4 DISCUSSION

4.1 Transcriptional profiling of differentiating neural progenitor cells

The mammalian brain contains specialized cells in the hippocampus and the subventricular zone capable of generating new neurons during the whole life. This novel insight has significant medical impact, since diseases involving neuronal death are difficult, if not impossible to treat so far. The newborn neurons are generated by neural progenitors, which constantly proliferate and thereby produce young neuroblasts. These neuroblasts migrate through the brain towards selected areas such as the olfactory bulb or the granule cell layer of the hippocampus. Upon arrival, they terminally differentiate and functionally integrate into the circuitry. However, the molecular biology that controls proliferation, migration, and differentiation of these cells is poorly understood.

The study presented here was performed to shed more light on the molecular processes that take place in these cells. To study neurogenesis in the adult SVZ, primary cultures of progenitor cells were established first. In analogy to the *in situ* situation, these progenitors divide *in vitro*, allowing to propagate the rather small population from the adult mouse brain. The progenitors were induced to migrate and differentiate into neurons and astrocytes by modification of the culture conditions. Thus, this cell culture system simulated the situation in the adult brain.

Large cDNA microarrays containing almost 14,000 clones were used to study the transcriptional changes that take place during the differentiation of neural progenitors. Since little was known about the molecular events, these experiments were done as screening assays to find genes with a functional role in the proliferation of the progenitors or in the migration and differentiation of their descendants. To preferentially identify genes with key regulatory functions and to be able to distinguish these from secondary effects, a time course study was done. Proliferating undifferentiated progenitors were compared to cells that had differentiated for 24, 48, or 96 hours.

Comparing the proliferating progenitors to differentiating cells revealed many genes whose expression changed during this process. Down-regulation of a transcript demonstrates its presence in the neurosphere cells and indicates that the encoded protein is relevant for the immature, proliferating state. In contrast, the products of genes which were found to be up-regulated during differentiation might have a function in the transition from immature to mature cell types or in the differentiated cells.

In general, almost all of the differentially expressed genes showed consistent up- or down-regulation throughout the time course. Therefore, an up- or down-regulation at 24 hours or

48 hours allowed to assume a further increase or decrease at later time points. To extract the dynamic behavior of the relevant gene expression changes during differentiation, a cluster analysis was performed. This groups genes according to the onset and dynamics of expression changes in a time course experiment. Here, the clones which were identified as being differentially expressed were grouped in ten clusters, five containing up-regulated and five containing down-regulated clones. Most clusters did not contain functionally related genes. However, cluster nine contained almost exclusively genes related to the cell cycle, and these were all early and strongly down-regulated. This supported the notion, that the progenitors stopped to divide upon induction of differentiation. Furthermore, it indicates that cell cycle exit is a very early event in the differentiation process. Cluster two contained transcripts that had changed significantly only after 96 h, and some of the corresponding genes are related to the cellular morphology and the cytoskeleton, or encode cell surface molecules. This demonstrates the acquisition of more mature cell types.

The heterogeneity of cultured neurospheres complicates the evaluation of the microarray results. Changes in expression of individual genes are unlikely to take place in all cells equally. Instead, the fold changes measured are probably due to much stronger expression changes in subpopulations of cells. However, it is desirable to attribute individual gene expression changes to certain cell types. For a few candidate genes the cell type expressing the gene of interest was studied *in vitro* by immunofluorescence. However, it was the purpose of this study to discover genes that are relevant for adult neurogenesis *in situ*. Therefore, it is necessary to analyze the distribution of the encoded proteins in the respective brain regions. This was done for four selected candidate genes.

Stem cells and neural differentiation have been studied with methods for large scale gene expression analysis before and in parallel to this study. Some of the genes identified in this study have been previously found to be specific to or enriched in neural progenitor cells derived from embryonic (D´Amour and Gage, 2003; Ivanova et al., 2002; Fortunel et al., 2003) or adult (Ramalho-Santos et al., 2002) tissue. Some of these studies compared neural progenitors to hematopoietic or embryonic stem cells with microarrays. Neurosphere cells from newborn mice were compared to cells differentiated for 24 hours by representational difference analysis and microarrays (Geschwind et al., 2001; Karsten et al., 2003; Easterday et al., 2003). These studies, however, identified less genes than found in this work, because the microarrays used by others contained less probes. In addition, they only compared the gene expression of undifferentiated cells to cells differentiated for 24 hours. However, the results presented here demonstrate that expression changes intensified over time, resulting in more clones displaying a significant change in transcript levels at later time points. Nonetheless, the identification of a gene in

independent studies is supportive for its functional role in adult neurogenesis. Therefore, previously published datasets were compared to this one, and genes that were identified in other studies as well were indicated in table one.

