4. Discussion

Over the last decade, many genes were discovered that are critical for muscle precursor cell development. Genetic analysis implicates only a few of these, for instance *Lbx1* and *c-Met*, in a control of the migration of muscle precursors (Bladt et al. 1995; Dietrich et al. 1999; Brohmann et al. 2000). A major problem in the study of muscle precursor development has been the fact that a pure population of these cells was not available, resulting in a difficulty to identify genes expressed in these muscle precursor cells. *Lbx1* is expressed exclusively in the population of long-range migrating muscle precursors and is a good marker for these cells (Jagla et al. 1995). In my work, I generated the $Lbx1^{GFP}$ mutant mouse strain that allowed to visualize the GFP-positive cells in the living embryo and to isolate the cells by FACS sorting. Using Affymetrix GeneChip analysis, I determined the gene expression profile of $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ muscle precursors. This made it possible to identify the genes expressed in migrating muscle precursor cells and to identify candidates whose expression is regulated by *Lbx1*.

4.1. Gene expression profiling of $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ muscle precursor cells

The use of Affymetrix GeneChip technology to produce gene expression profiles has many advantages, for example the fact that the expression of a large number of genes can be assessed in a single experiment. However, a certain ratio of false data may be generated in such a microarray experiment. These false positive/negative data might result from discrepancies in experimental conditions, array-to-array variability or possible differences in the genetic background of the animals whose cells are analyzed. To minimize erroneous data produced by the factors listed above, cRNA probes were generated from three independent cell pools obtained from $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ animals and the three probes were used for hybridization. Mean values obtained from the three independent hybridization experiments were obtained and analyzed statistically. To ensure the efficacy of Affymetrix gene profiling, 50 genes that were identified as being expressed in the muscle precursor cells of $Lbx1^{GFP/+}$ mice were selected randomly and their expression profiles were analyzed by *in situ*

hybridization. 48 out of these 50 genes showed an expression pattern that was consistent with the Affymetrix data, demonstrating the reliability of the Affymetrix gene expression profiling.

The expression profiling of *Lbx1*$^{GFP/+}$ muscle precursors reveals a number of novel but also known genes, whose role in the development of muscle precursor cell lineage was previously not described. Some of these have well known function in other cell lineages. For instance, the transcription factors *Sox4* and *Foxp1* are critical for proliferation and maturation of cardiac myocytes (Schilham et al. 1996; Wang et al. 2004). *Jumonji 1* (*Jmjd1*) encodes an AT-rich interaction domain transcription factor that is critical for the cell proliferation in the developing heart, neural tube, spleen and liver (Jung et al. 2005). In primary cardiac myocytes, *Jmjd1* was shown to attenuate differentiation by suppressing the expression of *Mef2*, a gene that promotes the differentiation of skeletal and cardiac muscle (Kim et al. 2005). *Jmjd1* might thus play a similar role in the skeletal muscle lineage and might regulate the balance between proliferation and differentiation of muscle precursor cells.

Muscle precursors produce many integral membrane proteins that are essential for cell-matrix and cell-cell adhesions, cell motility and guidance. Two protocadherin genes, the homologues of human *PCDH17* and *Pcdh18*, were found to be expressed in muscle precursor cells, and the expression of the *PCDH17* homolog was nearly absent in *Lbx1*$^{-/-}$ mutant mice. Protocadherins modulate the cell's affinity towards its neighbors, which is critical for migration. Loss of protocadherin-mediated cell-cell adhesions in *Lbx1*$^{-/-}$ mutant mice might affect the migratory capacity of muscle precursor cells and contribute to their aberrant migration.

During directed migration, cells have to receive and interpret signals from their environment and they have to interpret these signals. Up till now, two tyrosine kinase receptors, *c-Met* in mouse and *EphA4* in chick, were shown to control the migration of muscle precursors (Bladt et al. 1995; Swartz et al. 2001). In my gene profiling experiments, I identified several G-protein coupled (*CXCR4*, *CXCR5*, *Cmkor1*, *Edg2*, *Edg3*, *Gpr23*) and tyrosine kinase (*EphA4*, *EphB4*) receptors that are expressed in muscle precursor cells. *Edg2*, *Gpr23* and *Edg3* belong to a large group of G-protein

coupled receptors that are activated by the phospholipids lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), respectively. Both S1P and LPA are able to regulate cell migration and survival in different cell lines (Wang et al. 1999; Kupperman et al. 2000; Luquain et al. 2003). *Edg2* and *Edg3* expression was slightly upregulated in muscle precursor cell of *Lbx1*$^{-/-}$ embryos. However, the mutation of these genes does not lead to any obvious developmental abnormalities (Contos et al. 2000; Ishii et al. 2001). S1P and LPA can mediate their signals through multiple closely related receptors. Redundancy of receptors might be common, and explain very mild phenotypes observed after the mutations of these receptors.

