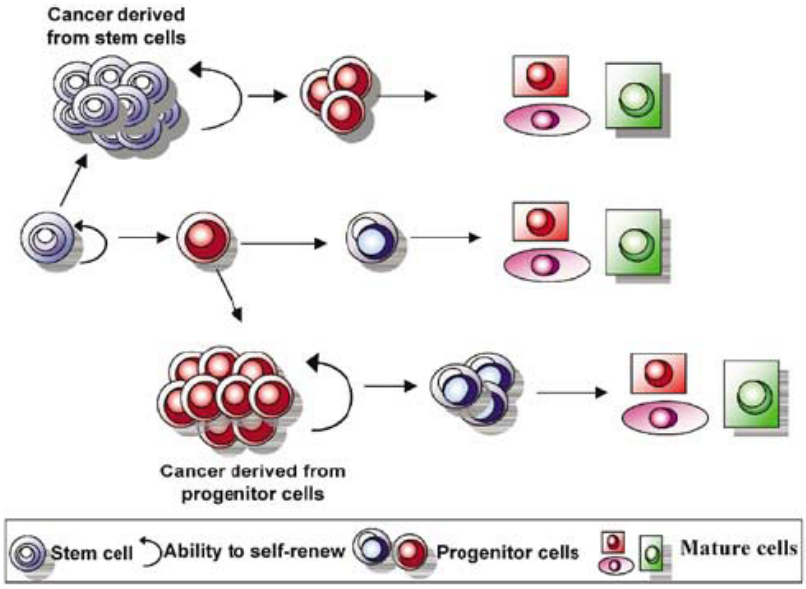


Figure 3 (Al-Hajj and Clarke, 2004): The origin of "cancer stem cells". CSCs derived from adult SCs can make use of the self-renewal machinery of adult SCs, whereas CSCs derived from progenitor cells have to overcome self-renewal restrictions by oncogenic mutations (Al-Hajj and Clarke, 2004).

The possibility of selectively isolating SCs of mammary gland would allow the study of the signaling pathways and mechanisms that regulate *proliferation*, *self-renewal*, survival and

differentiation of SCs and this in turn will give further insight in the mechanisms involved in breast cancer formation (Stingl *et al.*, 2006). One has to distinguish between the terms *self-renewal* and *proliferation*. The potential of self-renewal of a SC describes the kind of cell division in which either one or both daughter cells have the same capacity to proliferate and differentiate as the parental cell (Al-Hajj and Clarke, 2004). When progenitor cells proliferate, their daughter cells become more and more differentiated and their potential to proliferate diminishes with every cell cycle (Al-Hajj and Clarke, 2004).

The main problem regarding the CSC theory is, that the investigation of the properties and the analysis of the regulation of possible mammary SCs or possible mammary CSCs has simply been limited by a lack of methods for their isolation (Stingl *et al.*, 2006). Recent work of Dontu *et al*., 2003, Al-Hajj *et al.* (2003), Stingl *et al.* (2006) and Shackleton *et al.* (2006) rises the hope that a successful and specific isolation of SCs of the mammary gland and CSCs of the mammary gland might be accomplished in the near future.

A combination of surface markers ($CD24^{med}$ and $CD49f^{high}$) was used by Stingl *et al.* to isolate cells from the murine mammary gland and Stingl *et al.* referred to this subset of cells as "mammary repopulating units" (MRUs) (Stingl *et al.*, 2006). The definition of a MRU, or in other words the definition of a possible SC of the murine mammary gland was, that the cells had to be individually able to regenerate a murine mammary gland within 6 weeks *in vivo* while, at the same time, undergo ten or more symmetric cell divisions (Stingl *et al.*, 2006).

Shackleton *et al.* isolated cells from the murine mammary gland and showed that a single cell with the surface marker expression pattern $CD45^{-}CD31^{-}TER119^{-}CD29^{hi}CD24^{+}$ was able to regenerate a mammary gland *in vivo* (Shackleton *et al*., 2006). Their results revealed that cells

within the $CD45^{-}CD31^{-}TER119^{-}CD29^{hi}CD24^{+}$ subpopulation were multipotent and had self renewal capacity, proving the mammary gland SC quality of this isolated subpopulation.

Al-Hajj *et al.* found out by growing human breast cancer cells in mice, that a small subset of breast cancer cells was able to regrow a tumour in mice (Al-Hajj *et al.*, 2003). Al-Hajj *et al.* referred to these cells as "tumour initiating cells" (TICs), in comparision to "non tumour initiating" cells, and identified them as a $CD44^{+}CD24^{-\backslash low}Lineage^{-}$[1] (Al-Hajj *et al.*, 2003). Transplantation experiments revealed that as few as 100 TICs were able to regrow a tumour, whereas tens of thousands of "non tumour initiating" cells were not able to regrow a tumour in mice (Al-Hajj *et al.*, 2003). Their results also revealed that TICs undergo processes similar to differentiation and self-renewal as normal SCs (Al-Hajj *et al.*, 2003).

In addition to the two approaches described above, several researchers have developed a variety of methods to identify SCs of the mammary gland in mice: 5-bromo-2-deoxy-uridine (BrdU) label-retention studies for example, whereby in theory SCs of the murine mammary gland retain BrdU, while proliferative more active cells lose the label (Welm *et al.*, 2002). It also has been shown that stem cell antigen 1 expressing ($Sca1^{+}$) cells of the murine mammary gland contain a subpopulation of quiescent cells with low turn-over rates that show elevated outgrowth activity compared to Sca^{-} cells (Welm *et al*., 2002).

In attempts to isolate possible SCs of the human mammary gland, marker proteins for luminal cells such as epithelial membrane antigen (EMA) and epithelial specific antigen (ESA), as well as marker proteins for myoepithelial cells such as lymphoblastic leukaemia antigen (CALLA) were used in combination to enrich possible SC populations (Clayton *et al.*, 2004).

[1] "$Lineage^{-}$" is an abbreviation for $CD2^{-}CD3^{-}CD10^{-}CD16^{-}CD18^{-}CD31^{-}CD64^{-}CD140b^{-}$ cells.

In side populations, characterized by their ability to efflux the dye Hoechst 33342, breast cancer resistance protein (BCRP) was highly expressed (Clayton *et al.*, 2004). Using a combination of EMA, ESA, CALLA and BCRP, three possible SC populations of the human mammary gland were isolated, all of them were able to differentiate into luminal and myoepithelial cells (Clayton *et al.*, 2004). A study carried out by Alvi *et al.* showed that a subset of undifferentiated cells of the human mammary gland was able to efflux the dye Hoechst 33342 and outgrowths were observed in some cases when this subset of undifferentiated cells was injected into the cleared fat pad of mice (Alvi *et al*., 2002). This observation supports the possibility that the isolated side population had SC qualities (Alvi *et al*., 2002).

Although a lot of progress has been made over the past few years in isolating SCs of the mammary gland, the present methods still remain unspecific (Alvi *et al*., 2002 and Shackleton *et al.*, 2006). The isolation of potential stem/progenitor cells of the mammary gland is a complex task. On the one hand it has been shown that a $Sca1^{+}$ population exists within the mammary gland and that this population is enriched in slowly dividing, largely quiescent cells, in other words possible stem/progenitor cells of the mammary gland (Welm *et al*., 2002 and Woodward *et al*., 2005) But this also indicates that Sca1 might not solely be expressed on stem/progenitor cells, it is also possible that Sca1 is also present on more differentiated cells of the mammary gland. On the other hand the complexity lies in the heterogenous nature of stem/progenitor cells. For example, it has been reported that a distinct $Sca1^{+}$ side population of the murine mammary gland, which was also able to efflux the dye Hoechst 33342, was capable of repopulating the cleared fat pad of mice in transplantation experiments, indicating the stem/progenitor cell quality of the isolated side population (Coppock and Clarke, 2004 and Welm *et al*., 2002). On the other hand no $Sca1^{high}$ expression nor the ability to efflux the dye Hoechst 33342 were observed in other trials, where cells isolated from the murine

mammary gland with the surface marker expression pattern $CD45^{-}CD31^{-}TER119^{-}$ $CD29^{hi}CD24^{+}$ were able to regenerate a mammary gland *in vivo* (Shackleton *et al*., 2006).

The mammary gland comprises a complex mix of heterogenous cell types (Shackleton *et al.*, 2006) and even possible SCs or progenitor cells of the murine mammary gland exhibit different surface marker expression and behave different upon treatment with the dye Hoechst 33342 (Coppock and Clarke, 2004; Shackleton *et al*., 2006 and Welm *et al*., 2002), explaining the difficulties of specifically isolating possible stem/progenitor cells of the mammary gland.

The goal of this project is investigate the pattern of expression and the function of differentially expressed genes, which also might be stem/progenitor cell marker candidates using the model of HC11 cells. The results might contribute to a new approach in specifically isolating stem/progenitor cells of the mammary gland.

1.3 Potential HC11 marker genes

As an experimental model I made use of the prolactin (PRL) responsive murine mammary epithelial HC11 cell line that originated from COMMA-1D cells (Ball *et al.*, 1988), which in turn were derived from mammary tissue of mid-pregnant BALB/c mice (Danielson *et al.*, 1984). This cell line serves as a suitable model for differentiation as HC11 cells can be maintained in the undifferentiated stage, and they are also capable of differentiation in response to lactogenic hormones (Ball *et al.*, 1988; Hynes *et al.*, 1990 and Humphreys *et al.*, 1997), whereby this cell line mimics the different stages of mammary gland development (Danielson *et al.*, 1984). There are four stages in which HC11 cells can be maintained: undifferentiated, competent, induced and differentiated (Ball *et al.*, 1988 and Jankiewicz *et al.,*

2006). HC11 cells proliferate in an undifferentiated state, in media containing insulin (INS) and epidermal growth factor (EGF) (Ball *et al.*, 1988 and Jankiewicz *et al.,* 2006). After reaching 100% confluence the media containing INS and EGF is replaced by media containing just EGF. The activation of the EGF receptor promotes growth and inhibits differentiation (Taverna *et al.*, 1991). Furthermore, the activation of the EGF receptor in confluent cells transfers HC11 cells to a competent state, when HC11 cells produce extracellular matrix (ECM) proteins (Taverna *et al.*, 1991 and Chammas *et al.*, 1994) and gain the ability to respond to lactogenic hormones (Ball *et al.*, 1988). After removal of the media containing EGF, differentiation of HC11 cells can be induced by the hormone mix INS, glucocorticoid and PRL (Topper *et al.*, 1980). In this project dexamethasone (DEX), insulin (INS) and prolactin (referred to as DIP, see Material and Methods) is used to induce cellular differentiation of HC11 cells (Ball *et al.*, 1988). The differentiation of HC11 cells can be confirmed by detecting β-casein synthesis in the cells (Ball *et al.*, 1988). A detectable amount of β-casein is present in HC11 cells after 24 hours of DIP treatment and further accumulates during five days of treatment with the same lactogenic hormones (Ball *et al.*, 1988), when the cells are fully differentiated. In this project the DIP treatment lasts for 1 hour (see Material and Methods), and differentiation can therefore not be monitored by measuring the amount of β-casein in the cells. Furthermore 1 hour DIP treated HC11 cells are not fully differentiated (Shemanko, personal communication) and therefore the term "induced" will be used for 1 hour DIP treated HC11 cells.

Another important consideration is that the HC11 cell line cells are believed to have stem and/or progenitor cell characteristics, as they exist as undifferentiated cells and are able to differentiate (Cella *et al.*, 1996 and Hebbard *et al.*, 2000). The SC characteristics of the HC11 cell line are still being investigated to determine whether HC11 cells are more alike an adult SC or a progenitor cell. Markers that are specific for either SCs or progenitors are also

required to address this point. Experiments conducted in this laboratory showed that 99 percent of undifferentiated HC11 cells express CD24 and 25 percent to 50 percent of undifferentiated HC11 cells express Sca1 (Perotti, unpublished data). It has been reported that these proteins are expressed in a subpopulation of murine mammary epithelial cells, which showed elevated outgrowth activity *in vivo* (Shackleton *et al*., 2006 and Welm *et al*., 2002). Furthermore the injection of HC11 cells into the cleared fat pad of mice has led to regeneration of ductal-like structures (Humphreys and Rosen, 1997), proving the stem/progenitor cell quality of HC11 cells.

There is evidence that there are differences in the gene expression of several types of SCs and their differentiating progeny (Tanaka *et al.*, 2002 and Glover *et al.*, 2006). Using murine mammary epithelial HC11 cells as a model system, a gene array based on the NIA 15k cDNA library of developmentally expressed genes was carried out in collaboration with Dr. Peter Angel *et al.* (see appendix). Therein the gene expression HC11 cells was compared between the three stages undifferentiated, competent and induced (Shemanko, personal communication). The genes identified by the gene array by Dr. Angel *et al.* were ranked upon the highest levels of differential expression and upon the reproducibility of the qualitative comparision of their expression in undifferentiated, competent and induced HC11 cells. Out of the top eleven genes (see appendix) four genes that showed a higher expression in undifferentiated compared to induced HC11 cells were chosen (see Table 1, column "Fold change, undifferentiated vs. induced").

The selected genes are *Lgals1*, *Ran*, *Ppm1α* and *Rab14*. *Rab14* has been chosen because it was ranked third place on the gene array list (*Tcea1* and *Serbp1* were ranked first and second place; see next paragraph why these genes have been discarded). *Lgals1*, *Ran* and *Ppm1α* have been selected due to the fact that the three genes showed at least a 1.1-fold upregulation

between undifferentiated compared to competent and undifferentiated compared to induced HC11 cells and a difference as low as 0.9-fold between the expression of *Lgals1*, *Ran* and *Ppm1α* in competent compared to induced HC11 cells (see Table 1, column "Fold change"). The fact that the differences in gene expression between undifferentiated compared to competent and undifferentiated compared to induced HC11 cells are higher than the differences in gene expression between competent and induced HC11 cells for *Lgals1* and *Ppm1α* supports the possibility that these genes are downregulated by the differentiation process and that these genes might be stem/progenitor cell marker candidates. Another reason why these genes have been selected was their potential functional role in HC11 cells and possible stem/progenitor cells of the mammary gland. Galectin-1 (Gal-1), the *Lgals1* gene product, promotes proliferation in murine neural stem cells (mNSCs), (Sakaguchi *et al.*, 2006), whereas expression knockdown of *Lgals1* in endothelial cells inhibits proliferation and migration (Thijssen *et al.*, 2004), indicating an important functional role of galectin-1 in HC11 cells. It has been shown that Ppm1α abolishes transforming growth factor β1 (TGF-β1) signaling, a signaling pathway that causes growth arrest of mammary epithelial cells (García-Montero *et al.*, 2001). Furthermore Ppm1α abolishes bone morphogenic protein 2 (BMP-2) signaling by dephosphorylation of Smad1 (Duan *et al.*, 2006), and transforming growth factor-β (TGF-β) signaling by dephosphorylation of Smad2 and Smad3 (Lin *et al.*, 2006), which might indicate that Ppm1α positively influences proliferation of undifferentiated HC11 cells (see 1.3.3). The genes *Ran* as well as *Rab14* had furthermore been chosen due to the fact that these two candidates are supposed to be proto-oncogenes, indicating an important functional role of the two proteins in carcinogenesis (Azuma *et al.*, 2004 and Cheng *et al.*, 2005).