In the following, some very interesting candidate genes, their expression, and their potential functional involvement in adult neurogenesis are discussed.

4.2 PTPNS1 and CD47

4.2.1 Expression of *Ptpns1* and *Cd47*

The transcript level for *protein tyrosine phosphatase, non-receptor type substrate 1 (Ptpns1*, also called *Shps-1*, *Sirpα*, *Bit*, *P84*, *Mfr*) was increased and the transcript level for *cluster of differentiation 47* (*Cd47*, also called *Iap*) was decreased during neural progenitor cell differentiation *in vitro*. The encoded transmembrane proteins have been shown to constitute a cell-cell communication system in which they interact through their extracellular domains on adjacent cells (reviewed in Oshima et al., 2002). Of interest in the context of adult neurogenesis is their function in cellular aggregation and migration (Babic et al., 2000; Liu et al., 2002; Motegi et al., 2003).

The two proteins were detected in cells within the neurosphere and, to a lesser extent, in migrating cells close to the sphere. However, single cells that had migrated away from the spheres were negative for both proteins. In contrast, *in vitro* chain migrating cells contained PTPNS1 and CD47. These results indicated, that this system might be involved in the aggregation of the cells in neurosphere clusters or in the chain migration of neuroblasts.

Ptpns1 is expressed in a variety of CNS regions (Chuang and Lagenaur, 1990). Of interest for this study are *in situ* analyses that revealed *Ptpns1* expression in mitral and periglomerular cells of the olfactory bulb and in granule cells and other neurons of the hippocampus (Comu et al., 1997). Immunohistochemical experiments in this study revealed a similar distribution pattern for PTPNS1 and CD47 in areas of adult subventricular neurogenesis. The proteins were detected in GFAP-positive neural progenitor cells and in neuroblasts of the adult SVZ. In addition, both proteins were present in the RMS, the structure along which neuroblasts migrate to the olfactory bulb. Thus, findings from experiments with neurosphere cells *in vitro* corresponded to the situation *in situ*. Interestingly, expression and protein distribution of these candidates seem to coincide with areas of adult neurogenesis, namely in the SVZ and olfactory bulb and in the hippocampus.

4.2.2 PTPNS1 and CD47 in cell-cell interactions

PTPNS1 is a transmembrane protein and a member of the immunoglobulin (Ig) superfamily of proteins. The large extracellular part comprises three Ig-like domains and the small cytoplamic region contains four putative tyrosine phosphorylation sites and binding sites for SHP-2. SHP-2 is encoded by *Ptpn11*. The protein is a positive signal tranducer for several receptor tyrosine kinases and cytokine receptors. It binds to the cytoplasmic part of PTPNS1 and phosphorylates it (Fujioka et al., 1996; Kharitonenkov et al., 1997). The extracellular domain of PTPNS1 interacts with CD47 on neighboring cells (Seiffert et al., 1999; Jiang et al., 1999). CD47 also is an Ig family member. It consists of an Ig-like extracellular domain, five membrane-spanning domains, and a short cytoplasmic tail. Initially, it was identified as being physically associated with integrins on the plasma membrane and therefore called integrin-associated protein (IAP) (Brown et al., 1990).

The PTPNS1-CD47 system fulfills different functions. CD47 was described as a 'marker of self' on red blood cells, instructing macrophages not to digest red blood cells (Oldenborg et al., 2000). Red blood cells do not present class I MHC proteins, which are a well-known 'marker of self'. Instead, they present CD47, which is recognized by PTPNS1 on the macrophage surface. Red blood cells lacking CD47 were rapidly digested. Inhibition of the PTPNS1-CD47 interaction by an antibody specific to the extracellular domain of PTPNS1 caused digestion of CD47-positive red blood cells (Oldenborg et al., 2000). Macrophages strongly express *Ptpns1* (Veillette et al., 1998) and the interaction between CD47 and PTPNS1 works as a tethering step in the phagocytosis of apoptotic cells (Tada et al., 2003). Mice carrying a homozygous deletion of the PTPNS1 cytoplasmic domain presented a thromocytopenia (reduced platelet count), which resulted from an increased rate of clearance of circulating platelets (Yamao et al., 2002). These authors argued, that increased phagocytic activity of macrophages is likely responsible for this effect. However, the role of the PTPNS1-CD47 system in phagocytosis is not yet understood in detail.

For neural cells, purified PTPNS1 was shown to promote neuronal adhesion and neurite outgrowth of cerebellar cells *in vitro* (Chuang and Lagenaur, 1990). Correspondingly, expression of *Cd47* induced outgrowth of neurites in neuroblastoma cells and of dendrites in cultured cortical neurons, and association with PTPNS1 on the surface enhanced this effect (Miyashita et al., 2004; Numakawa et al., 2004). Thus, the heterophilic interaction of these two transmembrane proteins seems to support outgrowth of neurites and dendrites.

