1. Introduction

1.1. Generation of the skeletal musculature in vertebrate animals

Skeletal muscle of the vertebrate body is generated from the somites, epithelial structures that segregate from the paraxial mesoderm on both sides of the embryonic axis. Under the inductive influence of neighboring tissues, the epithelial somite generates the presumptive sclerotome and dermomyotome (Aoyama and Asamoto 1988; Christ et al. 1992; McMahon et al. 1998; Reshef et al. 1998). The sclerotome is generated by an epithelial-mesenchymal conversion, sclerotomal cells migrate around the neural tube and in a ventral direction, and will eventually form vertebrae and ribs (Christ and Ordahl 1995). The dermomyotome retains its epithelial morphology and gives rise to the dermis of the back and the skeletal musculature of the trunk.

The formation of myotome is a complex process and proceeds in two steps. Soon after the dermomyotome has formed, cells in the dorsal lip extend beneath the dermomyotome, exit the cell cycle, elongate and differentiate. These pioneer cells mark the appearance of the myotome (Denetclaw et al. 1997; Kahane et al. 1998b). The second wave of cells migrate from the rostral and caudal portions of the dorsal lip and colonize the myotome (Kahane et al. 1998a). Epaxial muscles, the deep muscles of the back, are generated by this dorsal myotome.

The ventral dermomyotome also generates muscle precursor cells. In the interlimb region, the ventral dermomyotome first elongates as an epithelial sheet, and subsequently cells delaminate and fuse with the myotome. Thus, the ventral myotomal compartment is formed that gives rise to hypaxial muscles of the body wall and of the intercostal regions (Ordahl and Le Douarin 1992; Cinnamon et al. 1999; Denetclaw and Ordahl 2000).

However, on particular axial levels, i.e. occipitally, cervically and at the levels of fore- and hindlimbs, the ventral dermomyotome develops in a different manner. In response to signals from the adjacent lateral plate mesoderm or the limb, cells of the ventral lip of dermomyotome undergo an epithelial-mesenchymal transition,

delaminate from the dermomyotome and migrate to particular sites of future muscle development, i.e. hypoglossal cord, branchial arches, diaphragm as well as the fore- and hindlimbs. These migrating cells represent migrating precursor cells of hypaxial muscle (Nishi 1967; Christ et al. 1977).