The reason why the genes *Serbp1*, *Wwc1*, *Ywhag* and *MNI07591* (see appendix) were discarded was that there was not enough information available about their functional role in

the mammary gland and/or other stem/progenitor cell systems. *Tmco1* has been discarded because this gene was not differentially regulated in undifferentiated compared to competent HC11 cells (see appendix, column "undifferentiated vs. competent"). *Tcea1* was ranked first place in the gene list upon the highest levels of differential expression and upon the reproducibility of the qualitative comparision of its expression in undifferentiated, competent and induced HC11 cells (see appendix). The reason why this gene was not chosen was that the number of spots, which represent the gene on the gene array plate, was lower than in case of *Lgals1*, *Ran*, *Ppm1α* (see appendix, column "# spots total"). The gene *Pef1* was among the chosen candidates, but it was not possible to design suitable primers for this gene without unwished secondary structures (see Material and Methods, 2.5) and for that reason the gene was discarded.

	Fold change		
Gene	undifferentiated vs. competent	competent vs. induced	undifferentiated vs. induced
Lgals1	1.5	-0.4	1.6
Ran	1.6	-0.9	1.1
Ppm1α	1.1	0.2	1.5
Rab14	0.7	2.8	1.5

Table 1: Comparision of gene expression in undifferentiated, competent and induced HC11 cells (data obtained from the gene array, see appendix).

1.3.1 *Lgals1*

Lectins are carbohydrate binding proteins that can be classified into four distinct families: C-type lectins, P-type lectins, pentraxins and galectins (Barondes *et al.*, 1994). Members of the galectin family share two characteristics: parts of the amino acid sequences are highly conserved within that family and they share the affinity for β-galactoside sugars (Barondes *et al.*, 1994). Over the past years the interest in galectins started to grow, and these proteins were

investigated in several species and in different context, resulting in a confusing variety for names for galectins (Barondes *et al.*, 1994). But a consensus has been achieved: *Lgals* (lectin, galactose-binding, soluble) is the abbreviation used for the genes encoding galectins and the numbering of genes is consistent with the numbering of proteins, for example: the gene *Lgals1* encodes the protein galectin-1 (Barondes *et al.*, 1994).

Galectin-1 is a homodimer with each subunit consisting of approximately 130 amino acids (Barondes *et al.*, 1994). Gal-1 is abundant in a wide variety of tissues, its active synthesis is often linked with specific developmental and physiological stages and it can be found in the cytosol (Barondes *et al.*, 1994), in cell nuclei, in the extracellular space and the translocation to the intracellular side of the cell membrane has been reported (Camby *et al.*, 2006). There is evidence that Gal-1 is differently expressed by normal and pathological tissues and it has a wide variety of biological functions (Camby *et al.*, 2006). It's interesting that in contrast to extracellular Gal-1, the activity of intracellular Gal-1 is mainly independent on the lectin activity (Camby *et al.*, 2006).

Positive effects of Gal-1 on cell migration have been reported for normal and cancer cells (Alge *et al.*, 2006; Camby *et al.*, 2002 and Rabinovich, 2005). Concerning the effects of Gal-1 on cell growth, there is evidence for positive and negative effects of Gal-1, highly dependent on the cell type, the amount of intracellular compared to extracellular Gal-1 and the relative distribution of monomeric and dimeric Gal-1 (Camby *et al.*, 2006).

In case of murine neural stem cells (mNSCs), Gal-1 promotes proliferation (Sakaguchi *et al.*, 2006), whereas expression knockdown of *Lgals1* in endothelial cells inhibits proliferation and migration (Thijssen *et al.*, 2004). Furthermore it has been reported that *Lgals1* is one of the genes tightly regulated during murine embryogenesis (Poirier *et al.*, 1992).

In a study carried out by Desriviéres *et al.* proteins were investigated, which were differentially expressed in undifferentiated and differentiated HC11 cells by employing two dimensional gel electrophoresis and mass spectrometry experiments (Desriviéres *et al.*, 2003). Sixty proteins were found to be differentially expressed in undifferentiated compared to differentiated HC11 cells (Desriviéres *et al.*, 2003). The reason why Gal-1 was not among these candidates may lie in the protein extraction procedure performed by Desriviéres *et al.*, which differs from the protein extraction protocol I used. In this project proteins were isolated from undifferentiated, competent, induced and differentiated HC11 cells by incubating the cells for 30 minutes in 1% NP40 lysis buffer containing the detergents sodium deoxycholate acid, a substance solubilising cellular and membrane components, sodium dodecyl sulfate (SDS) and Triton-X (see Material and Methods). The protein extraction carried out in the study by Desriviéres *et al.* was also undertaken with a 1% NP40 lysis buffer, but the buffer did not contain sodium deoxycholate, SDS and Triton-X and furthermore the incubation with the lysis buffer lasted just 15 minutes (Desriviéres *et al.*, 2003). These differences in the protein extraction protocols used in the two studies might explain why Desriviéres *et al.* did not detect the membrane bound protein Gal-1.

No data so far has been published for the functional role of Gal-1 in HC11 cells. However, taking into account the functions in other cell lines and SC systems, *Lgals1* might play an important role in the proliferation and migration process of HC11 cells.

1.3.2 *Ran*

The small GTPase Ran is a member of the Ras superfamily, it is primarily located in the nucleus of eukaryotic cells, cycles between a GDP (inactive) and a GTP-bound (active) state

(Nilsson *et al.*, 2002) and it is involved in cellular processes such as the regulation of cell cycle progression, nucleo-cytoplasmic transport and mitotic spindle assembly (López-Casas *et al.*, 2002). It has been shown that *Ran* is differently expressed during mouse embryogenesis (López-Casas *et al.*, 2002).

There is evidence that both the level of *Ran* mRNA and the protein level of *Ran* are increased in most cancer cell lines and cancer tissues, compared to normal surrounding cells or tissues and more than that, Ran is mostly undetectable in a wide variety of normal tissues (Azuma *et al.*, 2004). Generally, *in vitro* and *in vivo* studies have shown that autocrine growth factors such as EGF and receptors are frequently expressed in human malignancies (Murphy *et al.*, 2001). In case of cells from normal tissues it has been reported that the expression of *Ran* is dependent on the presence of EGF: the stimulation of the parasite *Setaria digitata* with murine EGF upregulates the expression of the nuclear GTPase Ran (Senarath Dissanayake, 2000). Except the last step of the HC11 cell treatment (see Material and Methods) of induced HC11 cells, the medium will contain EGF. Interestingly, *preproepidermal growth factor* (*EGF*) mRNA accumulates in HC-11 cells 16-24h after the combined hormone treatment with insulin (INS), dexamethasone (DEX) and prolactin (PRL) (Fang and Sheffield, 1998). Treatment of HC11 cells with individual hormones (INS, DEX or PRL) or combinations of two of the hormones did not effect *EGF* mRNA concentrations (Fang and Sheffield, 1998). That means in conclusion, that during the whole treatment of HC11 cells (see Material and Methods), except the last step which lasts just one hour, *Ran* expression might be upregulated due to the components of the media.

In a study by Ivanova *et al.* compared the gene expression profile of murine embryonic stem cells (mESCs), murine neural stem cells (mNSCs) and human hematopoietic stem cells

(hHSC) to their differentiated progeny (Ivanova *et al.*, 2002). The results evidently show that *Ran* is upregulated in hHSCs (Ivanova *et al.*, 2002).

Desriviéres *et al.* compared expression levels of selected genes in undifferentiated and differentiated HC11 cells and found that *Ran* is upregulated in differentiated HC11 cells (Desriviéres *et al.*, 2003). This result contradicts the information obtained from Peter Angel's gene array (see Table 1, column "Fold change, undifferentiated vs. induced"), because these results show that there is a higher amount of *Ran* mRNA in undifferentiated HC11 cells compared to the amount of *Ran* mRNA in induced cells. This is not necessarily a discrepancy since the treatment of HC11 cells with DIP-medium in the gene array by Dr. Peter Angel lasted just one hour compared to four days lactogenic hormone treatment in the experiments carried out by Desriviéres *et al.* (Desriviéres *et al.*, 2003).

Little is known about the functional role of *Ran* in mammary epithelial cells, but the fact that *Ran* is upregulated in hHSCs (Ivanova et al., 2002) might indicate that the GTPase Ran is important for SCs to remain in an undifferentiated state.

1.3.3 *Ppm1α*

Ppm1α (protein phosphatase 1A, magnesium dependent, alpha isoform) is a metal ion dependent protein serine/threonine phosphatase that dephosphorylates Smad1 (Duan *et al.*, 2006), Smad2 and Smad3 (Lin *et al.*, 2006), all members of the Smad transcription factor family (Massagué and Wotton, 2000). It has been reported that Ppm1α is primarily located in the nucleus, while there is no evidence for Ppm1α abundance in the extracellular space, (Lin *et al.*, 2006).

Members of the Smad protein family play an important role in transforming growth factor-β (TGF-β) signaling (Massagué and Wotton, 2000). A schematic illustration of TGF-β signaling is shown in Figure 4. The TGF-β ligand binds to a serine/threonine kinase, known as receptor type II, which in turn activates the kinase activity of receptor type I (Massagué and Wotton, 2000). In the next step, receptor type I phosphorylates a special member of the Smad family, called receptor-activated Smad (R-Smad) and this protein in turn recruits a Co-Smad (Massagué and Wotton, 2000).

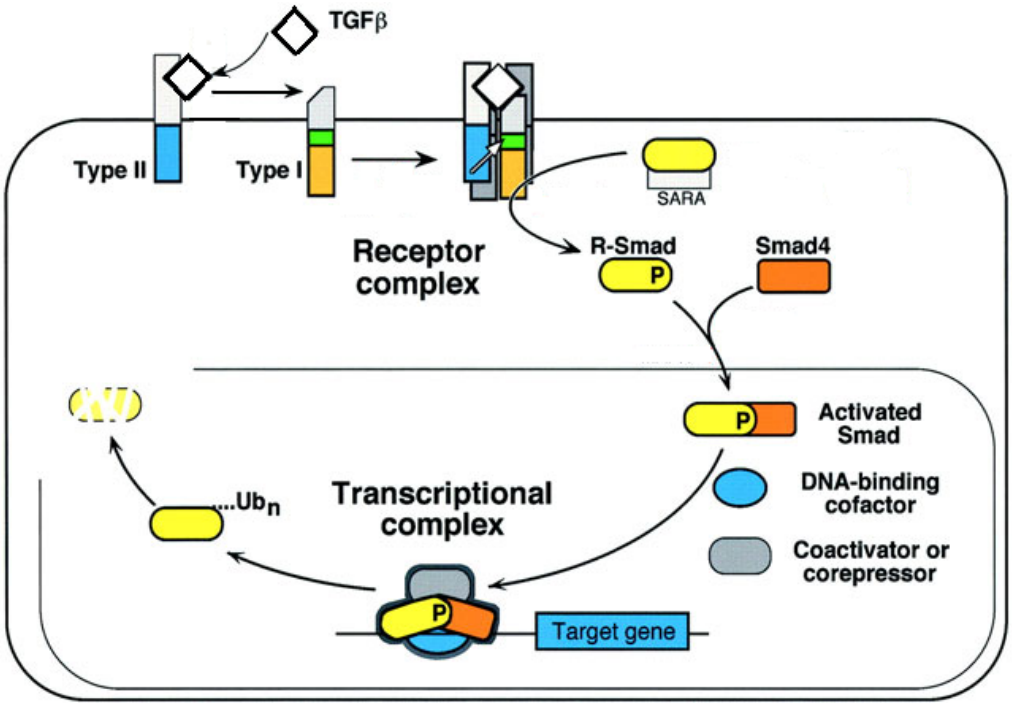


Figure 4 (Massagué and Wotton, 2000): Schematic illustration of the TGF-β/Smad signaling pathway. Upon activation of the TGF-β receptors, activated Smad proteins translocate into the nucleus and do their work as transcription factors (Massagué and Wotton, 2000).

In particular, the activation of Smad1 occurs upon the binding of the ligand bone morphogenic protein 2 (BMP-2) and the activation of Smad2 and Smad3 is dependent on the binding of TGF-β (Lai and Cheng, 2002). It is important to mention, that bone morphogenic

proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily (Duan *et al.*, 2006). The activation of Smad proteins (Smad1, Smad2 and Smad3) by phosphorylation allows these proteins to bind to Co-Smad Smad4 (Lai and Cheng, 2002). The complex, consisting of one R-Smad and Co-Smad Smad4, is thought to be involved in DNA binding and in the process of recruiting transcriptional cofactors (Massagué and Wotton, 2000).

There is evidence that Ppm1α abolishes BMP-2 signaling by dephosphorylation of Smad1 (Duan *et al.*, 2006), and TGF-β signaling by dephosphorlyation of Smad2 and Smad3 (Lin *et al.*, 2006). Loss of TGF-β responses has been linked to cancers and genetic diseases and therefore the interest in Ppm1α as an important target for new therapies has been grown over the past few years (Lin *et al.*, 2006).

The functions of Ppm1α in HC11 cells still remain elusive, but the facts that on the one hand TGF-β1 signaling causes growth arrest of mammary epithelial cells (García-Montero *et al.*, 2001) and on the other hand that Ppm1α abolishes BMP-2 signaling by dephosphorylation of Smad1 (Duan *et al.*, 2006), and TGF-β signaling by dephosphorylation of Smad2 and Smad3 (Lin *et al.*, 2006), might indicate that Ppm1α positively influences proliferation of undifferentiated HC11 cells.

1.3.4 *Rab14*

Rab GTPases in general are located at the cytoplasmic side (Martinez and Goud, 1997) of almost every membrane bound organelle and their major function is involved in the regulation of vesicle mediated trafficking between them (Burd and Collins 2004). Like Ran GTPases (see 1.3.2), Rab proteins are molecular switches, which cycle between a GDP (inactive) and a

GTP-bound (active) state (Burd and Collins, 2004). Recent studies revealed that mutations in Rab GTPases, as well as in proteins regulating them, are abundant in a variety of human diseases, including cancer (Cheng *et al.*, 2005).

No data so far has been presented for the role of *Rab14* in mammary epithelial cells. The facts that mutations in *Rab* genes are abundant in cancer cells (Cheng *et al.*, 2005) and that *Rab14* is upregulated in rat neural stem cells (NSCs) compared to their differentiated progeny rat oligodentrocyte precursor cells (OPCs) (Hu *et al.*, 2002) indicate that *Rab14* might regulate the proliferation of HC11 and/or stem/progenitor cells and were the reason why *Rab14* was selected for further investigation in this project.

1.3.5 *Id2*

Id (inhibitor of differentiation) helix-loop-helix (HLH) proteins function as dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors (Norton, 2000) and in general negatively influence cell differentiation and positively regulate cell proliferation (Mori *et al.*, 2000).

Id proteins belong to the HLH protein family of transcription factors and the HLH domain is necessary for homo- or heterodimerisation which in turn is essential for DNA binding[2] (Norton, 2000). Id proteins lack the DNA binding region, but they are capable to form dimers with other transcription factors, primarily with bHLH transcription factors (Norton, 2000). The formed Id-bHLH heterodimer complex (Figure 5) is not able to bind to DNA and

[2] The recognition sequence is CANNTG and generally known as "E-box" (Norton, 2000).

therefore Id proteins act as permanent negative regulators of HLH transcription factors (Benezra *et al.*, 1990).