PTPNS1 and CD47 are also involved in the transmigration of neutrophils (polymorpho-nuclear leukocytes) across endothelial and epithelial monolayers. This is a key step in host defence during an infection. Immune cells bind to endothelial or epithelial cells, transmigrate

through the cell layer, and migrate towards the site of infection. This association and subsequent transmigration was inhibited by antibodies against CD47 (Cooper et al., 1995; Parkos et al., 1996) or by CD47-specific peptides (Liu et al., 2004). In contrast, stable transfection of epithelial cells with *Cd47* enhanced transmigration (Liu et al., 2001). Mice without CD47 are indistinguishable from their normal littermates, but more susceptible to bacterial infections (Lindberg et al., 1996). Thus, interaction of CD47 with PTPNS1 is required for migration of immune cells to sites of infection. CD47 on endothelial and epithelial cells physically interacts with PTPNS1 on immune cells, and a PTPNS1-specific antibody partially inhibited transmigration (Liu et al., 2002). Interestingly, PTPNS1 was also detected on endothelial cells and found to interact with CD47 on B cells, thereby mediating their adhesion to the endothelial cells (Yoshida et al., 2002).

PTPNS1 and CD47 also are involved in another mode of cellular aggregation and migration. Expression of *Ptpns1* in a pro-B-cell line induced aggregation of the cells. This aggregate formation did not take place in *Cd47*-deficient cells and was inhibited by CD47-specific antibodies (Babic et al., 2000). Fibroblasts derived from the mutant mice without the cytoplasmic domain of PTPNS1 displayed a defect in cytoskeletal organization and cellular migration (Inagaki et al., 2000). This function in cell migration was further analyzed by Motegi et al. (2003), who showed that migration of human melanoma cells and of CHO cells was inhibited by purified extracellular domains of CD47. Migration was supported by overexpression of *Ptpns1* in these cells. Critical for migration was the interaction of PTPNS1 with the cytoplasmic kinase SHP-2. The authors presented a model, in which PTPNS1 not bound to CD47 on neighboring cells supports migration, whereas the interaction with CD47 causes migration arrest (Fig. 32).

Recently, Ohnishi et al. showed that the ectodomain of PTPNS1 is clipped off by metalloproteinases (2004). This ectodomain shedding disrupts the interaction of PTPNS1 with CD47 and thereby reverses the inhibition of cell migration. In cells, which are non-migrating due to an interaction of PTPNS1 with CD47, ectodomain shedding could provide a rapid mechanism to induce migration.

Aggregation and migration of cells take place in neurosphere cell cultures. *In situ* the neuroblasts migrate through the CNS. *Ptpns1* and *Cd47* are expressed in neurosphere cells as well as in the SVZ and the RMS. Taken together, the data about the PTPNS1-CD47 system and its involvement in cell aggregation and migration argue for an involvement of these proteins in adult neurogenesis.