CXCR4 and *CXCR5* belong to the chemokine receptor family and are able to elicit cellular responses such as proliferation, survival and migration. However, targeted inactivation of *CXCR5* does not result in any obvious developmental abnormalities (Forster et al. 1996). The mutation of *CXCR4* leads to multiple abnormalities arising from aberrant migration and maturation of different cell types in the vascular and central nervous systems (for more details see Introduction). The role of CXCR4 in development of the hypaxial muscle lineage was not analyzed previously.

Eph receptors and their ligands, the ephrins, are critical for axon pathfinding, for the migration of neural crest and endothelial cells, for vascular remodeling and cell positioning in the intestinal epithelium (reviewed in (Klein 2004; Poliakov et al. 2004). Two types of ephrins exist, the membrane associated ephrin A and the transmembrane ephrin B proteins. The receptor and the ephrin B proteins can transmit signals to the inside of cells and mediate attractive or repulsive cues. My data show that *EphA4*, *EphB4* and *Efnb2* are expressed in muscle precursor cells. The expression levels of these genes are not altered in muscle precursors of *Lbx1*$^{-/-}$ mutant embryos, showing that Lbx1 activity is not required for their expression. However, the expression pattern of *Efnb2* suggests an interesting function for this gene in development of muscle precursors. The expression of *Efnb2* is restricted to cells located in the dorsal muscle mass at early stages of migration. In development, muscle precursors delaminate from the somites and subsequently split into dorsal and ventral muscle masses (Schramm and Solursh 1990). The signals from the zone of polarizing activity and apical ectodermal ridge as well as the signals from limb

mesenchyme are required for appropriate splitting of the precursor cells into the two muscle masses (Hashimoto et al. 1999; Schweizer et al. 2004). The predominant expression of *Efnb2* in muscle precursors that colonize the dorsal limb mesenchyme suggests that *Efnb2* might play a role specifically in the development of dorsal muscle precursors.

In the chick, the tyrosine kinase receptor *EphA4* is expressed in the majority of muscle precursor cells and misexpression of Eph4 ligands indicates that the receptors interferes with the migration of the cells to inappropriate limb regions (Swartz et al. 2001). The expression patterns of *EphA4* in the limb of chick and mouse embryos are distinct. In mice, *EphA4* is expressed in delaminating muscle precursors and is downregulated shortly after the cells have migrated into the limb bud (Fig. 6C,D). In mice, other Eph receptors might overtake the function *EphA4* exerts in the chick.

The differential profiling experiments show that *c-Met* expression is downregulated but not extinguished in *Lbx1*$^{-/-}$ precursor cells. Decreased expression of *c-Met* in *Lbx1* mutant animals had been described previously (Schafer and Braun 1999). *c-Met* is critical for delamination and subsequent migration of muscle precursors and its downregulation might interfere with normal migration of these cells.

In conclusion, large-scale gene expression analysis of muscle precursor cell lineage reveals the expression of a set of genes whose contribution to the development of this lineage was not previously analyzed. Moreover, differential expression analysis reveals a number of genes, like *CXCR4*, *c-Met* and *Pcdh17*, that are deregulated in the *Lbx1* mutant and which might contribute to the changes in cell migration that are observed in *Lbx1*$^{-/-}$ animals. Future genetic and functional analysis of the genes identified in this screen will help to clarify the mechanisms important for migration of muscle precursor cells.

4.2. *Lbx1* is not sufficient to control the expression of *CXCR4*

The Affymetrix analysis showed that *CXCR4* is expressed in muscle precursors that migrate into the limb in wild-type embryos. CXCR4 expression is downregulated in