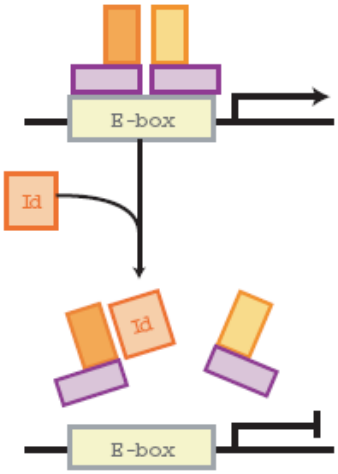


Figure 5 (Miyazono and Miyazawa 2002): Id proteins act as permanent negative regulators of HLH transcription factors (Benezra *et al.*, 1990). bHLH proteins form a heterodimer, whereby this complex is capable of binding to DNA and activating the transcription of E-box containing genes, whereas the Id-bHLH complex cannot bind to DNA and has therefore no transcription regulating functions (Miyazono and Miyazawa 2002).

Ectopic expression of *Id2* in mammary epithelial cells enhances differentiation and β-casein expression, whereas down-regulation of *Id2* negatively affects the differentiation process of mammary epithelial cells (Parrinello *et al.*, 2001). The observation that Id2 positively influences the differentiation process of mammary epithelial cells is also supported by the fact that *Id2* expression increases in confluent HC11 cells and remains high in differentiated HC11 cells (Jankiewicz *et al.*, 2006).

In the experiments I conducted, HC11 cells were stimulated for 1 hour with the lactogenic hormone mix DIP. Considering on the one hand that β-casein is present and detectable in HC11 cells after 24 hours of DIP treatment (Ball *et al.*, 1988) and on the other hand that *Id2* mRNA levels are upregulated in differentiated (Jankiewicz *et al.*, 2006) and induced[3] (1 hour

[3] The gene array carried out in collaboration with Dr. Peter Angel revealed a 3.2-fold higher Id2 expression in induced compared to undifferentiated HC11 cells.

DIP treated) HC11 cells, I made use of this gene as a useful tool to monitor the induction of HC11 cells by measuring and comparing *Id2* mRNA expression during the three treatment stages.

1.4 Objectives and Hypothesis

The two objectives of this project are to investigate the differential gene expression of potential stem/progenitor cell marker candidates in murine mammary epithelial cells (HC11) and to determine the functions of the potential stem/progenitor cell marker protein Gal-1 in HC11 cells.

Analysis of the results of the gene array that was performed in collaboration with Dr. Peter Angel *et al.* led me to the first working hypothesis, that the potential SC marker genes *Lgals1*, *Ppm1α*, *Ran* and *Rab14* are upregulated in undifferentiated compared to competent and induced HC11 cells (see Table 1, column "Fold change, undifferentiated vs. induced"). *Lgals1* is expected to be the most useful candidate for a potential stem/progenitor cell marker since galectin-1 (Gal-1), the *Lgals1* gene product, is abundant in the extracellular space (Camby *et al.*, 2006). It has been shown that Gal-1 promotes proliferation of mNSCs (Sakaguchi *et al.*, 2006), whereas expression knockdown of *Lgals1* in endothelial cells inhibits proliferation and migration (Thijssen *et al.*, 2004). This led me to the second working hypothesis: Gal-1 might play an important functional role in proliferation and migration of undifferentiated HC11 cells as well as in stem/progenitor cells of the mammary gland.

In order to achieve the first objective and test the hypothesis, I will investigate the gene expression of *Lgals1*, *Ran*, *Ppm1α* and *Rab14* in undifferentiated, competent and induced HC11 cells by polymerase chain reaction (PCR) experiments and validate the gene array data by real time PCR (RT-PCR) experiments. To confirm the gene expression pattern not only on an mRNA level, but also on a protein level, Western Blot analysis will be performed.

To determine the function of Gal-1 in HC11 cells and test the second hypothesis, *Lgals1* will be knocked down by cloning DNA encoding a short hairpin RNA (shRNA) sequence directed against *Lgals1* into an expression vector and transfecting HC11 cells. Knockdown of *Lgals1*, followed by proliferation and migration assays might reveal an important function of Gal-1 in HC11 cells, as well as in stem/progenitor cells of the mammary gland.

2. Material and Methods

2.1 HC11 cell culture

Roswell Park Memorial Institute Medium 1640 (RPMI 1640, Invitrogen, Burlington, Ontario) containing 10% heat inactivated fetal bovine serum (HI-FBS, Biofluids, Rockville, Maryland), 1% penicillin-streptomycin and 1% L-glutamine (Biowhitaker, Walkersville, Maryland) was the medium used for HC11 cell culture. HC11 cells in vitro were maintained in undifferentiated, competent, induced and differentiated stages, depending on the different hormone supplements in the RPMI medium.

HC11 cells were obtained from frozen stocks stored in HI-FBS with 10% dimethylsulfoxide (DMSO, Fisher, Ottawa, Ontario). After carefully thawing the frozen cell suspension at 37°C, the cells were plated in 7ml RPMI media containing the supplements mentioned above and additionally 0.01μg/ml epidermal growth factor (EGF, Oakville, Ontario) and 5μg/ml insulin (INS, Sigma, Oakville, Ontario) on a 10cm cell culture dish (Corning, Lowell, Massachusetts) and incubated at 37°C for two days. After the HC11 cells reached a 80% confluence, the cells were split (passaged) by washing the cells twice with 5ml sterile phosphate buffered saline, pH 7.4 (1 x PBS, containing 8.0g NaCl, 200mg KCl, 2.72g Na_2HPO_4 x $7H_2O$ and 240mg of

KH_2PO_4 per liter ddH_2O), treating each dish with 1ml Trypsin-EDTA (Invitrogen, Invitrogen, Burlington, Ontario) for 10min at 37°C and plating the cells again with a concentration of 7 x 10^5 cells/ml. The RPMI medium was changed every 2nd day.

To obtain undifferentiated HC11 cells, the cells were harvested after they grew up to a 60% confluence in RPMI medium containing 0.01μg/ml EGF and 5μg/ml INS. To obtain competent HC11 cells, cells were plated at a cell density of 7 x 10^5cells/ml and grown in RPMI medium containing 0.01μg/ml EGF and 5μg/ml INS four 4 days or until the cells reached a 100% confluence. HC11 cells were considered to be 100% confluent, when no empty spots on the cell culture dish were detectable when observed under a light microscope. Due to the high number of cells in confluent cell culture dishes the medium was changed every day. After reaching 100% confluence, RPMI medium containing EGF and INS was removed from the cell culture dish and the HC11 cells were washed three times with 5ml 1 x PBS. Cells were grown for another 4 days in RPMI media containing just EGF in order to transfer HC11 cells to a competent state (Taverna *et al.*, 1991 and Chammas *et al.*, 1994). After this treatment the RPMI medium was removed again, HC11 cells were washed three times with 5ml 1 x PBS and RPMI medium containing 0.1μM dexamethasone (DEX, Sigma, Oakville, Ontario), 5μg/ml insulin and 5μg/ml prolactin (PRL, Sigma, Oakville, Ontario), a lactogenic hormone treatment referred to as DIP, was added to the cell culture dish and the induced HC11 cells were harvested after one hour incubation at 37°C.

2.2 mRNA extraction

Messenger ribonucleic acid (mRNA) was extracted from undifferentiated, competent and induced HC11 cells using RNeasy® Mini Kit (Quiagen, Mississauga, Ontario). The media in

the cell culture dishes was removed and the cells were washed once with 5ml 1 x PBS and 1ml Trypsin-EDTA (Invitrogen, Burlington, Ontario) was added to each plate and incubated for 10min at 37°C. Cells were resuspended in 5ml RPMI medium and the suspension was centrifuged at 300 x g for 5min at 4°C. The supernatant was removed and cells were resuspended in 175µl ice cold RLN buffer (RNeasy® Mini Kit), transferred to a ribonuclease (RNase) and deoxyribonuclease (DNase) free polypropylene microcentrifuge tube (Diamed, Mississauga, Ontario) and incubated for 5min on ice. After centrifugation at 300 x g for 2min at 4°C, the supernatant was removed and transferred to a DNase and RNase free tube, 600µl RLT solution (RNeasy® Mini Kit) containing β-mercaptoethanol (10µl β-mercaptoethanol/ml RLT) was added to the tube and the solution was mixed by vortexing. In a next step 430µl 96% EtOH were added and the solutions were mixed by pipetting up and down. The content of the tube was transferred to a RNeasy® column with the provided 2ml collection tube attached, centrifuged at room temperature (RT) for 15sec at 10.000 x g and the flow through was discarded. 700µl RW1 (RNeasy® Mini Kit) buffer was added to the same column, the last centrifugation step was repeated and the flow through discarded. The column was then placed into a new 2ml collection tube and 500µl RPE (RNeasy® Mini Kit) solution was added, the last centrifugation step was repeated again and the flow through discarded. 500µl RPE (RNeasy® Mini Kit) solution was added and centrifuged at RT for 2min at 10.000 x g, the flow through was discarded and the column (RNeasy® Mini Kit) with the collection tube (RNeasy® Mini Kit) attached was centrifuged at RT for 1min at 10.000 x g. The precipitated RNA in the column (RNeasy® Mini Kit) was in a last step dissolved by adding 30µl of diethylpyrocarbonate (DEPC) treated, sterile, double distilled water (ddH_2O) to the column (RNeasy® Mini Kit) and centrifuging at RT at 10.000 x g for 1min. The concentration of the isolated mRNA was determined by photometric measurements at a wavelength of 260nm.

2.3 RNA electrophoresis

The quality of the isolated mRNA was determined by RNA electrophoresis of DNase treated and heat treated mRNA samples. The DNase treatment mixture consisted of 20µg isolated mRNA sample, 10µl of 10 x DNase buffer (Ambion, Streetsville, Ontario), 2µl DNase (Ambion, Streetsville, Ontario) and was adjusted to a final volume of 100µl with DEPC treated, sterile, double distilled water (ddH_2O). The heat treatment mixture differed from the mixture described above only in that way, that 2µl sterile DEPC treated ddH_2O were used instead of 2µl DNase.

1% formaldehyde agarose (FA) gels were prepared by suspending 0.8g UltraPure® agarose (Invitrogen, Burlington, Ontario) in 8ml 10 x FA buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), prepared in sterile DEPC treated ddH_2O, pH 7.0) and 72ml sterile DEPC treated DEPC ddH_2O. The agarose was melted in a microwave and the solution was cooled down to 65°C before 10µl 1% ethidium bromide (EtBr, Fisher, Ottawa, Ontario) and 1.4ml 37% formaldehyde were added. The solution was poured into the electrophoresis apparatus and the agarose was allowed to polymerize for 1h. After the gel hardened it was covered with 300 ml 1 x FA buffer (30ml 10 x FA, 264ml sterile DEPC treated water and 6ml 37% formaldehyde) and the gel was run without loaded samples for 30min at 70mA. 2µl of 5 x RNA loading buffer (48µl saturated bromophenol blue solution, 80µl of 0.5M EDTA pH 8.0, 720µl of 37% formaldehyde, 2000 µl glycerol, 3084 µl formamide, 4.0 ml 10 x FA buffer, 68 µl of DEPC treated ddH_2O, stored at 4ºC) were added to 8µl of DNase or heat treated samples and loaded into the gel. 1µl of the isolated mRNA samples were added to 2µl of 5 x RNA loading buffer and 7µl of DEPC

treated ddH_2O and loaded aswell. Gels were run at 70mA for 40 minutes and photographed under UV illumination.

2.4 Reverse transcription

To transcribe mRNA sequence into a complementary DNA (cDNA) sequence, 25µl of the DNase treated mRNA samples (see 2.3) were mixed with 2.5µl deoxythymidine oligonucleotides (500µg/ml Oligo dT) and 2.5µl deoxynucleotide triphosphates (dNTPs, 10mM) in a 2ml RNase free polypropylene microcentrifuge tube (Diamed, Mississauga, Ontario), incubated at 65°C for 5min in a water bath and quick chilled on ice after the heat treatment. In a next step 10µl 5 x First-Strand buffer (Invitrogen, Burlington, Ontario), 5µl 0.1M Dithiothreitol (DTT, Invitrogen, Burlington, Ontario) and 2.5µl sterile DEPC treated ddH_2O were added to the sample and incubated at 42°c for 2min. After that 1µl Superscript® II reverse transcriptase (Invitrogen, Burlington, Ontario) was added, the samples were incubated at RT for 10min and then incubated at 42°C for 50min at 300 revolutions per minute (rpm). In a last step, in order to inactivate the reverse transcription reaction, the samples were incubated in a water bath at 70°C for 10min.

2.5 Polymerase chain reaction (PCR)

PCR reactions were performed with a 25µl final volume of the reaction mixture, consisting of 1.5µl 50mM $MgCl_2$ (3mM $MgCl_2$ final concentration (FC)), 0.5µl 10mM dNTPs (200µM dNTPs FC), 0.4µl of each 12.5µM forward and reverse primer (200nM FC), 18.35µl sterile

ddH_2O, 1µl of Taq polymerase (Invitrogen, Burlington, Ontario), 2.5µl 1 x PCR buffer (Invitrogen, Burlington, Ontario) and 1µl of the synthesized cDNA sample in a MiniOpticon Real-Time PCR Detection System® (Biorad, Hercules, California). The first step of the PCR reaction temperature program was performed at 95°C for 3min, followed by 30sec at the same temperature. The annealing step was performed at 63°C for 30sec, followed by the extension step at 72°C for 30sec. The final step of the PCR reaction consisted of a 72°C heat treatment for 10min. The denaturation, annealing and extension steps were repeated 26 (*Lgals1*, *Ppm1α*, *Id2*) to 28 (*Ran*, *Rab14*) times.

Bioinformatics was used to design primers that were used for PCR experiments as well as for real-time Polymerase Chain Reaction (RT-PCR) experiments: a software tool termed Primer 3 was used to choose the sequence of the primers and the sequence of the amplification products (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The selected sequences were checked for unwanted structures using the Rensselaer and Wadsworth bioinformatics web server (http://www.bioinfo.rpi.edu/applications/mfold). Employing the BLAST software tool (National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/blast) the primers were checked for undesired amplification products.

Lgals1 (NM_008495.2) **Forward** 5' GCC AAG AGC TTT GTG CTG AA 3', **Reverse** 5' GGT CCC ATC TTC CTT GGT GTT AC 3'; **RAN** (NM_009391.3) **Forward** 5' AGT TTG CCC CCA ACC TTA GT 3', **Reverse** 5' TTT CCT CTC CTG CAC AAC CT 3'; **Ppm1α** (NM_008910.2) **Forward** 5' CGT AGC CAA GAT ATT GCA GCT G 3', **Reverse** 5' AGA ATC AAG GCA TGT TAG CCC A 3'; **Rab14** (NM_026697.3) **Forward** 5' ACA AAT CAC CCA TCG GGA CA 3', **Reverse** 5' TGG GAA ATG GGG TAT TGC AC 3'; **Id2** (NM_010496.2NM) **Forward** 5' GCC TTT TCA CAA AGG TGG AGC 3', **Reverse** 5' CAG

CAT TCA GTA GGC TCG TGT C 3'; **18s rRNA Forward** 5' GTA ACC CGT TGA ACC CCA TT 3', **Reverse** 5' CCA TCC AAT CGG TAG TAG CG 3'.