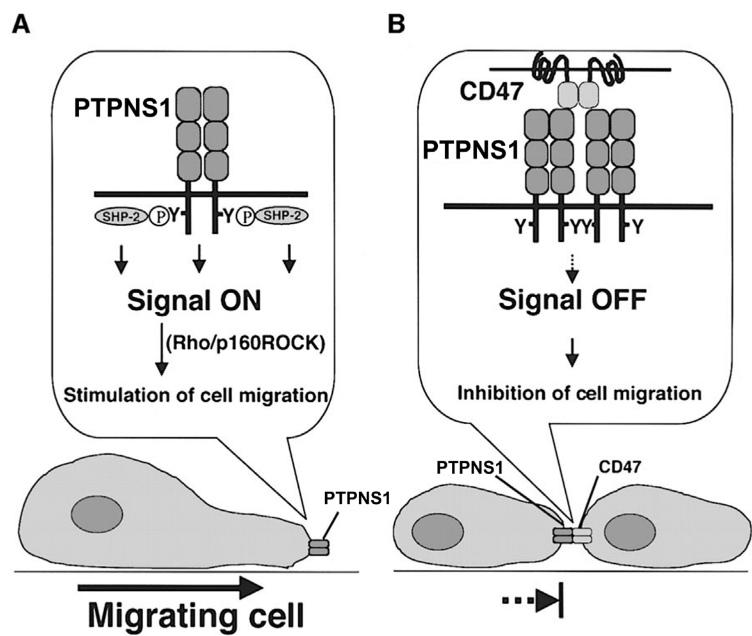

Figure 32. Model for the regulation of migration of melanoma and CHO cells by the PTPNS1-CD47 system. (A) During migration of an PTPNS1-expressing cell, PTPNS1 is tyrosine-phosphorylated in response either to integrin engagement by the extracellular matrix or to growth factor stimulation, resulting in the formation of an PTPNS1-SHP-2 complex that positively regulates cell migration through downstream signaling components including Rho and p160ROCK. (B) Interaction of a migratory PTPNS1-expressing cell with an adjacent CD47-expressing cell results in the engagement of PTPNS1 dimers by CD47 and consequent dephosphorylation of PTPNS1, the dissociation of SHP-2 from PTPNS1, impairment of Rho regulation, and cytoskeletal reorganization and inhibition of cell migration. Adopted from Motegi et al., 2003.

To study this presumed function of the PTPNS1-CD47 system in adult neurogenesis, fibroblasts stably expressing *Ptpns1* or *Cd47* were generated. Neurosphere cells were differentiated together with these fibroblasts, either in direct contact to them or in media conditioned by these cells. Attachment of the neurospheres, migration of the neuroblasts, and their differentiation were not altered by an excess of either PTPNS1 or CD47 presented to the progenitors. This could be due to technical limitations of the experimental system. The stably expressed proteins were glycosylated by the fibroblasts. However, PTPNS1 glycosylation is tissue-specific, with distinct patterns of glycosylation for neuronal and hematopoietic cells (van den Nieuwenhof et al., 2001). Thus, a neuronal cell line might be more suitable for these experiments. In addition, the cytoplasmic domain of PTPNS1, which recruits SHP-2 by four phosphorylated tyrosine residues, is important for the function of the extracellular domain (Sato et al., 2003). It is unclear, how these residues are phosphorylated in fibroblasts. Finally, the proteins might not have been transported to the cell surface properly, which could be tested by fluorescence-activated cell sorting.

4.2.3 PTPNS1 and CD47 - outlook

The PTPNS1-CD47 system likely plays a role in the cells of the adult neural progenitor niche. Two modes of regulating cell migration could be envisaged. An interaction of CD47 with PTPNS1 might be required for chain migration similar to the transmigration of neutrophils across endothelial cell layers (see section 4.2.2). Alternatively, the heterophilic interaction of PTPNS1 with CD47 might support cell-cell adhesion, and the interaction has to be broken for migration, as has been shown for melanoma cell migration (see section 4.2.2). Abolishment of this interaction could be achieved by increasing the abundance of one partner and decreasing that of the other partner. Thereby, the ratio of bound to unbound receptors would change. *In vitro*, the transcript for *Ptpns1* increased and for *Cd47* decreased. However, chain migrating neuroblasts contained both proteins. Signals did overlap for both proteins *in situ*, although the resolution was not sufficient to determine all corresponding cell types. This could be studied more efficiently with electron microscopy or with antibodies from different host species to co-localize both proteins in the CNS.

Ectodomain shedding could be a very efficient way to switch from a non-migrating to a migrating state. This could be demonstrated in differentiating progenitors by enrichment and purification of the released ectodomain from migrating neuroblasts *in vitro*. In addition, Ohnishi et al. used KB-R7785, a specific inhibitor of metalloproteinases that prevented shedding of PTPNS1 in CHO cells (2004). Application of this inhibitor might decrease migration of differentiating neurosphere cells *in vitro* or even neuroblasts *in situ*.

Very interesting results have been published concerning the function of CD47 in the CNS. Abundance of *Cd47* transcripts in the hippocampus positively correlates with increased memory function in rats, and the expression of *Cd47* was transiently induced upon certain learning tasks. Therefore, it was suggested that CD47 might be involved in the formation, but not the maintenance or storage of long-term memory (Huang et al., 1998; Lee et al., 2000). *Cd47*$^{-/-}$ mice displayed an impairment in memory formation and in hippocampal long-term potentiation (Chang et al., 1999). Injection of a CD47-specific antibody into the dentate gyrus of the hippocampus impaired memory retention in mice (Chang et al., 2001).

The functional involvement of CD47 in memory formation still has to be clarified. However, two scenarios seem possible. First, since the interaction of PTPNS1 and CD47 was shown to promote neurite outgrowth, the memory defect in *Cd47*$^{-/-}$ mice might be due to decreased neuronal maturation in the hippocampus. The affected cells could be newborn neurons, which are thought to function in memory formation. These newborn cells still have to develop neurites to functionally integrate into the circuitry. Second, newborn interneurons in the hippocampus need to migrate from the subgranular zone into the adjacent granule cell layer.