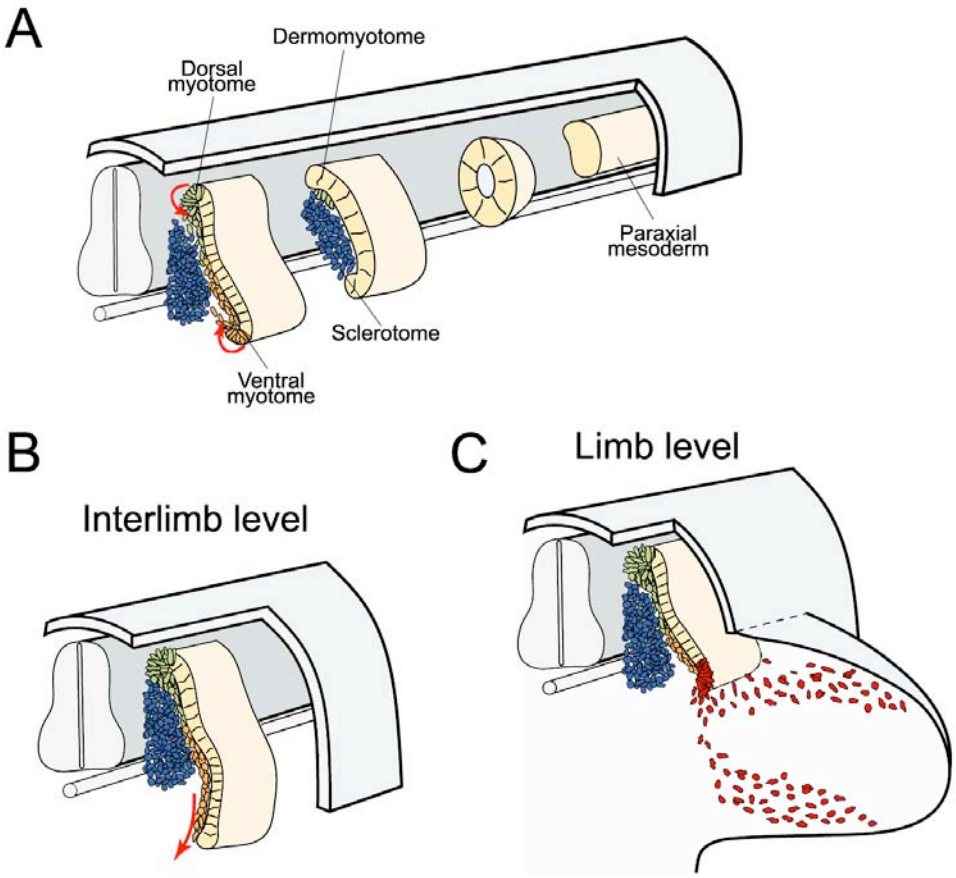

Figure 1. Schematic representation of somitogenesis in vertebrates.
(A) Somites segregate from paraxial mesoderm, and then become separated into sclerotome (blue) and dermomyotome (yellow). Dorsal and ventral dermomyotomal lips elongate and dissociate cells that give rise to dorsal (green) and ventral (orange) myotome, respectively. (B) In the interlimb region, ventral myotome and dermomyotome elongate to form the non-migrating precursor cells of hypaxial muscle. (C) On the limb levels, migrating hypaxial precursors (red) delaminate and migrate to their destined targets (Adapted from Buckingham et al. 2003).

The pool of muscle precursors in the limb subsequently splits to form dorsal and ventral muscle masses, the template of the future muscles in fore- and hindlimbs (Schramm and Solursh 1990). When the cells reach their target sites those, which arrive first start to differentiate and to fuse into multinucleated myotubes. Other precursors remain in an undifferentiated state and continue to proliferate. The secondary fibers encapsulate the primary fibers and constitute the bulk of skeletal muscle at birth (Duxson et al. 1989; Fredette and Landmesser 1991; Wigmore and

Evans 2002).

1.2. Molecular basis of migrating hypaxial muscle precursor cell development

Development of hypaxial muscle precursors proceeds in numerous, strictly controlled steps (specification, delamination, migration, proliferation, differentiation). In recent years, many molecules have been identified that regulate the specification, migration, proliferation and differentiation of these muscle precursor cells. Migrating muscle precursors can be identified by the expression of several markers like *Pax3*, *c-Met* and *Lbx1*. *Pax3* encodes a paired and homeodomain transcription factor and is expressed throughout the dermomyotome (Goulding et al. 1994; Daston et al. 1996). *c-Met* encodes a tyrosine kinase receptor that is expressed in the ventral dermomyotome at interlimb levels and in cells located at the dorsal tips of the dermomyotome (Yang et al. 1996). *Lbx1* encodes a homeodomain factor and is expressed exclusively in the migrating precursors and not in other types of muscle cells.

1.2.1. Specification of the precursor pool of hypaxial muscle

The establishment of the pool of migrating muscle precursors in the ventral dermomyotome is characterized by the expression of *Pax3* (Franz et al. 1993). Pax3 is a paired homeobox transcription factor and its expression in the dermomyotome is upregulated at the time when the muscle precursor cells are specified. In the spontaneous mutant *Splotch*, which carries a mutation of *Pax3*, the ventral dermomyotome is not correctly formed and therefore all hypaxial muscles are lacking. *Pax3* is therefore critical for the appropriate generation of the hypaxial muscle precursor pool (Franz et al. 1993; Daston et al. 1996; Epstein et al. 1996; Yang et al. 1996).