2.6 Real-time polymerase chain reaction (RT-PCR)

RT-PCR reactions were carried out in a 20µl final volume reaction mix, containing 0.4µl of each 12.5µM forward and reverse primer (200nM FC), 10µl 2 x Sybr-Green reaction buffer (Biorad, Hercules, California), 8.2µl sterile ddH_2O and 1µl synthesized cDNA in an iQ5 Real-Time PCR Detection System® (Biorad, Hercules, California). The primers and temperature program used for real-Time PCR experiments were the same as for PCR experiments. After the RT-PCR reaction, a melting curve program starting at 58°C to 96°C was run in order to confirm the purity of the amplified cDNA. All values were standardized (18S) before the amounts of amplified cDNAs were compared.

2.7 DNA electrophoresis

1% agarose-Tris-borate-EDTA (TBE) gels were prepared by adding 1.8g of UltraPure® agarose (Invitrogen, Burlington, Ontario) to 180ml 1 x TBE (10.8g of 2-amino-2-hydroxymethyl-1,3-propanediol (Tris base), 5.5g boric acid, and 4ml of 0.5 M Na_2EDTA (pH8, autoclaved) dissolved in 1l ddH_2O) and melting the agarose in a microwave for approximately 2min. After the molten agarose cooled down to 65°C, 15µl of 1% EtBr were added and the solution was poured into the gel electrophoresis apparatus where the agarose polymerized for 1h. Meanwhile 1µl 10 x DNA loading buffer (consisting of 50% glycerol,

0.4% bromphenol Blue and 0.4% xylene cyanol in sterile DEPC treated ddH_2O) was added to 9µl PCR reaction product and after the agarose polymerized, the electrophoresis apparatus was filled with 1.3l 1 x TBE buffer and 10µl of the prepared samples, consisting of PCR product and 10 x DNA loading buffer, were loaded into the wells of the gel. Gels were run at 120mA for 30min and photographed under UV illumination.

2.8 Western blot

After the medium of cell culture dishes containing undifferentiated, competent, induced or differentiated HC11 cells was removed, the cells were washed twice with 5ml 1 x PBS and the cells scraped with a rubber policeman in 1ml 1 x PBS, centrifuged at RT at 400 x g for 10min and resuspended in 100µl NP40 lysis buffer (5mM EDTA, 1% [w/v] NP40, 0.5% [w/v] sodium deoxycholate acid, 124.5 mM KCl, 5mM MgCl2, 10mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES, pH7.2) in 1x PBS) containing apropitin (0.5µg/ml), phenylmethanesulphonylfluoride (PMSF, 0.1µM), dithiothreitol (DTT, 1mM), sodium vanadate, phenylarsine oxide (PAO, 0.1mM), leucin (Leu, 1µg/ml), 1% Triton-X and 0.1% sodium dodecyl sulfate (SDS).

The cell suspension was incubated for 30min on ice and centrifuged at 13.400 x g, the supernatant was removed and both, the supernatant and the pellet were snap frozen in liquid nitrogen (N_2) and stored at -80°C. Using BioRad reagents, the protein concentration of the supernatant was determined by photometry at a wavelength of 595nm against a standard curve (Bovine Serum Albumin (BSA), Fisher, Ottawa, Ontario). The electrophoretic separation of the isolated proteins was performed using 15% acryl amide gels. The separation gel was prepared by mixing 3.75ml 30% acryl amide, 2.8ml 1M Tris (pH 8.8), 76µl 20% SDS,

0.925ml ddH_2O, 25µl 10% ammonium persulfate (APS) and 10µl tetra-methyl-ethylenediamine (TEMED). The mixture for the stacking gel consisted of 1.67ml 30% acryl amide, 1.25ml 1M Tris (pH6.8), 99µl 20% SDS, 7.03ml ddH_2O, 50µl 10% ammonium persulfate (APS) and 10µl tetra-methyl-ethylenediamine (TEMED).

50µg of isolated protein were diluted with 4 x sample buffer (2% [w/v] SDS, 10% [w/v] glycerol, 100mM DTT, 0.02% [w/v] bromophenol blue, 1M Tris-HCl (pH6.8)) to a final volume of 12µl, heated at 97°C for 5min and loaded into each well of the prepared gel. The protein electrophoresis was carried out for several hours at 25mA per gel. In a next step the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (2mA x cm^2 gel) by using a semi-dry system. Immunodetection was carried out employing antibodies (ABs) against Gal-1 (1µg/ml goat anti-mouse galectin-1 AB (R & D Systems, Hornby, Ontario) in 5% BSA dissolved in 1 x Tris-Buffered Saline (TBS, 8.8g NaCl, 0.2g KCl, 3g Tris base diluted in 1l ddH_2O; pH 7.4)), β-casein (0.8µg/ml anti-mouse β-casein AB (Santa Cruz, Santa Cruz, California) in 5% milk BSA dissolved in 1 x Tris-Buffered Saline-Tween (TBS-T, 8.8g NaCl, 0.2g KCl, 3g Tris base and 2.5ml 20% Tween adjusted to 1l with ddH_2O; pH7.4) and growth-factor receptor bound protein 2 (Grb2, BD Biosciences, Mississauga, Ontario, 0.25µg/ml anti-mouse Grb2 in 5% BSA dissolved in 1 x TBS-T). After the transference of the proteins the incubation of the PVDF membranes lastet over night at 4°C in case of the goat anti-mouse Gal-1 antibody and 1 hour at RT for all the other antibodies employed.

2.9 Ribonucleic acid interference (RNAi)

The Lgals1 shRNA sequence was designed by using bioinformatics programs (RNAi Explorer®, http://www.genelink.com/sirna/shRNAi.asp). The sequence was designed to be

inserted in pSuper vector (Oligoengine, Seattle, Washington), a vector containing ampicillin (AMP) and Geneticin® resistance for selection and the green fluorescence protein (GFP) sequence for detection.

The **Lgals1** (NM_008495.2) shRNA sequence: **Forward** 5′ CAA CAT GGA GGC CAT CAA CTA CAT TCA AGA GAT GTA GTT GAT GGC CTC CAT GTT GTT TTT 3′, **Reverse** 5′ AAA AAC AAC ATG GAG GCC ATC AAC TAC ATC TCT TGA ATG TAG TTG ATG GCC TCC ATG TTG 3′.

After annealing of the synthesized forward and reverse shRNA primers, the double stranded shRNA sequence was ligated into the pSuper vector. DH5α cells were transformed with pSuper vector containing *Lgals1* shRNA sequence, plated on agar plates (4.5g Bacto agar (BD), Mississauga, Ontario) dissolved in 300ml LB-medium (10g Bacto-tryptone, 5g yeast extract, 10g NaCl dissolved in ddH_2O, adjusted to pH 7.5 and adjusted to a final volume of 1l with ddH_2O) containing 0.1% ampicillin (AMP)), incubated over night at 37°C and grown colonies were screened for the pSuper vector containing the designed *Lgals1* shRNA sequence by isolating the plasmid using Mini Preparation and Midi Preparation (protocol see HiSpeed® Plasmid Midi Kit handbook (Qiagen, Mississauga, Ontario)) methods. The following protocol was used to isolate the plasmid by Mini Preparation: Each colony grown over night on the agar plates was transferred to a sterile 15ml High-Clarity Polypropylene Conical Tube (BD Falcon®, Mississauga, Ontario) containing 2ml LB-medium with 2% AMP. The cells were incubated over night at 37°C, transferred to a polypropylene microcentrifuge tube and centrifuged at 12.000 x g for 5min at 4°C. After the supernatant was removed, the cell pellet was resuspended in 100µl ice cold solution I (50mM glucose, 25mM Tris-HCl (pH 8.0) and 10mM EDTA (pH 8.0) in ddH_2O) by pipetting up and down, the suspension was further mixed by vortexing and incubated at RT for 5min. In a next step 200µl freshly

prepared solution II (0.2m NaOH, 1% SDS in ddH_2O) were added, the tube was inverted and the cells were incubated on ice for 5min. After that 150µl ice cold solution III (5M potassium acetate (KOAc) and 5M glacial acetic acid in ddH_2O) were added, the tube was inverted and the mixture was incubated on ice for 10min. A centrifugation step at 12.000 x g for 5min at 4°C followed and the supernatant was transferred to a new polypropylene microcentrifuge tube. The DNA was precipitated by adding 900µl 96% ethanol (EtOH) and an incubation at RT for 2min followed. After another centrifugation step (12.000 x g for 5min at 4°C) the precipitated DNA was washed twice with 900µl 70% EtOH and dried at RT for several hours. The quality of the isolated plasmid was determined using DNA electrophoresis. The isolated DNA had been sent to sequencing and it was confirmed that the vector contained the inserted *Lgals1* shRNA sequence and therefore the plasmid was employed in HC11 transfections (see 2.10).

2.10 Transfection of HC11 cells

HC11 cells were transfected using the Calcium Phosphate Transfection method. HC11 cells were plated at a cell density of 4.5 x 10^5cells/plate in 7ml RPMI medium containing 0.01µg/ml EGF and 5µg/ml INS. After the HC11 cells reached a confluence of 50% the medium was removed 6h prior to transfection, the cells washed twice with 1x PBS and 7ml of Dulbecco's Modified Eagle's Medium without serum (DMEM, HyClone, Logan, Utah) were added.

In a polypropylene microcentrifuge tube 2µg purified plasmid (see 2.8) were mixed with 18µg Sheared Salmon Sperm DNA (Eppendorf, Mississauga, Ontario) and the volume was

adjusted to 450µl with ddH_2O. 50µl 2.5M sterile filtered $CaCl_2$ were added to the solution containing DNA and this mixture was added dropwise to a polypropylene microcentrifuge tube which contained 500µl 2x HBS (50mM Hepes, 280mM NaCl and 1.5mM Na_2HPO_4 in ddH_2O). After incubation for 20min at RT this solution was added dropwise to 50% confluent HC11 cells. After incubation of the HC11 cells for 6h at 37°C, the DMEM medium was removed, the cells washed twice with 1x PBS and 7ml RPMI medium containing 0.01µg/ml EGF and 5µg/ml INS were added. After a 24h incubation period, the medium was changed to RPMI medium containing 0.01µg/ml EGF, 5µg/ml INS and 92.8µg/ml Geneticin® (Gibco, Burlington, Ontario) and the transfection efficiency was determined by detecting the fluorescent GFP signal.

2.11 Fluorescent activated cell sorting (FACS)

Fluorescent activated cell sorting (FACS) was used to quantify the percentage of Sca1 and/or Gal-1 expressing HC11 cells. HC11 cells were plated at a cell density of 7 x 10^5 cells/ml in 7ml RPMI 1640 medium containing 10% HI-FBS, 1% penicillin-streptomycin, 1% L-glutamine 0.01µg/ml EGF and 5µg/ml INS and after the cells reached a 90% confluence the cells were washed three times with 1x PBS, 1ml of Trypsin-EDTA was added and the cells were incubated for 10min at 37°C. After that the cells were resuspended in 5ml of RPMI 1640 medium containing the supplements listed above and the cell suspension was transferred to a 15ml High-Clarity Polypropylene Conical Tube and centrifuged at 300 x g for 5min at RT. In a next step the HC11 cells were washed twice with 5ml 3% PBS/BSA (8.0g NaCl, 200mg KCl, 2.72g Na_2HPO_4 x $7H_2O$ and 240mg KH_2PO_4 dissolved in 1l sterile ddH_2O, pH adjusted to pH 7.4 and 30g BSA added) and after counting cells and another centrifugation step (300 x

g for 5min at RT) the cells were resuspended in 3% PBS/BSA containing 1% paraformaldehyde (PFA, Sigma, Oakville, Ontario; 0.1g paraformaldehyde dissolved in 10 ml 3% PBS/BSA) at a cell density of 2 x 10^6 cells/100µl. After incubation for one hour at RT, the cell suspension was washed twice with 5ml 3% PBS/BSA and stored in the fridge for one day in 5ml 3% PBS/BSA.

To stain the cells with ABs 100µl of the cell suspension were transferred to a polypropylene microcentrifuge tube and centrifuged at 300 x g for 5min at RT, the supernatant was removed, 100µl 3% PBS/BSA containing 10µl Sca1 AB (FITC anti-mouse Ly-6A/E BD Biosciences Pharmigen, New Jersey, USA) was added, the cells resuspended and incubated for 30min at 4°C in the fridge. After another centrifugation step (300 x g for 5min at RT) the supernatant containing the AB was removed and the pellet washed three times with 3% PBS/BSA. In a next step the cell suspension was centrifuged at 300 x g for 5min at RT, 100µl 3% PBS/BSA containing 1µl Gal-1 AB were added, the cells resuspended and incubated for 30min at 4°C in the fridge. After that the washing and centrifugation steps, as described above, were repeated. In a next step 100µl 3% PBS/BSA containing 1µl of the secondary AB for Gal-1 (Alexa Fluor 647 donkey anti-goat IgG Molecular Probes, Oregon, USA) were added to the cell pellet, the cells were resuspended and the suspension was incubated for 30min at 4°C in the fridge. The last step of the sample preparation for FACS analysis consisted of centrifuging the cells at 300 x g for 5min at RT, washing HC11 cells three times with 3% PBS/BSA and resuspending the cell pellet in 400µl 3% PBS/BSA.

3. Results

The genes investigated, *Lgals1*, *Ran*, *Ppm1α* and *Rab14*, were among the top eleven candidates in the gene list, which were ranked upon the highest levels of differential expression and upon the reproducibility of the qualitative comparision of their expression in undifferentiated, competent and induced HC11 cells (see appendix). *Id2* was not part of the gene list and the reason why it had been chosen is its role as a valuable tool to monitor the induction of cellular differentiation of 1 hour DIP treated HC11 cells.

The mRNA from undifferentiated cells was isolated from 60% confluent HC11 cells, whereas the mRNA from competent, induced and differentiated HC11 cells was isolated from 100% confluent cells.

3.1 Polymerase chain reaction (PCR)

In order to qualitatively confirm the differential gene expression of *Id2, Lgals1*, *Ran*, *Ppm1α* and *Rab14* in HC11 cells, mRNA extractions were performed from three independently cultured undifferentiated, competent, induced and differentiated HC11 cells, the sequence was

transcribed into a complementary DNA (cDNA) sequence and all samples were run twice in independent PCR experiments.

3.1.1 *Id2*

The treatment of competent HC11 cells with the lactogenic hormone mix DEX, INS and PRL for one hour results in an upregulation of *Id2* expression in induced compared to competent and undifferentiated HC11 cells (Figure 6a[4] and Figure 6b) and therefore monitoring the expression of *Id2* over the three treatment stages is a useful tool to confirm the induction of HC11 cells.

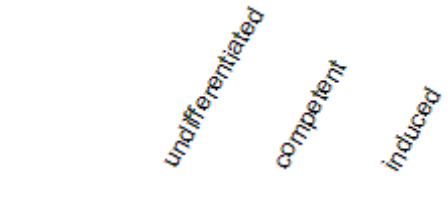


Figure 6a

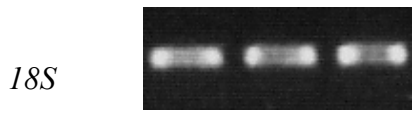


Figure 6b

Figure 6a: PCR experiment results for *Id2*. *Id2* is higher expressed in induced compared to competent and undifferentiated HC11 cells. **Figure 6b**: The loading control *18S* is similarly expressed during the three treatment steps.