Thus, if the PTPNS1-CD47 system is involved in migration of adult neuroblasts, decreased migration leading to a reduced supply of newborn neurons might also contribute to the memory defect. Further experiments are needed to answer these questions. The mouse models mentioned above might serve as valuable tools for these studies. In addition, if newborn interneurons contribute to memory formation in the hippocampus and the olfactory bulb, the mouse models mentioned above might also suffer from a defect in odor memory. Immunohistochemical studies of their SVZ and RMS anatomy and cellular composition, as well as behavioral tests on their odor memory might detect such a phenotype.

It remains an interesting notion, that mutations in *PTPN11*, the gene encoding SHP-2, cause Noonan syndrome and juvenile leukemia (Tartaglia et al., 2001; Tartaglia et al., 2003). Patients with this syndrome frequently present, among malformations of the face and the heart, a mild mental retardation. Gain of function mutations leading to an increased tyrosine phosphatase activity of SHP-2 caused an increased activation of the RAS-ERK pathway and were sufficient to establish a condition similar to the Noonan syndrome in a mouse model (Araki et al., 2004). It is unknown, whether adult neurogenesis or, more specifically, PTPNS1 function and phosphorylation are affected in Noonan patients.

4.3 Molecules relevant for adult neural progenitor cells

Proliferation and migration of neural progenitors in the adult SVZ has to be tightly regulated. This regulation involves intracellular mechanisms, such as transcriptional activity and signal transduction, and intercellular mechanisms, such as communication through cell-cell contacts and secreted factors. In this section, a few identified differentially expressed genes are discussed. Among them is *Fabp7*, a gene with a very interesting expression pattern. In addition, the probable involvement of insulin-like growth factor binding proteins and the importance of transcriptional activity are discussed.

This study identified secreted protein factors, whose genes are expressed in dividing progenitors, but down-regulated during differentiation. These are for example heparin-binding EGF-like growth factor (HB-EGF), neural epidermal growth factor-like like 2 (NELL2), and pleiotrophin. These factors were shown previously to have distinct effects on proliferation and differentiation of multiple cell types.

HB-EGF is a transmembrane protein, whose ectodomain is shedded off and thereby secreted into the surrounding tissue. The ectodomain was shown to bind the EGF receptor and the ERBB4 receptor (Higashiyama et al., 1991; Elenius et al., 1997). The interaction with the EGF receptor was shown to stimulate proliferation of neural progenitors and to enhance neuronal

survival *in vitro* (Kornblum et al., 1999). HB-EGF could act as an autocrine factor on the neural progenitor cells themselves and at the same time support the survival of migrating neuroblasts. Concerning the localization *in situ*, conflicting reports exist. Whereas Nakagawa et al. detected the *Hb-Egf* transcript in the subventricular zone (1998), Kornblum et al. did not (1999). Immunohistochemical analysis demonstrated HB-EGF in several migration pathways and at the ventricular surface (Caric et al., 2001).

NELL2 is a secreted glycoprotein produced by CNS neurons. *Nell2* expression was localized to neural germinal zones, and was shown to peak during embryonic development of the CNS (Kim et al., 2002; Nelson et al., 2002). The protein was shown to promote survival of hippocampal and cortical neurons *in vitro* (Aihara et al., 2003) and to induce motor neuron differentiation of CNS progenitor cells in *in ovo* transfected chicken embryos (Nelson et al., 2004).

4.3.1 Pleiotrophin

Pleiotrophin (PTN), also called heparin binding growth-associated molecule (HB-GAM), is a secreted cytokine with multiple functions (for review see Deuel et al., 2002; Muramatsu, 2002). The distribution of the *pleiotrophin* transcript and the corresponding protein in the CNS have been analyzed in detail (Nakamoto et al., 1992; Vanderwinden et al., 1992; Rauvala et al., 1994; Matsumoto et al., 1994; Silos-Santiago et al., 1996). *Pleiotrophin* transcription was shown to be precisely regulated during development. In the brain, expression peaks in the immediate postnatal period, when glial and neuronal cells are actively differentiating. The protein was found in association with radial glial processes during embryonic development (Rauvala et al., 1994; Matsumoto et al., 1994), suggesting a functional role in cell migration or axon guidance.

Many different functions have been reported for PTN. In fact, it was named pleiotrophin (Greek pleios = many) to reflect its diverse activities (Li et al., 1990). For example, the protein was proposed to stimulate cellular proliferation, neurite outgrowth, process outgrowth in glial progenitors, formation and plasticity of neuronal connections, inhibition of long-term potentiation in the hippocampus, and angiogenesis (for review see Deuel et al., 2002; Muramatsu, 2002). Notably, PTN was shown to stimulate migration of embryonic neurons by interaction with one of its receptors, the transmembrane protein-tyrosine phosphatase PTPζ (Maeda and Noda, 1998). Furthermore, the promotion of neurite outgrowth by PTN is likely mediated through another receptor, syndecan-3 (Raulo et al., 1994). Syndecan-3 is a transmembrane proteoglycan that binds to extracellular ligands and regulates intracellular signaling and cytoskeletal organization during developmental processes. It is localized at axons in the developing brain (Kinnunen et al., 1998; Hsueh and Sheng, 1999).