Lbx1$^{-/-}$ muscle precursors, which might reflect a function of *Lbx1* in control of *CXCR4* expression. However, the differential expression observed does not address the question whether *CXCR4* is controlled by Lbx1 directly, or whether the change in expression is caused by other, indirect mechanisms. To start to address this, I analyzed the expression of *CXCR4* in the precursors that migrate along the hypoglossal cord and observed *CXCR4* expression in both wild-type and *Lbx1*$^{-/-}$ mutant embryos. These data indicate that Lbx1 does not suffice to control *CXCR4* expression. I hypothesized therefore that local signals provided by the limb are important for *CXCR4* expression. In *Lbx1* mutant embryos, the majority of muscle precursor cells destined for the limb fail to invade this tissue; a few muscle precursor cells arrive delayed at this site and colonize only the ventro-proximal domain of the limb bud. These few muscle precursor cells that are able to reach the limb in *Lbx1* mutant occupy positions comparable to those observed for these cells in control mice, but nevertheless these cells do not express *CXCR4*. Thus, even those muscle precursor cells that reached the limb are unable to induce *CXCR4* expression in *Lbx1* mutant mice, indicating that the change in position of the muscle precursor cells can not account for the downregulated *CXCR4* expression. I conclude therefore that *Lbx1* in limb muscle precursor cells is important, but not a sufficient to control *CXCR4* expression.

4.3. *CXCR4* is expressed in a subpopulation of migrating muscle precursor cells

I show here that the chemokine receptor *CXCR4* is expressed in migrating muscle precursor cells in limb of chick and mouse, while SDF1 transcripts are found in the limb mesenchyme. CXCR4 protein in muscle precursors is present only after delamination from the dermomyotome, and is observed on forelimb levels only in those muscle precursor cells that had entered the limb. Expression in precursor cells is thus observed only after *Pax3* or *Lbx1* expression is initiated. *Pax3* and *Lbx1* are the earliest markers for long-range migrating muscle precursors and they function in delamination and migration of these cells. The late appearance of CXCR4 in muscle precursor cells suggests that this receptor cannot be required for delamination or for the first step of migration, the invasion into the mesenchyme of the limbs or branchial arches. Pax3^{+} or Lbx1^{+} muscle precursor cells in the limb are heterogeneous with

respect to *CXCR4* expression, and $Pax3^+/Lbx1^+/CXCR4^+$ and $Pax3^+/Lbx1^+/CXCR4^-$ cell populations exist. Moreover, *CXCR4* is exclusively expressed in MyoD-negative cells, and consequently only in undifferentiated precursor cells. Differentiated muscle precursors are believed to lose their long-range migration potential. *CXCR4* is thus expressed during the time window in development of muscle precursor cells in which they possess migratory capacity. However, even before the onset of differentiation many of $Lbx1^+$ cells does not express *CXCR4*, and a complete overlap between *Lbx1* and *CXCR4* expression domains was not observed at any developmental stage. This might suggest that the differentiation state is not the only factor that determines if a muscle precursor cell expresses *CXCR4*.

Endogenous *SDF1* expression is observed in the mesenchyme of the limb close to the positions occupied by precursor cells. Along the route and target of those muscle precursor cells that migrate into the tongue anlage, *SDF1* expression was only observed at the target, i.e. in the floor of the first branchial arch. The distribution of the endogenous *SDF1* transcripts indicates that migrating muscle precursors can encounter the factor during their migration.

4.4. *CXCR4* is important for migration and survival of muscle precursor cells

Ectopic application of SDF1 in the limb of chick embryos resulted in the attraction of CXCR4-positive muscle precursor cells towards the SDF1 source. The redistribution of $CXCR4^+$ cells was more pronounced than the redistribution of $Pax3^+$ cells, which is consistent with the existence of two $Pax3^+$ cell populations, one population that expresses CXCR4 and responds to SDF1, and a second population that is CXCR4-negative and non-responsive. Additionally, differentiation of muscle precursor cells was suppressed by ectopic SDF1. Thus, SDF1 can maintain the undifferentiated state and provide directional cues for migrating muscle precursor cells.

The loss-of-function mutation of *CXCR4* in mice affects the distribution of muscle precursor cells. The number of Lbx1-positive precursor cells in the dorsal limb was reduced at E10.75 and the reduction in cell numbers was more pronounced in the distal than in the proximal limb of *CXCR4* mutant mice. No significant differences in proliferation or differentiation rates were observed in muscle precursors of *CXCR4*

mutant embryos and these mechanisms can therefore not account for the changed distribution of muscle precursors. However, I found that the survival of muscle precursor cells was impaired. Nevertheless, the impaired survival cannot account for the change in distribution only, since enhanced apoptosis of limb cell was observed only proximally. It appears thus that a reduced migratory capacity is responsible for decreased cell numbers at distal positions in the dorsal limb. The cell population that migrates towards the tongue anlage was affected in a similar manner. I conclude therefore that CXCR4 provides signals that control the migration and the survival of migrating muscle precursor cells.