The specification of $Pax3^+$ precursors requires signals from the dorsal ectoderm and lateral plate mesoderm (Kenny-Mobbs and Thorogood 1987; Fan and Tessier-Lavigne 1994; Cossu et al. 1996; Pourquie et al. 1996; Dietrich et al. 1998). Wnt proteins have been suggested to be the ectodermal signals that play a role in the development of the lateral dermomyotome (Roelink 1996; Dietrich et al. 1998). Bmp4, a member of the TGFß superfamily, is provided by the lateral plate mesoderm and induces the

expression of genes characteristic for ventral dermomyotome. However, ectopic Bmp4 was able to induce the characteristics of ventral dermomyotome only if combined with signals provided by the lateral plate mesoderm, suggesting that additional factors are necessary to specify hypaxial muscle precursors (Dietrich et al. 1998).

1.2.2. Genes that control delamination of muscle precursor cells

The key molecules known to control delamination are scatter factor/hepatocyte growth factor (SF/HGF) and its receptor, the Met tyrosine kinase. Scatter factor is expressed in lateral plate mesoderm close to all somites that form migratory cells and can induce delamination of the ventral dermomyotome when applied ectopically into the interlimb mesenchyme of chick embryo (Brand-Saberi et al. 1996; Heymann et al. 1996). Genetic inactivation of *SF/HGF* or *c-Met*, the receptor for SF/HGF, prevents delamination of myogenic precursors from the ventral dermomyotome, but does not interfere with fate specification (Bladt et al. 1995; Dietrich et al. 1999).

1.2.3. Genes involved in migration and proliferation of muscle precursor cells

Transplantations of somites in the limb region result in muscle patterns corresponding to the limb segment (wing or leg) independent of the origin of the grafted somite (Chevallier et al. 1977; Lance-Jones 1988). Thus, initially muscle precursor cells do not contain any positional information and the migration of muscle precursor cells to their final positions is controlled by extrinsic signals provided by the tissue that they colonize. However, muscle precursor cells must be able to receive and interpret these signals.

SF/HGF and its receptor c-Met play roles not only in delamination, but also in migration of muscle precursor cells. In chick, SF/HGF increases the motility of myogenic precursors and ectopic application of SF/HGF causes an accumulation of muscle precursors in the area of high SF/HGF concentrations (Scaal et al. 1999). In mice, *c-Met* is expressed in migrating muscle precursors, and *SF/HGF* is expressed along the route that is used by the muscle precursor cells for migration (Dietrich et al.

1999). Gab1 encodes a docking protein that can directly bind the activated c-met receptor and that transduces c-met signals. Mutation of Gab1 leads to impaired ability of muscle precursors to migrate (Sachs et al. 2000).

The tyrosine kinase receptor EphA4 and its ligand ephrin-A5 were implicated to play a role in muscle precursor cell migration. Ectopic application of ephrin-A5 in the chick prevents the migration of muscle precursors into ephrin-A5-positive areas. EphA4 is transiently expressed in migrating muscle precursor cells, and might be the receptor that recognizes ephrin-A5 in the limb (Swartz et al. 2001). However, loss of function mutations in Eph4 do not interfere with migration of muscle precursors; similarly, a phenotype in the muscle lineage was not described in mice with a loss of function mutations in ephrin-A5.