Neurosphere cells express the genes for both PTN and its receptor syndecan-3, and both transcripts decreased during differentiation. This study demonstrated the presence of PTN in the adult SVZ, where it was found in different cell types, including astrocytes and neuronal cells (see section 3.5.1). The adult neural progenitors share similarities with radial glial cells and may be derived from them, which is outlined below (see section 4.4). Thus, the presence of PTN in embryonic and adult neurogenic niches strongly suggest a specific function therein, e.g. in cellular proliferation, migration, or differentiation in the SVZ. This makes PTN one of the most interesting genes discovered by this microarray study. However, to determine the precise mode of action, further functional studies are required.

4.3.2 Brain fatty acid binding protein

The finding that the transcript for the brain fatty acid-binding protein (FABP7/ BFABP / BLBP) decreased during neural progenitor cell differentiation revealed *Fabp7* expression by the progenitors. This was confirmed by immunofluorescence analysis on neurosphere cells. In addition, the protein was detected in type B cells of the adult SVZ. The distribution of the *Fabp7* transcript and the corresponding protein in the murine CNS had been studied before (Feng et al., 1994; Kurtz et al., 1994). The transcript and the protein had been detected in areas of neuronal differentiation. More specifically, *Fabp7* is expressed in the CNS columnar neuroepithelium at embryonic day 10. From e12 to e14, it becomes enriched in radial glial cells at the ventricular surface, which extend radial processes through the ventricular wall to the pial surface. This expression parallels the appearance of a broad layer of MAP2-positive neuronal cells in the intermediate zone of the ventricular wall. At later stages, FABP7 is no longer present in the ventricular zone, but is restricted to cells attached to the pial surface. This change in expression reflects a transition from radial glial cells to astrocytes. In the adult CNS, FABP7 has been detected in Bergmann glial cells of the cerebellum, in radial glial cells spanning the granule cell layer of the hippocampus, in the glia limitans, and in some astrocytes throughout the CNS. In summary, FABP7 is characteristic for radial glial cells and expressed at the peak of neuronal differentiation and migration in all regions of the developing forebrain and the cerebellum. The presence of FABP7 in the adult progenitor cell niche was unknown so far.

Subcellular localization studies by immunoelectron microscopy demonstrated that FABP7 is present in the nucleus as well as the cytoplasm. Interestingly, the application of anti-FABP7-antibodies to primary cell cultures lead to an inhibition of glial and neuronal differentiation, but did not show any effect on cell proliferation or adhesion (Feng et al., 1994).

Proteins of the FABP family bind to fatty acids, eicosanoids and retinoids. Thereby, they contribute to the fatty acid metabolism. In addition, they have been shown to carry small

hydrophobic signaling molecules between cellular compartments (Hauerland and Spener, 2004). However, the precise function of FABP7 has not been demonstrated yet. Based on the expression pattern in the brain, the subcellular localization, and the differentiation blockade due to the antibody, Feng et al. (1994) speculated that it might be required for the establishment of the radial glial fiber system in the developing brain. This could be mediated by interaction with and transport of a small hydrophobic molecule with a morphogenic signaling activity.

The finding of *Fabp7* expression in type B cells from the subventricular zone raises questions about its function in this context. Radial glial cells function as neuronal progenitors in the embryonic ventricular zone (see section 4.4). Since FABP7 is present in these embryonic progenitors and in the type B neural progenitors of the adult SVZ, it is likely to contribute to neurogenesis by association with a hydrophobic signaling molecule. Unfortunately, the substrate of FABP7 is not known. All this makes *Fabp7* a very interesting candidate for further studies on the regulation of cellular fates in the SVZ.

4.3.3 Insulin-like growth factor binding proteins

Expression changes of genes encoding insulin-like growth factor binding proteins (IGFBPs) were among the strongest effects observed in the microarray experiments. In mammals there are six IGFBPs, four of which are synthesized in the brain: IGFBP2, IGFBP3, IGFBP4, and IGFBP5 (D'Ercole et al., 1996). These IGFBPs bind to insulin-like growth factor 1 (IGF1) with high affinity and thereby regulate its bioavailability. IGF1 is a peptide hormone of the IGF family, which is comprised of three proteins: insulin, IGF1, and IGF2. They excert their functions through specific association with outer membrane receptors that contain tyrosine kinase domains to transmit extracellular signals into the cell (Fig. 33). A significant crosstalk between the various receptors and ligands exists.