[4] The mRNA extractions were performed from three independently cultured sets of undifferentiated, competent, induced and differentiated HC11 cells, the sequence was transcribed into a complementary DNA (cDNA) sequence and all samples were run twice in independent PCR experiments. Figure 6a to Figure 11b show the PCR results for *Id2, Lgals1*, *Ran*, *Ppm1α* and *Rab14* of one replicate of one set of isolated undifferentiated, competent and induced/differentiated HC11 mRNA.

3.1.2 *Lgals1*

Lgals1 is upregulated in undifferentiated compared to competent, induced and differentiated[5] HC11 cells. Figure 7a and Figure 7c show the PCR results for *Lgals1* and the loading control *18S*. This qualitative expression pattern for *Lgals1* matches the expected results from Dr. Angel's gene array (see Table 4, row "*Lgals1* (gene array)").

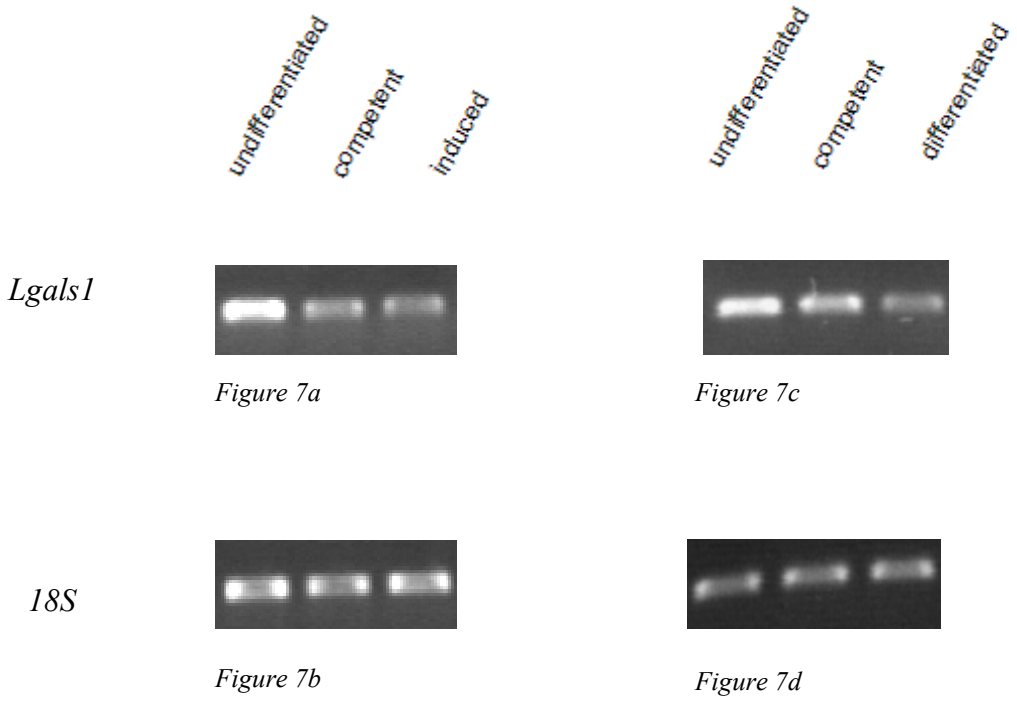


Figure 7a *Figure 7c*

Figure 7b *Figure 7d*

Figure 7a and **Figure 7c**: PCR results for *Lgals1*. Undifferentiated HC11 cells show a higher expression of *Lgals1* compared to competent, induced and differentiated HC11 cells. **Figure 7b** and **Figure 7d**: *18S* is constantly expressed in undifferentiated, competent, induced and differentiated HC11 cells.

During the PCR experiments conducted in this study the observation was made that small changes in the confluence of undifferentiated HC11 cells have a major impact on the comparision of *Lgals1* expression in the three stages of HC11 cell treatment. This raised the

[5] Complementary deoxyribonucleic acid (cDNA) samples from undifferentiated, competent and differentiated (four days DIP treated) HC11 cells used in this PCR experiment were provided by Dr. Perotti.

possibility that the regulation of *Lgals1* expression might be driven by cell confluence and not by the differentiation process itself.

To prove that the downregulation of *Lgals1* expression is regulated by differentiation, the mRNA expression of *Lgals1* of undifferentiated HC11cells (harvested at a confluence of 50%, 80% and 90%), competent and induced HC11 cells was compared in PCR experiments. The results (see Figure 8a) revealed that the amount of expressed *Lgals1* in undifferentiated HC11 cells increases with confluence, which in turn proves that the downregulation of *Lgals1* in competent and induced HC11 cells is regulated by differentiation, since mRNA of competent and induced HC11 cells was harvested from 100% confluent HC11 cells (see Material and Methods).

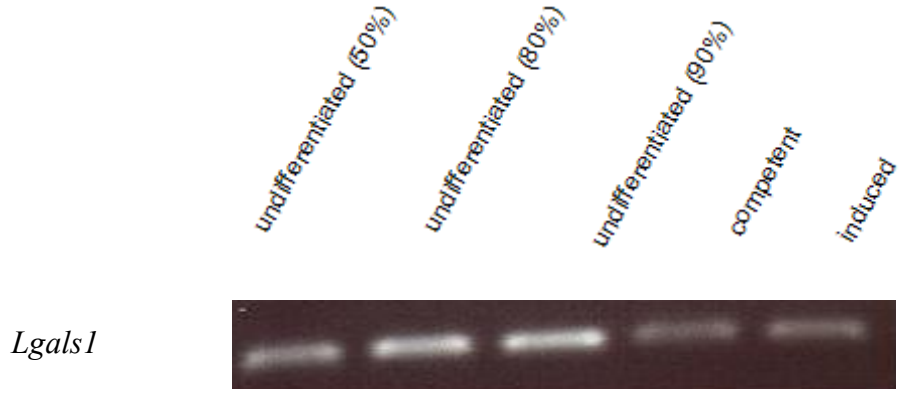


Figure 8a

Figure 8b

Figure 8a: *Lgals1* expression in undifferentiated, competent and induced HC11 cells is regulated by differentiation. The amount of expressed *Lgals1* in undifferentiated HC11 cells increases with confluence. **Figure 8b**: *18S* is constantly expressed in undifferentiated (50%, 80% and 90% confluent), competent and induced HC11 cells.

3.1.3 *Ran*

Ran is approximately 2-fold upregulated in undifferentiated HC11 cells compared to competent and induced HC11 cells (Figure 9a). Compared to the intensities of the bands of *18S* (26 cycles, Figure 9b), *Lgals1* (26 cycles, Figure 7a), *Ppm1α* (26 cycles, Figure 10a) and *Id2* (26 cycles, Figure 6a) amplification, the band intensities of the amplified *Ran* (28 cycles, Figure 9a) and *Rab14* (28 cycles, Figure 11a) sequence is very weak. This proves that the overall amount of expressed *Ran* and *Rab14* mRNA in HC11 cells is lower compared to the amount of expressed mRNA of the genes listed above.

The results of the qualitative comparison of expressed *Ran* in undifferentiated, competent and induced HC11 cells go along with the expected results from Dr. Angel's gene array (see Table 5, row "*Ran* (gene array)").

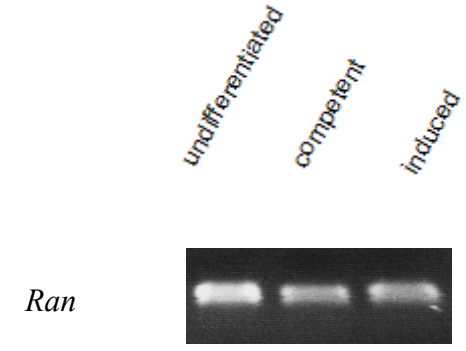


Figure 9a

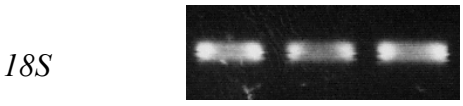


Figure 9b

Figure 9a: PCR results for *Ran*. Undifferentiated HC11 cells show a higher expression of *Ran* compared to competent and induced HC11 cells. **Figure 9b**: *18S* is constantly expressed in undifferentiated, competent and induced HC11 cells.

3.1.4 *Ppm1α*

Ppm1α is similarly expressed in undifferentiated and induced HC11 cells. *Ppm1α* seems to be slightly upregulated in competent HC11 cells (Figure 10a), which might be evidence that *Ppm1α* expression is slightly differently regulated during the three treatment stages or this expression pattern might be a result of unspecific EtBr binding during the DNA electrophoresis procedure. The expected expression pattern of *Ppm1α* in undifferentiated, competent and induced HC11 cells from Dr. Angel's gene array could not be confirmed (see Table 2, column "Fold change").

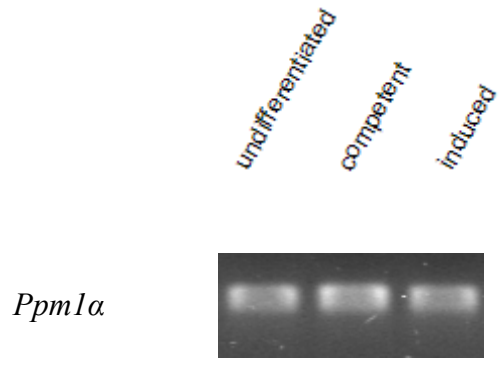


Figure 10a

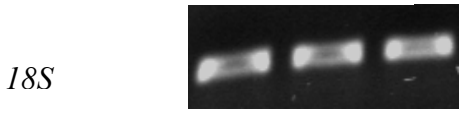


Figure 10b

Figure 10a: PCR results for *Ppm1α*. *Ppm1α* is similarly expressed in undifferentiated, competent and induced HC11 cells. **Figure 10b**: *18S* expression is constant during the three treatment stages.

	Fold change		
Gene	undifferentiated vs. competent	competent vs. Induced	undifferentiated vs. induced
Ppm1α	1.1	0.2	1.5

Table 2: Comparision of *Ppm1α* expression in undifferentiated, competent and induced HC11 cells.

3.1.5 *Rab14*

Figure 11a and Figure 11b show the PCR experiment results for *Rab14*. No differences in *Rab14* expression during the three treatment stages were found (Figure 11a). The expected expression pattern of *Rab14* in undifferentiated, competent and induced HC11 cells from Dr. Angel's gene array could not be confirmed (see Table 3, column "Fold change").

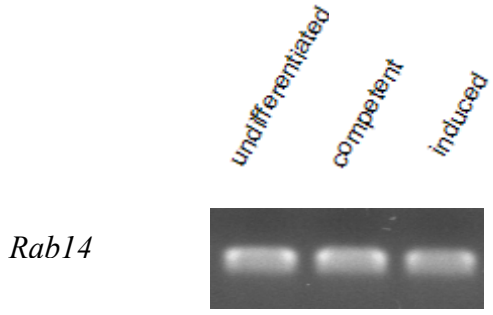


Figure 11a

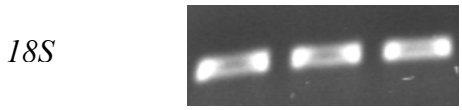


Figure 11b

Figure 11a: *Rab14* expression in undifferentiated, competent and induced HC11cells. *Rab14* is similarly expressed in undifferentiated, competent and induced HC11 cells. **Figure 11b**: Constant *18S* expression during the three treatment steps.

	Fold change		
Gene	undifferentiated vs. competent	competent vs. induced	undifferentiated vs. induced
Rab14	0.7	2.8	1.5

Table 3: Gene array data for the comparision of *Rab14* expression in undifferentiated, competent and induced HC11 cells.

3.2 Real-time polymerase chain reaction (RT-PCR)

3.2.1 *Lgals1*

Lgals1 is 4.1-fold upregulated in undifferentiated compared to induced HC11 cells and 3.5-fold upregulated in undifferentiated compared to competent HC11 cells (Figure 12). The mentioned differences of *Lgals1* expression during the three treatment stages are significant ($P < 0.05$).

	Fold change		
	undifferentiated vs. competent	competent vs. induced	undifferentiated vs. induced
***Lgals1* (gene array)**	1.5	-0.4	1.6
***Lgals1* (RT-PCR)**	3.5 (P = 0.008)	0.7	4.1 (P = 0.001)

Table 4: Comparision of expressed *Lgals1* in undifferentiated, competent and induced HC11 cells.

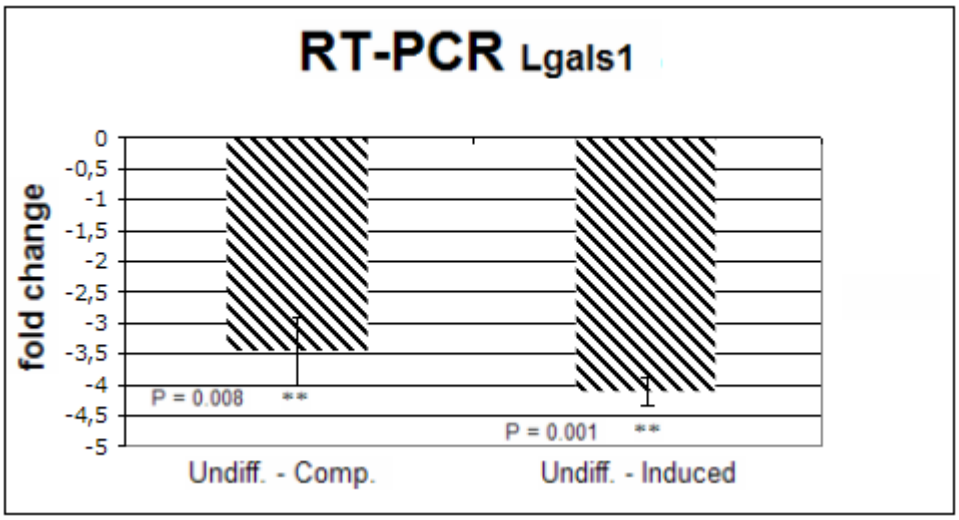


Figure 12: RT-PCR results for *Lgals1*. *Lgals1* is significantly ($P < 0.05$) upregulated in undifferentiated HC11 cells compared to competent and induced HC11 cells.

3.2.2 *Ran*

The upregulation of *Ran* in undifferentiated HC11 cells was confirmed in two independent RT-PCR experiments (see Figure 13 and Figure 14). Two possibilities contribute to high standard deviation values: firstly, pipetting errors might have occurred and secondly, *Ran* might be very sensitive to small methodical irregularities during the HC11 cell culture procedure and mRNA extraction. Thus the results of the RT-PCR experiments revealed a less significant difference ($P < 0.07$) in *Ran* expression between undifferentiated and induced HC11 cells than in case of *Lgals1* ($P < 0.05$) (see Figure 13, Figure 14 and Table 5, row "*Ran* (RT-PCR)")).

	Fold change		
	undifferentiated vs. competent	competent vs. induced	undifferentiated vs. induced
***Ran* (gene array)**	1.5	-0.4	1.6
***Ran* (1. RT-PCR)**	1.7 (P = 0.18)	0.2	1.9 (P = 0.08)
***Ran* (2. RT-PCR)**	1.1 (P = 0.18)	0.2	1.3 (P = 0.06)

Table 5: Comparision of *Ran* expression in undifferentiated, competent and induced HC11cells.