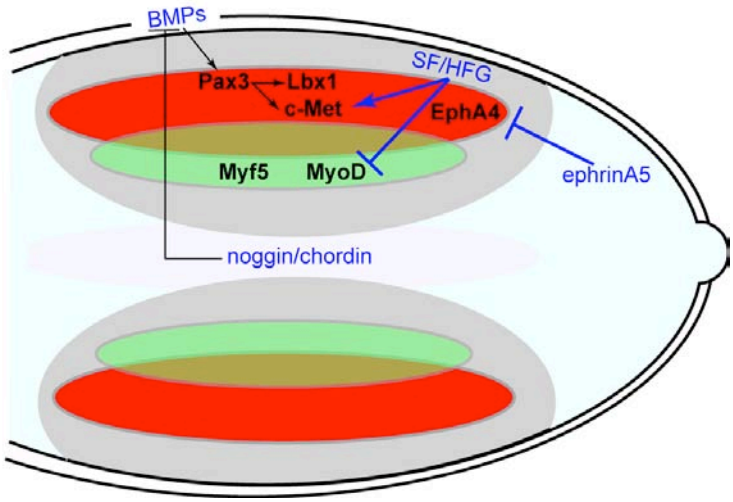

Figure 2. Signals that control migration and proliferation of the muscle precursors in the limb. The migration of muscle precursor cells (shown in red) is positively regulated by *Lbx1* and *c-Met*, which are under the control of *Pax3*. The c-Met ligand, SF/HGF (gray areas), is expressed along the migrating route and at the target; it supports migration of muscle precursors and provides them with proliferation and survival signals. Entrance to inappropriate areas is prevented by EphA4/ephrinA5 signaling. Once muscle precursors start to differentiate, they switch on the expression of the myogenic regulatory genes, *Myf5* and *MyoD* (green). In the limb, these cells are located further centrally than the undifferentiated precursors. Bmp signals from the surface ectoderm promote *Pax3* expression and repress myogenic differentiation; their influence is antagonized by noggin/chordin signals emanating from centrally located mesenchyme (Adapted from Francis-West et al. 2003).

Lbx1 expression is induced shortly after the migratory cell population is established. Expression is maintained during migration of the muscle precursor cells and is downregulated shortly after precursor cells differentiate. In mice that lack *Lbx1*, muscle precursors are correctly specified and delaminate from the lateral edges of the

dermomyotome at forelimb level but do not move into the limb buds (Schafer and Braun 1999; Brohmann et al. 2000; Gross et al. 2000). Interestingly, in *Lbx1*$^{-/-}$ mutant embryos muscle precursors that migrate along another routes into the diaphragm and hypoglossal cord find their targets. This phenotype indicates that lbx1 controls the expression of a gene that recognizes or interprets guidance cues that direct migrating precursor cells. Furthermore, the different subpopulations of migrating muscle precursors cells appear to encounter distinct cues that allow migration and guidance.

Pax3 is an important regulator of muscle development. In the migrating lineage, neither *Lbx1* nor *c-Met* are expressed in the dermomyotome in *Splotch* mutant embryos, demonstrating that *Lbx1* and *c-Met* act genetically downstream of *Pax3* (Epstein et al. 1996; Dietrich et al. 1999). Various lines of evidence indicate that *c-Met* is a direct target for Pax3 (Epstein et al. 1996; Relaix et al. 2003; Keller et al. 2004). A role of Pax3 in the control of the *Lbx1* promoter is also indicated by the altered *Lbx1* expression in the *Splotch* mice; it is however clear that *Pax3* is not sufficient to drive the expression from *Lbx1* promoter, and additional signals are required (Mennerich et al. 1998).

Only a limited number of cells migrate to the limb and a proliferation step is necessary to reach the number of cells required to form limb muscle. Many genes that maintain the migratory state of muscle precursors also promote their proliferation and survival. For instance, increased activity of *Pax3* and *c-Met* leads to formation of hypertrophic musculature (Relaix et al. 2003; Keller et al. 2004). *Six1* directly regulates the proliferation in muscle precursor cells. In *Six1*$^{-/-}$ mice muscle precursor cells are correctly specified, but a severe reduction in the size of the muscle precursor cell population was observed. Detailed analysis revealed that Six1 contributes to the Six1-Eya1-Dach complex, which activates the expression of *c-myc*, a gene that is a critical for proliferation (Li et al. 2003).

Members of the insulin growth factor family are thought to promote proliferation, since the *Igf1* mutant mice develop a muscle hypoplasia (Powell-Braxton et al. 1993) and gain of function of *Igf1* leads to enlarged muscles due to increased myoblast proliferation (Mitchell et al. 2002). In chick, an increased proliferation rate of muscle

precursors could be also achieved by ectopic FGFs and BMPs (Hannon et al. 1996; Floss et al. 1997; Amthor et al. 1999). *Myostatin* encodes a gene of the TGFβ superfamily. Its mutation causes an increased muscle size in the adult, and the gene is thought to regulate the time point of differentiation. Spontaneous mutation of *myostatin* has also been observed in cattle, where they were selected by breeders interested in increasing the meat production (reviewed in (Lee 2004).