Analysis of *CXCR4* on a *Gab1*$^{-/-}$ genetic background confirmed the role of *CXCR4* in migration and survival of muscle precursor cells. Gab1 is an important signal transduction molecule downstream of the c-Met receptor, which binds and recruits other signal transduction components to activated Met (for a review see (Birchmeier et al. 2003; Gu and Neel 2003). In *Gab1* mutant mice, muscle precursor cells reach their targets, the first branchial arch and the forelimb, however the number of muscle precursors at these sites is reduced (this study and (Sachs et al. 2000). In *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ mice, a further decrease in the number of precursor cells that reach the forelimb was observed, and this reduction in cell numbers is more pronounced than in either of the single mutant mice. Furthermore, migrating muscle precursor cells do not reach the floor of the first branchial arch in *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ double mutant mice; this target is colonized in *Gab1*$^{-/-}$ and *CXCR4*$^{-/-}$ single mutant mice. Similar to an analysis of *CXCR4* single mutants, proliferation and differentiation of muscle precursors were not significantly altered when *Gab1*$^{-/-}$ and *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ mutant mice were compared, and increased apoptosis was observed in the limbs and branchial arches of *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ embryos. Hence, in agreement with the results obtained by comparison of wildtype and *CXCR4*$^{-/-}$ mutant mice, the comparison of the *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ and *CXCR4*$^{-/-}$ mice support the notion that *CXCR4* controls migration and survival of muscle precursor cells. The alterations in development of muscle precursors caused by mutation of *CXCR4* or *Gab1* relate to the same processes; both mutations result in impaired migration and survival, but not proliferation or differentiation of muscle precursor cells. Moreover, these data demonstrate that the *Gab1* and *CXCR4* mutations interact genetically.

In spite of the fact that the dorsal limb of CXCR4 mutant embryos lacks 35% of precursor cells compared to control mice, I observed no significant differences in organization or size of different muscle groups in control and *CXCR4*$^{-/-}$ mice at later developmental stages when defined muscles have differentiated. Thus, the changes in precursor numbers were compensated at subsequent developmental stages. The deficit in precursor numbers of *Gab1*$^{-/-}$ and *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ mutants can however not be compensated, which is reflected in a change in the size or even the absence of particular muscle groups in the limbs at later developmental stages. The limb and tongue muscles of *CXCR4*$^{-/-}$*Gab1*$^{-/-}$ embryos were stronger affected than those of *Gab1*$^{-/-}$ embryos, which is consistent with more pronounced reduction in the numbers of precursor cells in *CXCR4;Gab1* double mutants than in *Gab1* single mutants at earlier developmental stages.

My data indicate that a correlation exists between the severity of the deficits in the number of precursor cells observed at earlier developmental stages and the reduction in the size of differentiated muscles. I attempted to estimate this critical number of precursor cells, which ensures the generation of muscle of normal size. For this, numbers of precursor cells and size of the differentiated muscles in the different limb domains of the different mutant strains were compared. The dorsal distal limb domain of *CXCR4*$^{-/-}$ mice lacks 35% of muscle precursors, which did not affect the size of extensor muscle. The 60% reduction in the precursor numbers in the ventral proximal limb domain of *Gab1*$^{-/-}$ mice resulted in only a small size reduction of flexor muscles. This indicates that the critical number corresponds to about half of the number of precursors present in the limb of wild-type animals.

A balance between proliferation and differentiation of the myogenic cells controls the growth of differentiated muscle (Amthor et al. 1999). In none of the mutant strains analyzed here did I observe a change in the proliferation rate of muscle precursors at E10.75. Compensation appears thus to occur at later developmental stages. Compensatory mechanisms might use a prolongation of the proliferative phase of the precursors, or a delayed differentiation of muscle precursors at late developmental stages. Compensatory mechanisms might operative during a limited time period only, in which a favorable environment is provided in the developing limb.

4.5. A genetic interaction of Gab1 and CXCR4

Tyrosine kinase receptors and chemokine receptors are two important classes of molecules implicated in the regulation of cell migration, proliferation and survival. Gab1 transduces intercellular signals from activated tyrosine kinase receptors, while CXCR4 uses G-proteins to mediate its signaling. Mutation of *Gab1* and *CXCR4* results in impaired migration and survival of migrating muscle cells, although the *Gab1* mutation has a more prominent effect on the development of the muscle lineage than the *CXCR4* mutation. In addition, I observe a genetic interaction between these two loci: the muscle lineage of *CXCR4;Gab1* double mutant mice is more strongly affected than in single *CXCR4* or *Gab1* mutants.