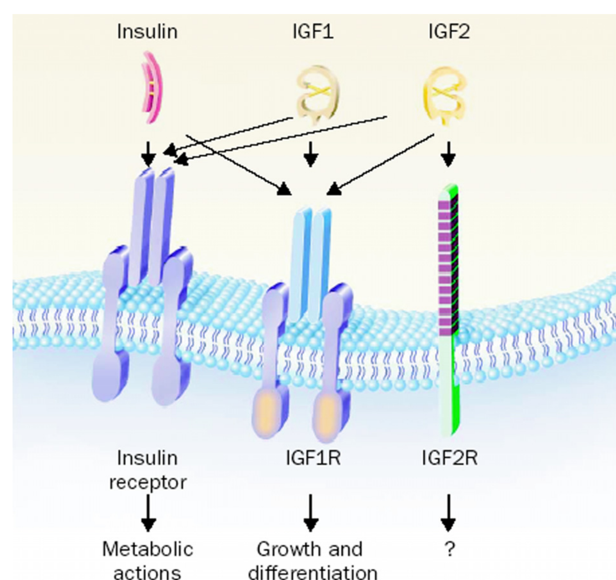

Figure 33. Binding of circulating insulin and IGFs to target cells. Insulin and IGF1 receptor are structurally similar and are both tyrosine kinases. IGF2R differs structurally from the insulin receptor and the IGF1R, and is thought to function primarily as a scavenger receptor for IGF2. Insulin binds to its own receptor and to IGF1R. IGF1 binds to the IGF1R and the insulin receptor. IGF2 associates with all three receptors. Adopted from Fürstenberger and Senn, 2002.

IGF1 is present everywhere in the body from embryonic development into adulthood and it functions as a growth factor in many organs. It plays a major role in CNS development (D´Ercole et al., 1996) and stimulates neurogenesis, oligodendrogenesis, synaptogenesis, and cell migration (D´Ercole et al., 2002; Hsieh et al., 2004). *IGF1* knock-in mice have enlarged brains, whereas mice with reduced or ablated IGF1 or IGF1 receptor synthesis display retarded brain growth (reviewed in D´Ercole et al., 2002).

In this study *Igfbp2* and *Igfbp4* were strongly down-regulated during differentiation. They are both known to inhibit IGF actions. *Igfbp5* on the other hand increased in expression and its product is known to potentiate IGF1 effects. In addition, IGFBP5 can itself act as a growth hormone. These expression changes indicated that the differentiating cells were converted into an IGF-responsive state during neural differentiation. According to RT-PCR analyses (see section 3.5.3), *Igf1* was expressed in the progenitors, but the transcript decreased during differentiation. The transcripts for the IGF1 receptor and the insulin receptor were detected in cells before and after differentiation and did not significantly change in expression.

These findings are somewhat contradictory. One the one hand, genes encoding for factors which potentiate IGF1 action were induced and those for inhibitory factors were down-regulated. On the other hand, *Igf1* expression itself was reduced. Several notions suggest possible ways to explain this inconsistency. First, respective RNA levels were measured, not functional proteins. A change in transcript level does not necessarily mean that the amount of functional protein is proportionally changed. Second, the proteins mentioned here are presumably not all players in this system, but rather represent a selection. Maybe more factors contribute to the instructive effects caused by insulin and IGF1. *Igfbp3*, for example, was found to be expressed in the periventricular germinal zones of developing mice (Easterday et al., 2003). Third, the RNA pools studied were from heterogeneous cell cultures containing neuronal and glial cells at different developmental states. To elucidate the consequences of insulin and IGF1 signalling, expression has to be assigned to individual cell types *in vitro* and *in situ*. In conclusion, this microarray approach certainly identified the *Igfbps* to be differentially regulated during neural progenitor cell differentiation. A detailed understanding of the role of insulin and IGF1 signaling in this context awaits further studies.

4.3.4 Transcriptional regulators

The alterations that a stem cell or a commited progenitor undergoes during its development towards a terminally differentiated cell are mainly mediated by changes in the transcriptome. Therefore, proteins that contribute to the regulation of transcription are of special interest. These can be DNA binding transcription factors, proteins with a function in the transcriptional

machinery, or proteins involved in chromatin structure and dynamics. Those systems are not independent from each other, but rather form a multi-layered and interlinked network that ensures proper gene expression. The complexity of gene regulation is just beginning to be unraveled.