1.2.4. Differentiation of muscle precursor cells

When muscle precursor cells reach their target sites, they switch on the expression of the myogenic regulatory factors (MRF). These factors, MyoD, Myf5, myogenin and MRF4, are transcription factors of the helix–loop–helix family, and their appearance in myogenic precursors mark the onset of differentiation (Braun et al. 1989; Edmondson and Olson 1989; Rhodes and Konieczny 1989; Wright et al. 1989). Terminal differentiation of myogenic cells is characterized by the expression of genes that encode contractile proteins like desmin or the myosin heavy chain (Sweeney et al. 1989). In mice that carry targeted mutation of the *MyoD* or *Myf5* genes, no obvious defects in skeletal muscle are observed (Braun et al. 1992; Rudnicki et al. 1992). However, mice lacking *MyoD* and *Myf5* show a complete absence of myoblasts and muscle fibers. Thus, at least one of these factors is required to determining the myogenic differentiation program. Activation of *myogenin* and *MRF4* appears to be dependent on the preceding expression of *MyoD* and/or *Myf5* (Rudnicki et al. 1993). Inactivation of *myogenin* leads to prenatal death due to severe defects in secondary, but not primary muscle fibers (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). Together, these data suggest a model in which *MyoD* and *Myf5* act to determine the myoblast lineage while *myogenin* and *MRF4* are important for differentiation and maintenance of the terminally differentiated state (Megeney and Rudnicki 1995; Rudnicki and Jaenisch 1995).

Expression of MRFs is tightly regulated by diverse extracellular signals. The growth of embryonic muscle is a balance between proliferation and differentiation of muscle precursors (Amthor et al. 1998). Consequently, the growth factors FGFs or IGFs, that maintain the proliferative state of muscle precursors, suppress the differentiation

process (Powell-Braxton et al. 1993; Amthor et al. 1999). BMPs and myostatin were shown to regulate muscle differentiation negatively (Pourquie et al. 1996; Amthor et al. 1998; Langley et al. 2002). On the other hand, expression of the BMP antagonists, noggin and chordin, in the limb mesenchyme promote the differentiation of muscle precursors by blocking the BMP signaling (Zimmerman et al. 1996; Reshef et al. 1998; Amthor et al. 1999).

1.3. CXCR4 receptor

Chemokines (chemotactic cytokines) represent a family of small secreted proteins (8–13 kDa). Based on the relative position of the first two of four cysteine residues, chemokines are classified in four main subfamilies, CXC (α), CC (β), CXXXC, and C chemokines. The α-chemokines activate the CXC receptors (CXCR), β-chemokines signal through the CC receptors (CCR) and fractalkine mediates its effects through the CX3CR1 receptor. Eighteen chemokine receptors have been cloned so far including six CXC, 10 CC, one CX3C and one XC receptor (Baggiolini 1998; Zlotnik and Yoshie 2000). CXCR4 was cloned by five separate groups as an orphan G-protein coupled receptor and several different names like LD78, NPY receptor, LESTR and fusin were in use (Federsppiel et al. 1993; Herzog et al. 1993; Jazin et al. 1993; Nomura et al. 1993; Loetscher et al. 1994). Based on high homology to the CCR1 and IL-8 receptors, Loetscher et al. suggested that this receptor might belong to chemokine receptor family. Later CXCR4 was identified as an HIV1 co-receptor and was found to recognize the stromal cell derived factor-1 (SDF1) as a ligand (Bleul et al. 1996; Feng et al. 1996; Oberlin et al. 1996).