The genetic interaction between *CXCR4* and *Gab1* can indicate that either (some) downstream signaling events employed by these molecules are similar, or alternatively that CXCR4 and Gab1 signaling control two distinct endpoints that are both important in cell migration. Cell migration relies on the ordered disruption and reformation of cell adhesion sites, as well as on cytoskeletal dynamics (Ridley et al. 2004). Gab1 and CXCR4 signaling might for instance control cell matrix attachment and cytoskeletal rearrangements, respectively, but might nevertheless cooperate to regulate cell migration. Alternatively, the genetic co-operation of *Gab1* and *CXCR4* might relate to the fact that both utilize similar signaling cascades. Upon ligand-binding, G-protein coupled receptors like CXCR4 trigger the dissociation of a G-protein complex into subunits, which results in activation of Ras/MAPK, PI3K/Akt and PLCγ/PKC pathways and that controls cell migration, survival and proliferation (reviewed in (Mellado et al. 2001). Recently, this classical signaling pathway of chemokine receptors was reported to be accompanied by stimulation of tyrosine phosphorylation cascades (Daub et al. 1996). G-αi, which is used by CXCR4 to transmit signals, directly binds and activates the c-Src tyrosine kinase, thereby eliciting the activation of signaling cascades typical of tyrosine kinases (Ma et al. 2000). Gab1 contains multiple docking sites for SH2 domain containing proteins like Shp2, the p85 subunit of PI3kinase, Src, Crk and PLC. Activated c-Src is able to bind Gab1 directly and induces its phosphorylation at tyrosine residues; this facilitates Gab1 signaling (Chan et al. 2003). In particular, activation of Ras/MAPK and

PI3K/Akt signaling cascades were reported to occur in response to tyrosine phosphorylation of Gab1 (reviewed in (Birchmeier et al. 2003; Gu and Neel 2003). Experiments performed on cultured cells demonstrate many examples when tyrosine kinase and chemokine receptors work together to regulate cell migration. For instance, HGF and SDF1 cooperate to elicit chemotaxis in cultured rhabdomyosarcoma cells, and PI3K/Akt activities are required for this response (Jankowski et al. 2003). c-Kit and CXCR4 signals attract hematopoietic precursor cells, act synergistically and both receptors rely on MAPK activity to transduce the chemotactic signals (Dutt et al. 1998). In conclusion, the genetic interaction that I observed for *Gab1* and *CXCR4* might reflect the fact that the signal transduction cascades used by these molecules converge on identical effectors to control migration and survival of muscle precursor cells.

4.6. Outlook

Despite great advances in the understanding of the molecular mechanisms that controls specification, differentiation and growth of cells of migrating muscle precursors, as yet there is little information available about the genes that determine their migratory ability. In my work, I isolated the muscle precursor cells from $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ mice, and analyzed the genes expressed in these muscle precursor cells by the Affymetrix GeneChip microarray technology. A set of genes was identified that are known to affect cell migration or motility and might also control migration of muscle precursors cells. Interestingly, many of these, for instance *Epnb2*, *EphA4*, *FoxM1*, *Pcdh17* and *CXCR4*, are not expressed in all, but only in a subpopulation of muscle precursor cells, suggesting a more heterogenous nature of muscle precursor cells that was previously recognized. A role of *CXCR4* in development of the muscle lineage was not studied previously and I therefore chose *CXCR4* for further functional analysis. I show here that *CXCR4* is expressed in undifferentiated muscle precursor cells and regulates their migration and survival. Mutation of *Gab1*, an adaptor molecule that mediates tyrosine kinase signals, results in impaired migration and survival of muscle precursor cells. It appears therefore that both *CXCR4* and *Gab1* contribute to similar processes in the development of muscle precursors. Furthermore, *CXCR4* genetically cooperates with *Gab1*, since migrating muscle precursors of *CXCR4;Gab1* double mutants are more strongly affected than in

either of the single mutants. However, migration of muscle precursor cells is severely impaired, but not completely abolished, in *CXCR4;Gab1* double mutant mice. This indicates that muscle precursor cells encounter additional signals, different from those provided by CXCR4 and Gab1, during their migration. Several G-protein coupled and tyrosine kinase receptors, for instance *CXCR5*, *Cmkor1*, *Gpr23*, *EphA4* and *EphB4*, were identified to be expressed in muscle precursor cells in a course of gene profiling experiments. Future functional studies of these genes might reveal additional key molecules that regulate migration of muscle precursor cells.