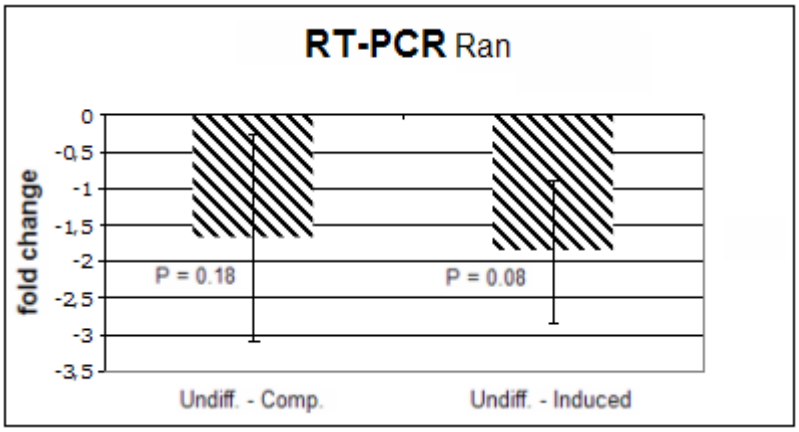


Figure 13

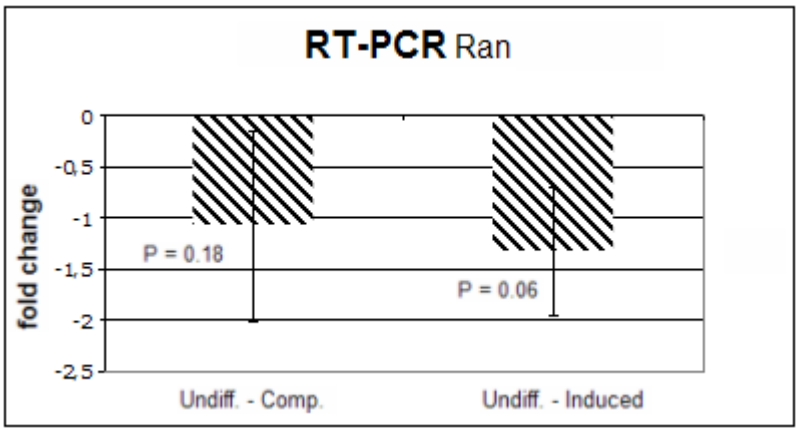


Figure 14

Figure 13 and **Figure 14**: RT-PCR results for *Ran* expression. *Ran* is upregulated in undifferentiated compared to competent (P = 0.18) and induced (P = 0.08) HC11 cells.

3.3 Western blot

Western blot experiments were carried out in order to qualitatively confirm the differential gene expression of *Lgals1* not only on an mRNA level, but also on a protein level. Protein extractions were performed from three independently cultured sets of undifferentiated, competent, induced and differentiated HC11 cells and Western blot experiments were repeated twice for each set of isolated proteins. Figure 15a to Figure 15d show one replicate of the Western blot results for *Lgals1* for one out of three sets of proteins isolated from undifferentiated, competent and induced/differentiated HC11 cells.

Lgals1 is not only upregulated in undifferentiated compared to induced HC11 cells on the mRNA level, but also on the protein level (Figure 15a). The lactogenic hormone treatment for induced HC11 cells lasts just for one hour, which leads to the assumption that the Gal-1 synthesis might not be completed within that short time span. Therefore Gal-1 expression was also compared between undifferentiated, competent and differentiated HC11 cells. Western

blot experiments revealed that Gal-1 is upregulated in undifferentiated compared to competent and differentiated HC11 cells (Figure 15c).

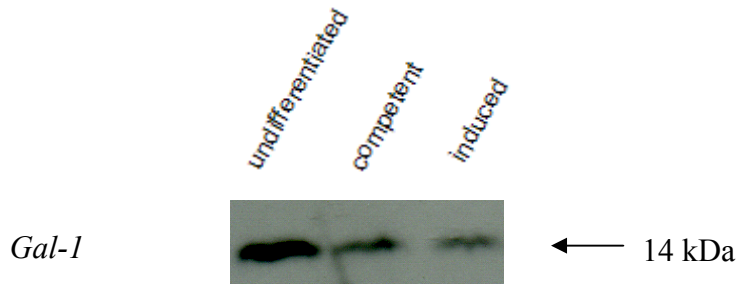


Figure 15a

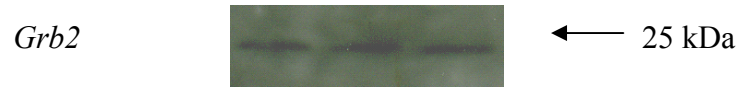


Figure 15b

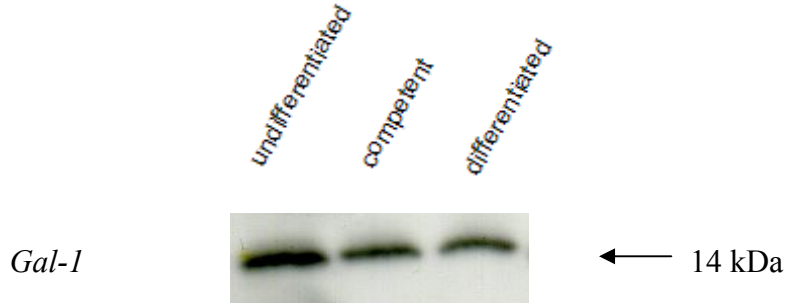


Figure 15c

Figure 15d

Figure 15: Gal-1 expression in undifferentiated, competent, induced and differentiated HC11 cells. Gal-1 is upregulated in undifferentiated HC11 cells compared to induced (**Figure 15a**) and differentiated (**Figure 15c**) HC11 cells. The expression of the loading control growth factor receptor-bound protein 2 (Grb2) for undifferentiated, competent, induced and differentiated HC11 cells is shown in **Figure 15b** and **Figure 15d**.

As mentioned above PCR and Western blot experiments were employed to qualitatively investigate the expression pattern of selected genes in undifferentiated, competent, induced

and differentiated HC11 cells. In order to obtain quantitative information out of this experiments ImageJ®, a program capable to calculate area and pixel value statistics of intensity thresholded objects *(*http://rsb.info.nih.gov/ij/*),* was employed. The results of this analysis are shown in Table 6 and revealed that the differential expression of *Lgals1* in undifferentiated, competent, induced and differentiated HC11 cells on the mRNA level are comparable to the differential *Lgals1* expression on the protein level.

type of experiment	**undifferentiated vs. competent** (Fold change)	**undifferentiated vs. induced** (Fold change)	**undifferentiated vs. differentiated** (Fold change)
PCR	**1.5**	**1.7**	**-**
PCR	**-**	**1.5**	**2.2**
Western blot	**-**	**1.3**	**1.7**

Table 6: Quantitative analysis of *Lgals1* expression in undifferentiated, competent, induced and differentiated HC11 cells with ImageJ®.

3.4 *Lgals1* knockdown in HC11 cells

In order to determine the function of HC11 cells, *Lgals1* was knocked down using RNAi. Figure 16 shows HC11 cells 24 hours after transfection. The transfection efficiency can be estimated by green fluorescence protein (GFP) expression and corresponds to approximately 20 percent, which is a high transfection efficiency for HC11 cells.

There is evidence that Gal-1 promotes proliferation (Sakaguchi *et al.*, 2006) and knockdown of *Lgals1* expression negatively influences migration (Thijssen *et al.*, 2004) of certain cell

types. Functional assays with parental HC11 cells and *Lgals1* knockdown cells will be carried out and these experiments will shed more light on the functional role of Gal-1 in HC11 cells in general and might give further insight into the molecular machinery of how proliferation in HC11 cells and stem/progenitor cells of the mammary gland is governed. Nonetheless, *Lgals1* knockdown still remains to be confirmed by PCR and Western blot experiments before functional assays will be performed.

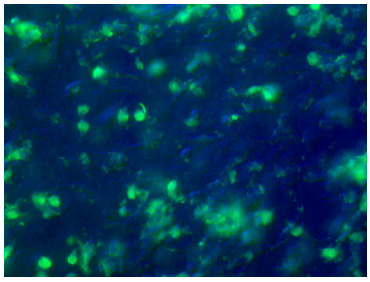


***Figure 16a**: HC11 cells transfected with the empty vector.*

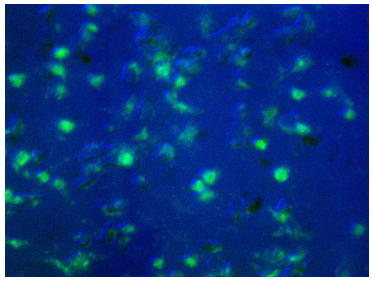


***Figure 16b**: HC11 cells transfected with a vector containing Lgals1 shRNA.*

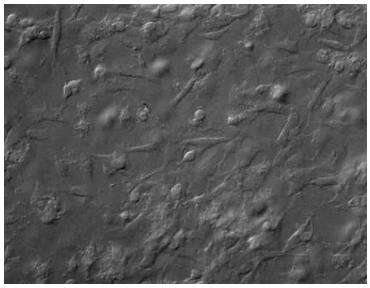


Figure 16c

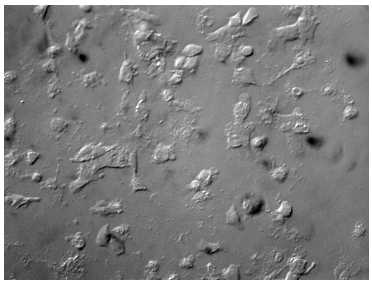


Figure 16d

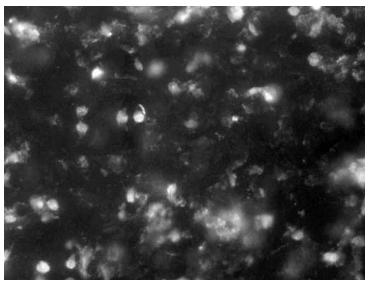


Figure 16e

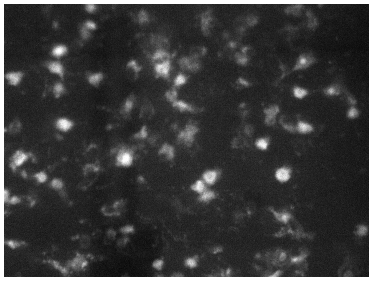


Figure 16f

Figure 16: Green fluorescent protein (GFP) expression of transfected HC11 cells, 24 hours post transfection. The transfection efficiency is higher in case of HC11 cells containing the empty vector (**Figure 16a**) compared to HC11 cells containing the vector with the inserted *Lgals1* shRNA sequence (**Figure 16b**). **Figure 16c** (control vector) and **Figure 16d** (vector containing *Lgals1* shRNA) show transfected HC11 cells observed under a light microscope. **Figure 16e** (empty vector) and **Figure 16f** (vector containing *Lgals1* shRNA) show the detected GFP signal.

3.5 Fluorescent activated cell sorting (FACS) analysis of undifferentiated HC11 cells

FACS experiments were carried out with undifferentiated HC11 cells in order to determine the percentage of HC11 cells expressing Gal-1 and/or Sca1. Based on the consideration that $Sca1^+$ cells are supposed to represent a progenitor cell population within the murine mammary gland and that about 20% of the total cell population of the mammary epithelium are $Sca1^+$ (Welm *et al*., 2002), this experiment might shed more light on the possibility that HC11 cells comprise a stem/progenitor cell subpopulation and on the hypothesis that Gal-1 might be a potential stem/progenitor cell marker.

In a first step it was interesting to determine the percentage of HC11 cells that express Sca1. The results revealed that 60.85% out of the cells gated expressed Sca1 (see Table 7, row "$Sca1^+$" and Table 10, column "$Sca1^+$ [%]"). Taking into consideration that $Sca1^+$ cells are supposed to represent a progenitor cell population within the murine mammary gland (Welm *et al*., 2002), the percentage of Gal-1 expressing HC11 cells was expected to be lower than the percentage of Sca1 expressing HC11 cells, which would support the possibility that Gal-1 might be a potential stem/progenitor cell marker. The results revealed that 90.12% out of the gated HC11 cells expressed Gal-1 (see Table 8, row "$Gal\text{-}1^+$" and Table 10, column "$Gal\text{-}1^+$ [%]"). The last step consisted of determining the percentage of Gal-1 and Sca1 expressing HC11 cells. 60.77% of the gated double stained HC11 cells expressed Gal-1 and Sca1 (see Table 9, row "$Sca1^+Gal\text{-}1^+$" and Table 10, column $Gal\text{-}1^+Sca1^+$ [%]). The FACS experiment carried out with unlabeled HC11 cells (negative control) revealed that 99.96% of the gated cells were detected as unstained (see Figure 20).

	replicate 1 [%]	replicate 2 [%]	replicate 3 [%]
Sca1$^+$	59.98	59.64	59.44

	mean (repl. 1)		mean (repl. 2)		mean (repl. 3)	
Quadrant	X Mean	Y Mean	X Mean	Y Mean	X Mean	Y Mean
lower right (LR)	230.92	8.32	232.44	8.53	230.33	8.49

***Table 7**: FACS results for single labeled HC11 cells expressing Sca1. All samples were run in triplicates. The data on the top represents the percentage of HC11 cells expressing Sca1 out of the cells gated. The data below show the mean intensities of the detected signal of Sca1 (X Mean). The detected intensity of replicate 1 is illustrated in **Figure 17a** and **Figure 17b**.*

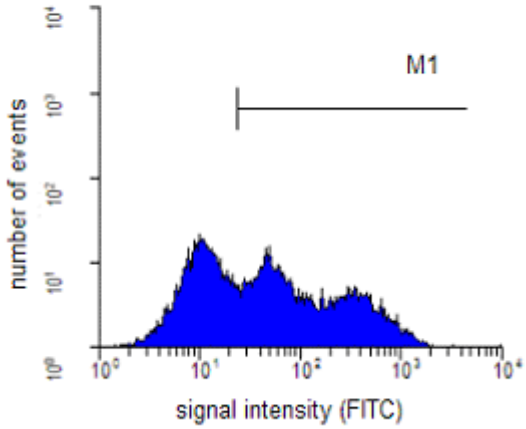


Figure 17a

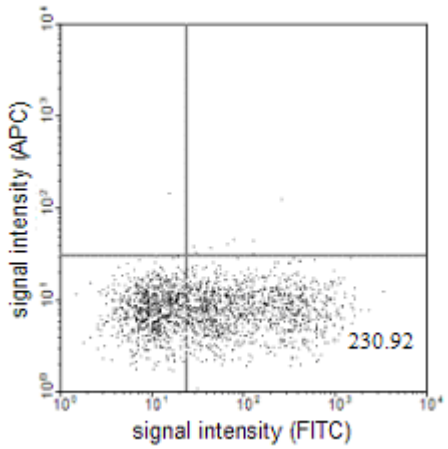


Figure 17b

***Figure 17a:** FACS histogram of Sca1 (FITC) stained undifferentiated HC11 cells (replicate 1, see Table 7). **Figure 17b:** Distribution of Sca1 (FITC, X Mean) stained undifferentiated HC11 cells (replicate 1, see Table 7). The mean intensity of the cells expressing Sca1 (replicate 1) is indicated in the lower right quadrant.*

	replicate 1 [%]	replicate 2 [%]	replicate 3 [%]
Gal-1$^+$	88.42	91.74	90.19

	mean (repl. 1)		mean (repl. 2)		mean (repl. 3)	
Quadrant	X Mean	Y Mean	X Mean	Y Mean	X Mean	Y Mean
upper left (UL)	6.00	378.36	5.08	441.25	5.13	345.04