1.3.1. CXCR4 structure and downstream components of CXCR4 signaling pathway

Seven transmembrane helices make up a core domain of the CXCR4 receptor. Conformational changes in this domain are believed to be responsible for receptor activation. Binding of SDF1 to CXCR4 triggers the dissociation of a heterotrimeric Gαβγ protein into the Gβγ subunit complex and the Gαi subunit. Signals mediated by the Gβγ subunit involve the activation of the Ras/MAPK, PI3K/Akt and PKC

signaling pathways. Chemokine activation of MAPK stimulates the transcription of genes important for cell proliferation and might regulate cytoskeletal changes necessary for cell migration (Peppelenbosch et al. 1993; Hill and Treisman 1995). Activation of the PI3K pathway results in generation of 3-phosphorylated lipids that act as second messengers for downstream effectors such as the AKT pathway (Shimizu and Hunt 1996; Vicente-Manzanares et al. 1999). This activation has been implicated in integrin adhesiveness, cell migration, polarization, and chemotaxis (Dekker and Segal 2000; Li et al. 2000). Other PI3K effectors include the small GTPases, such as Rho, Rac, and Cdc42, which participate in regulation of the actin cytoskeleton and cell adhesion (Kaibuchi et al. 1999; Serrador et al. 1999).

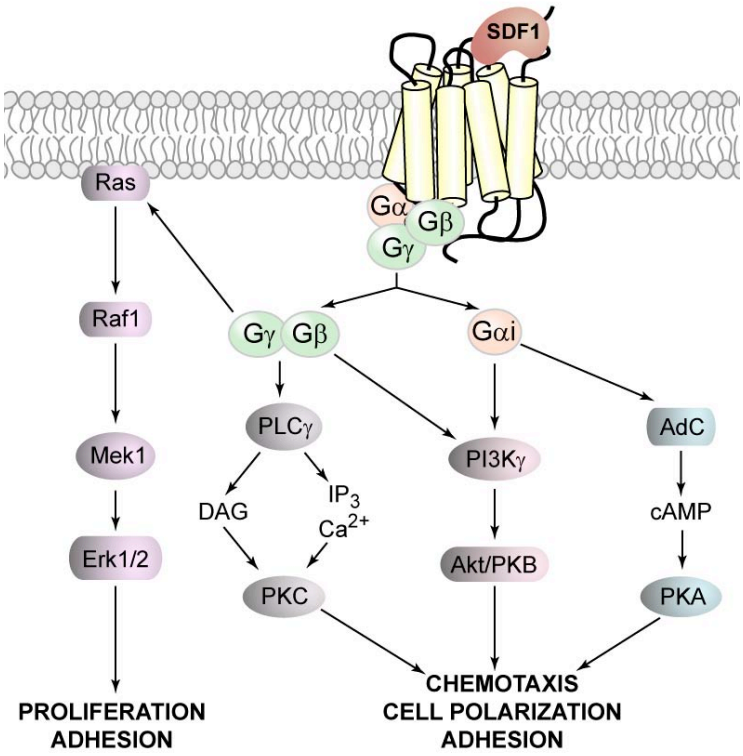

Figure 3. Signaling pathways activated by CXCR4/SDF1 and their biological role.
Upon ligand binding, CXCR4 receptor activate several downstream cascades like Ras/MAPK, PI3K/Akt, PKA and PKC signaling pathways, which elicit various biological response (cell polarization and chemotaxis, cell proliferation, cell adhesion).

The free Gβγ complex also activates PLC, leading to formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Jiang et al. 1996). IP_3 accumulation mobilizes Ca^{2+} from intracellular stores and is essential for polarization of migrating cells. Together with Ca^{2+}, DAG activates various protein kinase C (PKC) isoforms.
Gαi activates PI3K/Akt and adenylate cyclase. Adenylate cyclase activation leads to the accumulation of intracellular cAMP, which activates protein kinase A (PKA).

Both PKC and PKA trigger events vital for cell motility and chemotaxis (Jiang et al. 1996; Wu et al. 1998).

1.3.2. The function of CXCR4 in developing and adult organism

Chemotaxis is an important mechanism that controls the migration of different cell lineages in developing and adult organisms. The first biological role of the chemokine receptor CXCR4 emerged in the regulation of infectious and inflammatory processes. Parenchymal tissues at the site of inflammation upregulate SDF1, which is recognized by CXCR4 and controls lymphocyte trafficking towards the inflammation site (Buckley et al. 2000; Gonzalo et al. 2000; Gear and Camerini 2003). However, an impoved understanding of CXCR4 biological function was achieved by the analysis of the hematopoetic system of CXCR4 mutant mice. Hematopoietic precursor cells express CXCR4 and migrate into fetal liver and bone marrow, the sites where SDF1 is expressed. CXCR4/SDF1 signals were shown to regulate the proliferation and retention of hematopoietic precursor cells at these sites (Nagasawa et al. 1996; Ma et al. 1998; Zou et al. 1998; Ishii et al. 1999). The correct immunological response also depends on CXCR4 function. In the adult organism, lymphocytes move between blood and lymphoid tissues through vascular endothelium. The transendothelial migration relies on the expression of adhesion molecules that are activated by CXCR4-mediated signals (Kim and Broxmeyer 1999).