The aim of this microarray study was not to explain the regulatory networks and the interactions of key regulatory factors in a wholistic or systems biology approach. It was rather intended to find genes whose role in this system had not been revealed so far. Several genes that potentially contribute to the transcriptional regulation underlying the neural differentiation were found. Some of these have been described in the contex of neural development before. Others have not been studied so far, but encode for proteins which are similar to transcription regulatory factors. A number of these differentially expressed genes are excellent candidates for further functional studies.

Oligodendrocyte transcription factor 2 (*Olig2*) is a helix-loop-helix transcription factor whose expression decreased during differentiation. It is expressed by oligodendrocyte precursors and differentiated oligodendrocytes and is the key mediator of oligodendrocyte specification (Takebayashi et al., 2002). However, *Olig2* is also expressed by neuronal progenitors in the VZ and the SVZ of the embryonic CNS (Takebayashi et al., 2000). OLIG2 signals were detected in BrdU-positive, fast proliferating progenitors in the SVZ, but not in PSA-NCAM- or GFAP-positive cells (Hack et al., 2004). It is required for the proliferation and self-renewal of neurosphere cells *in vitro* (Hack et al., 2004). This study found *Olig2* to be down-regulated during differentiation. Taken together, these data suggest that Olig2 may act in progenitor cells *in situ* and mediate their proliferation.

Inhibitor of DNA binding 4 (*Id4*) was up-regulated during differentiation. Members of the ID protein family act as negative regulators of helix-loop-helix transcription factors, which are essential for cell determination and differentiation in diverse cell types (reviewed in Norton, 2000). Studies of *Id4* expression in the developing and adult CNS and in cultured neural cells indicated that ID4 contributes to the regulation of mammalian CNS development (Jen et al., 1997; Stewart et al., 1997). ID4 regulates stem cell proliferation and differentiation during embryonic cortical neurogenesis and *Id4*-deficient mice have a reduced brain size (Yun et al., 2004). Up-regulation of this transcriptional regulator during *in vitro* neurosphere differentiation suggests a role in the differentiation of the progenitor cells.

4.4 Similarities to radial glial cells

In the embryonic brain, radial glial cells reside in the ventricular zone and extend long radial processes through the developing cortex to the brain surface which serve as migration scaffolds for newborn neurons. In addition, radial glial cells themselves give rise to neurons. In the adult brain, neurogenesis is strongly reduced, taking place only in discrete regions such as the hippocampus and the SVZ. The neurogenic progenitor cells in the SVZ are astrocytes, and were suggested to be descendants of the radial glial cells in the embryonic ventricular zone (Alvarez-Buylla et al., 2001; Tramontin et al., 2003; Doetsch, 2003; Götz, 2003). The radial glial cells were shown to progressively transform into mature astrocytes during the first two postnatal weeks and to be absent from the ventricular zone thereafter (Tramontin et al., 2003).

The neurosphere cells used in this study originated from postnatal day seven mice. According to the model of transition from embryonic to adult neurogenesis outlined above, the neurosphere cells analyzed in this study could correspond to either radial glial cells, to adult neural progenitors, to an intermediary cell type between the radial glial and astrocyte progenitors, or to a mixture of radial glial cells, adult neural progenitor cells, and possibly also intermediary cell types. The transition from radial glial cells to adult neural progenitors was not described in enough detail yet to allow to decide, which of the alternatives mentioned above is true. However, it is informative to inspect the marker proteins for the relevant cell types. Radial glial cells of the rodent ventricular zone synthesize FABP7, tenascin C, and pleiotrophin (Malatesta et al., 2003), but are negative for GFAP (Sancho-Tello et al., 1995). Adult progenitors from the SVZ are GFAP-positive (Doetsch et al., 1997). In addition, tenascin C is present in the adult SVZ and at elongated astrocytes in the RMS (Jankovski and Sotelo, 1996), indicating that adult neural progenitor cells synthesize this protein.

Microarray data and immunofluorescence analyses of this study demonstrated expression of *Gfap*, *Fabp7*, *tenascin C*, and *pleiotrophin* in the neurosphere cells derived from postnatal day seven mice. This places the cells between radial glial cells and SVZ astrocytes, and suggests that they correspond to a transitional state. Of interest in this context are findings from the immunofluorescence studies on adult brains sections, which demonstrated FABP7 in SVZ and RMS astrocytes, and pleiotrophin in different cell types in the SVZ, including SVZ astrocytes. This indicates, that not only radial glial cells and possibly cells in a transitional state, but also neural progenitor cells in the adult brain share these markers. Although one has to interpret the presence of molecular markers with caution, since they might specify a cellular state rather than a lineage, these results are consistent with the proposed lineage relationship of radial glial cells and adult SVZ stem cells.