***Table 8**: FACS results for single labeled HC11 cells expressing Gal-1. All samples were run in triplicates. The data on the top represents the percentage of HC11 cells expressing Gal-1 out of the cells gated. The data below show the mean intensities of the detected signal of Gal-1 (Y Mean). The detected intensity of replicate 1 is illustrated in **Figure 18a** and **Figure 18b**.*

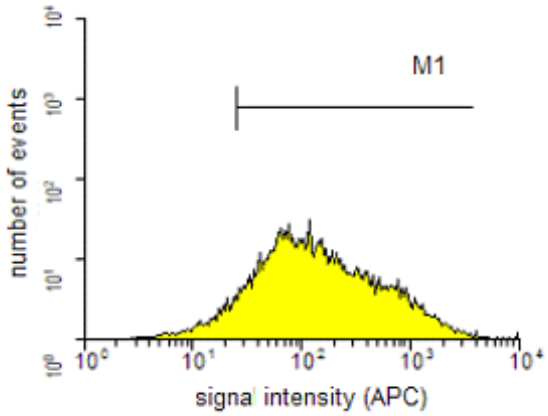
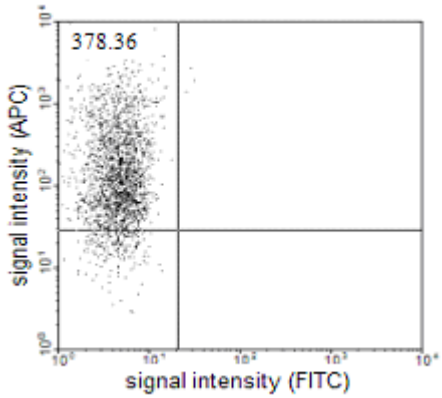


Figure 18a

***Figure 18a:** FACS histogram of Gal-1 (APC) stained undifferentiated HC11 cells (replicate 1, see Table 8). **Figure 18b:** Distribution of Gal-1 (APC, Y Mean) stained undifferentiated HC11 cells (replicate 1, see Table 8). The mean intensity of the cells expressing Gal-1 (replicate 1) is indicated in the upper left quadrant.*



	replicate 1 [%]	replicate 2 [%]	replicate 3 [%]
Sca1$^+$	64.87	64.04	65.97
Gal-1$^+$	88.73	90.64	90.18
Sca$^+$Gal-1$^+$	60.73	60.13	61.80

	mean (repl. 1)		mean (repl. 2)		mean (repl. 3)	
Quadrant	X Mean	Y Mean	X Mean	Y Mean	X Mean	Y Mean
upper left (UL)	13.04	155.05	13.00	153.24	13.18	151.17
upper right (UR)	226.98	419.70	234.17	436.02	229.57	413.12
lower right (LR)	198.98	19.25	205.57	18.56	200.87	19.76

***Table 9**: FACS results for double labeled HC11 cells expressing Sca1 and Gal-1. All samples were run in triplicates. The data on the top represents the percentage of HC11 cells expressing Sca1 and /or Gal-1 out of the cells gated. The data below show the mean intensities of the detected signal of Gal-1(Y Mean) and Sca1 (X Mean). The detected intensities of replicate 1 are illustrated in **Figure 19a**, **Figure 19b** and **Figure 19c**.*

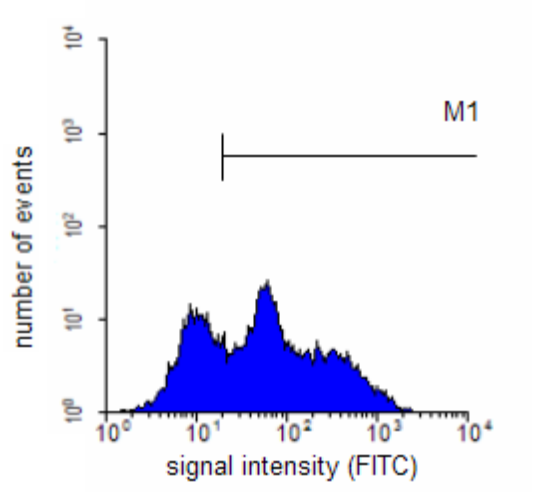


Figure 19a

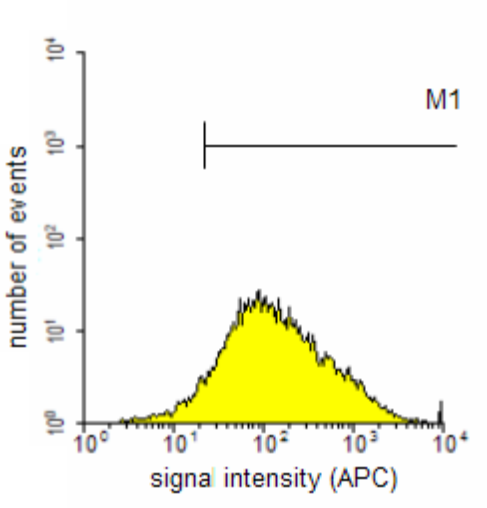


Figure 19b

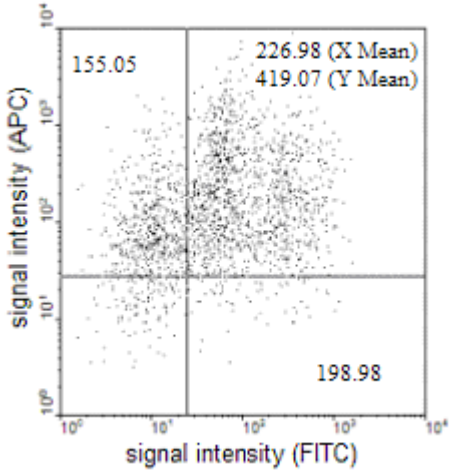


Figure 19c

***Figure 19a:** FACS histogram of Sca1 (FITC) stained undifferentiated HC11 cells (replicate 1, see Table 9). **Figure 19b:** FACS histogram of Gal-1 (APC) stained undifferentiated HC11 cells (replicate 1, see Table 9). **Figure 19c:** Distribution of Sca1 (FITC, X Mean) and Gal-1 (APC, Y Mean) stained undifferentiated HC11 cells (replicate 1, see Table 9). The mean intensity of the cells expressing Gal-1 (Y Mean, APC) is indicated in the upper left quadrant, the mean intensity for Sca1 (X Mean, FITC) expressing cells is indicated in the lower right quadrant and the mean intensities for Sca1 (X Mean, FITC) and Gal-1 (Y Mean, APC) expressing undifferentiated HC11 cells can be found in the upper right quadrant.*

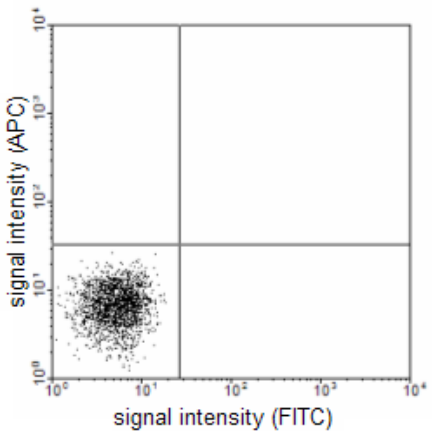


Figure 20

Figure 20: *FACS results for unstained undifferentiated HC11 cells (negative control). This sample was not run in triplicates (just one sample). The FACS results for unlabeled HC11 cells revealed that 99.96% of the gated cells were detected as unstained.*

The FACS experiment carried out with unstained HC11 cells revealed that 99.96% of the HC11 cells gated were detected as unstained. Figure 20 illustrates this result: almost every detected signal is located in the lower left quadrant.

The results for single and double stained HC11 cells with Gal-1 and/or Sca1 are summarized in Table 10.

Gal-1$^+$ [%]	**Sca1$^+$** [%]	**Gal-1$^+$Sca1$^+$** [%]
90.12 $^+$/- 0.66	60.85 $^+$/- 0.61	60.77 $^+$/- 0.90

Table 10: FACS results for single and double labeled HC11 cells with Sca1 and Gal-1. The results show the percentage of HC11 cells expressing Sca1 and /or Gal-1 out of the cells gated.

The results of the FACS experiment also revealed that $Sca1^{+}$ HC11 cells can furthermore be divided into a $Sca1^{high}$ and a $Sca1^{low}$ HC11 cell population (see Figure 21 and Table 11). For the calculations the program WinMDI® was employed. Figure 21 shows three peaks, whereby the peak on the left represents the Sca^{-} population out of the undifferentiated HC11 cells gated and the two peaks on the right represent the Sca^{+} population. With WinMDI® it was possible to set new marker boundaries. Marker M1 (see Figure 21) represents all Sca^{+} cells out of the HC11 cells gated, whereas Marker 2 represents the Sca^{high} population. Subtraction of the percentage of Sca^{+} and Sca^{high} HC11 cells resulted in the percentage of Sca^{low} HC11 cells (see Table 11).

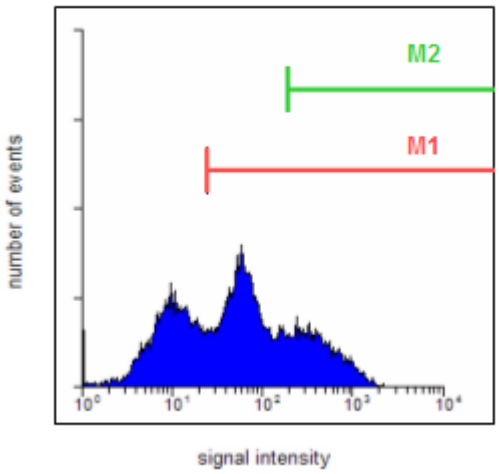


Figure 21: HC11 cells expressing Sca1. The peak on the left illustrates the number of $Sca1^{-}$ HC11 cells out of the cells gated in the FACS experiment, the peak in the middle represents the $Sca1^{low}$ HC11 population and the peak on the right illustrates the $Sca1^{high}$ HC11 population.

	$Sca1^{high}$ [%]	Sca^{+} [%]	Sca^{low} [%]
replicate 1	23.13	60.57	37.5
replicate 2	22.74	60.42	37.68
replicate 3	23.58	61.55	37.91
average [%]	**23.15**	**60.85**	**37.70**
st. dev [$^{+}$/- %]	0.46	0.61	0.21

Table 11: Percentage of $Sca1^{high}$ and $Sca1^{low}$ expressing HC11 cells out of the cells gated.

4. Discussion and Future Work

4.1 Gene expression in HC11 cells

The genes *Lgals1*, *Ran*, *Ppm1α* and *Rab14* were ranked among the top eleven candidates in the gene array upon the highest levels of differential expression and upon the reproducibility of the qualitative comparision of their expression in undifferentiated, competent and induced HC11 cells (see appendix). *Id2* was chosen due to its role as a valuable tool to monitor the induction to cellular differentiation of 1 hour DIP treated HC11 cells.

4.1.1 *Id2*

The confirmed upregulation of *Id2* mRNA in induced compared to competent and undifferentiated HC11 cells is evidence for the actual induction of HC11 cells to cellular differentiation (Figure 6a).

4.1.2 *Lgals1*

Reverse transcriptase PCR is a suitable tool for the qualitative comparision of gene expression in undifferentiated, competent, induced and differentiated HC11 cells. Two out of four investigated genes were found to be higher expressed in undifferentiated compared to competent and induced HC11 cells. These two genes are *Lgals1* and *Ran* (see 4.1.3).

There are three reasons why *Lgals1* was analyzed for its role as a potential stem/progenitor cell marker: in first place, according to the results of the gene array, *Lgals1* is upregulated in undifferentiated compared to competent and induced HC11 cells (see Table 4, row "*Lgals1* (gene array)"). Secondly, Gal-1 is located in the extracellular space, which makes it suitable for a cell marker and thirdly there is evidence that Gal-1 promotes proliferation in other SC systems (Sakaguchi *et al.*, 2006), indicating an important functional role of Gal-1 in SCs.

The PCR results for *Lgals1* confirm the data from the gene array (see Table 4, row "*Lgals1* (gene array)"): *Lgals1* is upregulated in undifferentiated compared to competent and induced HC11 cells and it is similar expressed in competent and induced HC11 cells (Figure 7a). The induction of HC11 cells consists of a one hour treatment with the lactogenic hormone mix DIP (see Material and Methods). All the changes that occur in HC11 cells during this last treatment step can be seen as the early response to the stimulation of cellular differentiation. Therefore it was also interesting to find out if *Lgals1* is upregulated in differentiated compared to undifferentiated and competent HC11 cells, which would indicate that Gal-1 plays an important role in HC11 differentiation. The PCR results revealed that *Lgals1* is not upregulated in differentiated compared to undifferentiated and competent HC11 cells (Figure 7c).

In a next step the relative expression differences of *Lgals1* in undifferentiated, competent and induced HC11 cells were quantified by real-time PCR (RT-PCR) experiments. These experiments revealed that *Lgals1* is significantly ($P < 0.05$) 4.1-fold upregulated in undifferentiated compared to induced HC11 cells ($P = 0.001$) and significantly 3.5-fold upregulated in undifferentiated compared to competent HC11 cells ($P = 0.008$) (Figure 12 and Table 4, row "*Lgals1* (RT-PCR)"). The RT-PCR results mainly confirm the expected expression pattern of *Lgals1* qualitatively, but not quantitatively (see Table 4, column "Fold change"). The differences between the expression of *Lgals1* in undifferentiated compared to competent and undifferentiated compared to induced HC11 cells as a result of RT-PCR experiments are much larger than the data from the gene array indicates (see Table 4, column "Fold change").

The differences between the results of the two independent experiments might have several reasons: firstly, different mRNA samples were used in the two experiments. Secondly, different serums were used during the cell culture procedure for HC11 cells during this project and during the experiments for the gene array. Thirdly, undifferentiated HC11 cells were harvested for mRNA extraction with a 60% confluence in this project compared to 50% confluent HC11 cells harvested for the experiments conducted by the group of Dr. Angel. Since the percentage of cell confluence is estimated by observing the cells under a light microscope, it is likely that the cells for this project and the cells for the gene array experiments were harvested at a slightly different confluence. During this project the observation was made, that small changes in the confluence of harvested undifferentiated HC11 cells have a major impact on the comparision of *Lgals1* expression in undifferentiated, competent and induced HC11 cells (see 3.1.2). PCR experiments revealed that the amount of expressed *Lgals1* in undifferentiated HC11 cells drastically increases with confluence (see 3.1.2). This might explain the discrepancies between the results of the quantitative

comparision of *Lgals1* expression derived from experiments in this project and from the experiments conducted by Dr. Angel and proves that the downregulation of *Lgals1* in competent and induced HC11 cells is regulated by differentiation (see Figure 8a).

In a last step in order to investigate *Lgals1* expression in undifferentiated, competent and induced HC11 cells, the amount of expressed Gal-1 in HC11 cells was analyzed by Western blot experiments. Gal-1 was found to be higher expressed in undifferentiated compared to competent and induced HC11 cells (Figure 15a). Although differences in *Lgals1* expression in undifferentiated, competent and induced HC11 cells can be detected by monitoring the changes of *Lgals1* mRNA expression, it is possible that Gal-1 synthesis is not completed within one hour of DIP treatment and therefore changes in Gal-1 expression in induced HC11 cells might not be detectable. This is the reason why Gal-1 levels were compared in undifferentiated, competent and differentiated HC11 cells. The results show that Gal-1 is upregulated in undifferentiated compared to competent and differentiated HC11 cells (Figure 15c).