The genetic analysis of the *CXCR4* or *SDF1* has revealed the important role of these molecules in other cell lineages. Mice with targeted deletion of these genes generally die in *utero* with different defects in cardiovascular and central nervous systems. Both *CXCR4* and *SDF1* mutant embryos display cardiac ventricular septum defects and a deficit in vascularization of the gastrointestinal tract (Tachibana et al. 1998). Furthermore, mutation of CXCR4 signaling results in aberrant migration of distinct cell lineages during embryogenesis. For instance, CXCR4 provides chemotactic cues for hippocampal and cortical neurons. Consequently, inactivation of *CXCR4* leads to misrouting or decreased motility of hippocampal and neocortical neuronal precursors. In contrast, in the cerebellum *CXCR4* is essential to retain the cerebellar granule cells in the external granular layer. In the absence of CXCR4, these cells emigrate

prematurely (Ma et al. 1998; Zou et al. 1998; Bagri et al. 2002; Lazarini et al. 2003). In addition, CXCR4/SDF1 was shown to control the migration of primordial germ cells in fish, birds and mammals (Doitsidou et al. 2002; Molyneaux et al. 2003; Stebler et al. 2004). Finally, metastatic cells are directed to organs that express *SDF1*. For instance, metastasis of human breast cancer cells to preferred sites correlates with the expression of the CXCR4 receptor in these tumor cells, and with the expression of the SDF1 in the organ, which the metastatic cells invade (Muller et al. 2001). Frequently, not only migration of the affected cells, but also their growth and/or survival are impaired if they do not receive the appropriate CXCR4/SDF1 signal. This effect of CXCR4/SDF1 on growth and survival might be direct or indirect. For instance, hippocampal neuronal precursors receive the proliferation/survival signal from the CXCR4 signaling cascade (Bagri et al. 2002). In contrast, cerebellar granule cells proliferate under the influence of Sonic hedghog (Shh), but CXCR4 is necessary to retain these cells in Shh expressing areas (Ma et al. 1998; Zou et al. 1998).

1.4. The purposes of the project

Lbx1 is expressed in long-range migrating precursor cells and is essential for their migration to particular target sites (Schafer and Braun 1999; Brohmann et al. 2000; Gross et al. 2000). In my work, I determined the gene expression profile of migrating muscle precursor cells. For this, I generated a new $Lbx1^{GFP}$ mutant mouse strain. From the $Lbx1^{GFP}$ allele, functional Lbx1 protein cannot be produced and instead green fluorescent protein (GFP) is generated. This newly established mouse strain made it possible to visualize muscle precursor cells in living embryos and to isolate such cells by fluorescence-activated cell sorting (FACS). I isolated muscle precursors cells from $Lbx1^{GFP/+}$ and $Lbx1^{GFP/GFP}$ mice and analyzed their expression profiles using Affymetrix GeneChips. Among the genes expressed in migrating muscle precursors, I identified the chemokine receptor *CXCR4*. My further functional analysis showed that *CXCR4* plays an essential role in the development of migrating muscle precursor cells. Ectopic application of the CXCR4 ligand, SDF1, in chick embryos attracts muscle precursor cells and suppresses their differentiation. In $CXCR4^{-/-}$ mice, I observed an incorrect distribution of muscle precursor cells in the limb and the branchial arches, which was accompanied by an impaired survival of the cells. Moreover, I showed that

CXCR4 interacts genetically with *Gab1*, the gene encoding an adaptor protein that mediates the signals of tyrosine kinases.