As mentioned above PCR and Western blot experiments were employed to qualitatively investigate the expression pattern of selected genes in undifferentiated, competent, induced and differentiated HC11 cells. In order to obtain quantitative information out of this experiments, ImageJ®, a program capable to calculate area and pixel value statistics of intensity thresholded objects *(*http://rsb.info.nih.gov/ij/*),* was employed. The results of this analysis are shown in Table 6 and revealed that the differential expression of *Lgals1* in undifferentiated, competent, induced and differentiated HC11 cells on the mRNA level are comparable to the differential *Lgals1* expression on the protein level.

In order to determine the function of Gal-1 in HC11 cells, *Lgals1* was knocked down by cloning a DNA encoding a short hairpin RNA sequence directed against *Lgals1* into an expression vector and transfecting HC11 cells (Figure 16). Based on the facts that Gal-1 promotes proliferation (Sakaguchi *et al.*, 2006) and knockdown of *Lgals1* expression negatively influences migration (Thijssen *et al.*, 2004) of certain cell types, proliferation and migration assays with parental HC11 cells and *Lgals1* knockdown cells will be carried out after the *Lgals1* knockdown has been confirmed by PCR and Western blot experiments.

FACS experiments were carried out to determine the potential of Gal-1 as a stem/progenitor cell marker. 90.12% of all gated undifferentiated HC11 cells expressed Gal-1, whereas only 60.85% expressed Sca1 and 60.77% of the cells expressed Gal-1 and Sca1 (see Table 10). In a study carried out by Stingl *et al.*, murine mammary glands were enzymatically digested and $CD49f^{+6}$ cells were sorted out using FACS (Stingl *et al.*, 2006). The results revealed that more than half of the mammary repopulating units[7] (MRUSs) of all MRUs comprised in the mammary gland were found within the $CD49f^{high}Sca1^{low}$ population at a sevenfold higher frequency than the frequency of MRUs in the cell suspension obtained directly from the digestion (Stingl *et al.*, 2006). In contrast, no MRUs were found within the $CD49f^{high}Sca1^{high}$ population (Stingl *et al.*, 2006). These results evidently prove the existence of $Sca1^{high}$ and $Sca1^{low}$ cells within the mammary gland and indicate that stem or progenitor cells of the mammary gland are more enriched in the $Sca1^{low}$ population compared to the $Sca1^{high}$ population. In the FACS experiment conducted in this project 37.70% of all gated cells showed a low expression of Sca1 (see Table 11, column "$Sca1^{low}$ [%]"). Based on the information above, the results indicate that 37.70% ($Sca1^{low}$) of all gated HC11 cells are supposed to be more alike stem/progenitor cells than the $Sca1^{high}$ or $Sca1^{-}$ population.

[6] CD49f is a protein expressed by epidermal SCs and is used to enrich potential stem/progenitor cells of the murine mammary gland (Stingl *et al.*, 2006).

[7] Mammary repopulating units (MRUs) are cells of the mammary gland with self-renewal potential and the ability to regenerate new mammary tissue when injected into the cleared fat pad of mice (Stingl *et al.*, 2006).

In summary, these experiments revealed that Gal-1 has the potential as a marker protein to enrich stem/progenitor cells of the mammary gland and functional assays carried out with HC11 *Lgals1* knockdown cells might reveal important functions of Gal-1 in mammary epithelial cells.

4.1.3 *Ran*

The two facts that *Ran* is upregulated in hHSCs, in comparision to their differentiated progeny (Ivanova *et al*., 2002) and the higher expression of *Ran* in undifferentiated compared to competent and induced HC11 cells (see Table 5, row "*Ran* (gene array)") were the reasons why *Ran* was selected for further investigation for its role as a potential stem/progenitor cell marker.

Using PCR for qualitative comparision, it was shown that *Ran* followed the expected expression scheme (Figure 9a and Table 5, row "*Ran* (gene array)"). Even the slight downregulation of *Ran* expression in competent compared to induced HC11 cells was confirmed by PCR experiments. The results of two independent RT-PCR experiments quantitatively confirmed the results of the gene array ($P < 0.09$) (see Table 5, row "*Ran* (RT-PCR)", column "undifferentiated vs. induced").

The results of the first RT-PCR experiment (Figure 13) showed a 1.3-fold upregulation of *Ran* expression in undifferentiated compared to induced HC11 cells (see Table 5, row "*Ran* (1. RT-PCR)"). The reason for the variation in the expression for *Ran* might be a result of dilution or pipetting errors during sample preparation. Therefore the RT-PCR experiment was repeated (see Figure 14 and Table 5, row "*Ran* (2. RT-PCR)"), unfortunately the results

showed equally high standard deviation values as the results of the first experiment (see Figure 13 and Table 5, row "*Ran* (1. RT-PCR)")) and a less significant difference ($P < 0.07$) in *Ran* expression between undifferentiated and induced HC11 cells than in case of *Lgals1* ($P < 0.05$).

One explanation for the high standard deviation values is that *Ran* expression might be very sensitive to small methodical irregularities during the HC11 cell culture procedure and the following mRNA extraction. This consideration is supported by the fact, that the same cDNA samples were used for the comparision of *Ran* expression and for the comparison of *Lgals1* expression in undifferentiated, competent and induced HC11 cells, whereby the standard deviation values in case of *Lgals1* expression are relatively small (Figure 12) compared to the standard deviation values of *Ran* expression (Figure 13 and Figure 14). Based on the RT-PCR results for *Ran* it was decided that this gene will not be further investigated for its possible role as a stem/progenitor cell marker.

4.1.4 *Ppm1α*

The PCR experiment results for *Ppm1α* revealed that this gene is similarly expressed in undifferentiated, competent and induced HC11 cells (Figure 10a), which does not go along with the expression scheme I expected from the gene array (see Table 2, row "*Ppm1α*").

The reason for the discrepancies of the results for *Ppm1α* in this project compared to the results from the gene array might have several reasons: firstly, the mRNA samples I used were different from the mRNA samples the gene array was based on. Secondly, different serums for the cell culture procedure were used in this project and in the cell culture

procedure for the gene array and thirdly, which might be the most important consideration, the undifferentiated HC11 cells could have been harvested at a slightly different confluence in the two trials, which might have a major impact on the expression of *Ppm1α* in undifferentiated HC11 cells. Not able to confirm the expected expression pattern, it was decided that *Ppm1α* will not be further investigated.

4.1.5 *Rab14*

PCR experiments revealed that *Rab14* is similarly expressed in undifferentiated, competent and induced HC11 cells (Figure 11a). The reasons why I could not confirm the results from the gene array (see Table 3, row "*Rab14*") are the same as mentioned in 4.1.4.

4.2 Summary

The aim of this project was to investigate differentially expressed genes in HC11 cells, which might be stem/progenitor cell marker candidates. Polymerase chain reaction experiments revealed that out of four genes only *Lgals1* and *Ran* were upregulated in undifferentiated compared to competent and induced HC11 cells. These findings were confirmed and quantified with real-time PCR experiments. Western Blot experiments confirmed the upregulation of galectin-1 (Gal-1), the *Lgals1* gene product, in undifferentiated compared to competent, induced and differentiated HC11 cells. FACS experiments revealed that Gal-1 expression overlaps with Sca1 expression and therefore could be used to enrich stem/progenitor cells of the mammary gland. DNA encoding a shRNA sequence directed

against *Lgals1* was designed to investigate the functional role of Gal-1 by using RNAi in a next step.

In this study Gal-1 was identified as a marker protein that potentially could be used to enrich possible stem or progenitor cells of the mammary gland and functional assays with *Lgals1* knockdown cells need to be performed and will shed more light on the functional role of Gal-1 in HC11 and stem or progenitor cells of the mammary gland.

5. Future Work

In order to further determine the quality of galectin-1 (Gal-1), the *Lgals1* gene product, as a potential stem/progenitor cell marker, $Sca1^{+}Gal\text{-}1^{+}$ HC11 cells will be sorted by fluorescent activated cell sorting (FACS) and functional assays will be performed with $Sca1^{+}Gal\text{-}1^{+}$ HC11 cells, as $Sca1^{+}$ cells of the murine mammary gland are considered to be potential mammary epithelial stem/progenitor cells (Welm *et al*., 2002). Gal-1 might be involved in HC11 proliferation and migration, therefore performing proliferation and migration assays with $Sca1^{+}Gal\text{-}1^{+}$ HC11 cells in comparision to parental HC11 cells will shed more light on the functional role of Gal-1 in HC11 cells.

Another approach to determine the function of Gal-1 in HC11 cells will be pursued by carrying out proliferation and migration assays with HC11 *Lgals1* knockdown cells, after the gene knockdown has been confirmed by PCR and Western blot experiments. The results of the functional assays with HC11 *Lgals1* knockdown cells, as well with $Sca1^{+}Gal\text{-}1^{+}$ HC11 cells in comparision to parental HC11 cells will not only provide information about the function of Gal-1 in HC11 cells, but also give further insight of the possible functional role of Gal-1 in stem/progenitor cells of the mammary gland.

The role of Gal-1 as a potential stem/progenitor cell marker can furthermore be determined by transplanting Gal-1^+ HC11 cells and parental HC11 cells into the cleared fat pad of mice, since it has been shown that HC11 injection has led to a partial regeneration of a murine mammary gland (Humphreys and Rosen, 1997). It is expected that, in comparision to parental HC11 cells, a lower Gal-1^+ HC11 cell number is required to partially regenerate a mammary gland in mice.

6. Abbreviations

%	percent
°C	degree Celsius
mA	milliampere
g	gram
mg	milligram
µg	microgram
l	liter
ml	milliliter
µl	microliter
mM	millimol per liter
µM	micromol per liter
nM	nanomol per liter
cm	centimeter
cm^2	sqaure centimeter
µm	micrometer
h	hour
min	minute
sec	second

AB	antibody
AMP	ampicillin
APS	ammonium persulfate
bHLH	basis helix-loop-helix
BMP	bone morphogenic protein
BrdU	5-bromo-2-deoxy-uridine
BSA	bovine serum albumin
CSC	cancer stem cell
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DEX	dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic
EGF	epidermal growth factor
ECM	extracellular matrix
EtBr	ethidium bromide
EtOH	ethanol
FC	final concentration
Id2	inhibitor of DNA binding 2
FA	formaldehyde agarose
FACS	fluorescent activated cell sorting
Gal-1	galectin-1
GDP	guanosin diphosphate
GFP	green fluorescent protein
Grb2	growth-factor receptor bound protein 2
GTP	guanosin triphosphate
GTPase	guanosin diphosphatase
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
HI-FBS	heat inactivated fetal bovine serum
HLH	helix-loop-helix
H_2O	hydrogen hydroxide
INS	insulin
KCl	potassium chloride
KH_2PO_4	potassiumdihydrogenphosphate
Leu	leucin
Lgals1	lectin, galactose binding, soluble 1
mESC	murine embryonic stem cell
$MgCl_2$	magnesium chloride
mHSC	murine haematopoietic stem cell
mNSC	murine neural stem cell
mRNA	messenger ribonucleic acid
MRU	mammary repopulating unit
NaCl	sodium chloride
Na_2HPO_4	disodiumhydrogenphosphate
Oligo dT	deoxythymidine oligonucleotides
OPC	oligodentrocyte precursor cells
PAO	phenylarsine oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
PI3K	phosphoinositide 3-kinase
PFA	paraformaldehyde
PMSF	phenylmethanesulphonylfluoride
Ppm1α	protein phosphatase 1a
PRL	prolactin
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
rpm	revolutions per minute

RPMI	Roswell Park Memorial Institute Medium
RT	room temperature
RT-PCR	real-time polymerase chain reaction
SC	stem cell
Sca1	stem cell antigen 1
SDS	sodium dodecyl sulfate
Serbp1	serpine1 mRNA binding protein
shRNA	short hairpin ribonucleic acid
TBE	tris/borate/EDTA buffer
TBS	tris-buffered saline
TBS-T	tris-buffered saline-Tween
Tcea1	transcription elongation factor A (SII)
TEMED	tetra-methyl-ethylenediamine
TGF-β	transforming growth factor-β
TIC	tumour initiating cells
TRIS base	2-amino-2-hydroxymethyl-1,3-propanediol
Wwc1	WW, C2 and coiled-coil domain containing 1
Ywhag	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide

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8. Appendix

GeneSymbol or Gene Name	growing vs. competent	# Spots	Mean Intensity	norm. Log Ratio A	norm. Log Ratio B	Final Mean of norm. Log Ratios	Std.Dev.	similar on both slides	regulated G/C	competent vs. induced	# Spots	Mean Intensity	norm. Log Ratio A	norm. Log Ratio B	Final Mean of norm. Log Ratios	Std.Dev.	similar on both slides	regulated C/I	growing vs. induced	# Spots	Mean Intensity	norm. Log Ratio A	norm. Log Ratio B	Final Mean of norm. Log Ratios	Std.Dev.	Regulated on both slides	regulated G/I	# Spots total	2x Yes (11 genes)
Tcea1		4	410	1,5	0,5	**1,0**	0,5	yes	1		1	426	0,0	-1,0	**-1,0**	--	--	0		3	469	1,4	2,0	**1,7**	0,3	yes	1	8	**1**
Serbp1		3	481	0,7	1,5	**1,1**	0,4	yes	1		1	1199	2,7	0,0	**2,7**	--	--	1		4	376	1,1	1,1	**1,1**	0,0	yes	1	8	**1**
Rab14		2	435	1,0	0,4	**0,7**	0,3	yes	1		1	1244	2,8	0,0	**2,8**	--	--	1		3	374	1,9	1,2	**1,5**	0,4	yes	1	6	**1**
Tmco1		4	375	0,3	-0,2	**0,0**	0,3	yes	0		1	846	2,2	0,0	**2,2**	--	--	1		4	318	1,7	1,2	**1,4**	0,3	yes	1	9	**1**
Wwc1		4	384	1,0	1,5	**1,2**	0,3	yes	1		1	1000	2,4	0,0	**2,4**	--	--	1		3	343	1,7	1,7	**1,7**	0,0	yes	1	8	**1**
Ppm1a		4	679	1,0	1,2	**1,1**	0,1	yes	1		3	940	-0,4	0,8	**0,2**	0,6	no	0		4	511	1,3	1,7	**1,5**	0,2	yes	1	11	**1**
Pef1		4	3559	0,7	1,4	**1,0**	0,4	yes	1		4	4046	-0,5	0,4	**-0,0**	0,4	yes	0		4	2794	2,1	1,1	**1,6**	0,5	yes	1	12	**1**
Ywhag		4	1563	0,8	1,1	**0,9**	0,1	yes	1		4	1832	-0,5	0,2	**-0,1**	0,3	yes	0		4	1350	1,3	1,1	**1,2**	0,1	yes	1	12	**1**
Lgals1		4	3079	1,3	1,6	**1,5**	0,2	yes	1		4	1844	-0,5	-0,3	**-0,4**	0,1	yes	0		4	3253	1,9	1,2	**1,6**	0,3	yes	1	12	**1**
Ran		4	1219	1,1	2,0	**1,6**	0,5	yes	1		4	925	-1,0	-0,9	**-0,9**	0,0	yes	0		4	1449	1,2	1,1	**1,1**	0,1	yes	1	12	**1**
MNI07591		3	979	0,5	1,4	**0,9**	0,4	yes	1		3	703	-0,7	-0,6	**-0,6**	0,0	yes	0		4	754	1,1	1,3	**1,2**	0,1	yes	1	10	**1**

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