CXCR4 & SDF1 are part of the core mechanism regulating migration of muscle precursor cells to the developing limb

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Abstract

The ligand CXCR4 and its receptor SDF1 have been associated on several occacions with the formation of the limb musculature, specifically SDF1/CXCR4 signaling has been implicated to be associated with migration of the limb muscle precursor cells. In this thesis I will provide an overview of SDF1/CXCR4 signaling and how this pleiotropic signaling cascade could be involved in the regulation of the core mechanism regulation migration of limb muscle precursor cells. These interactions point to a top role of FGFs, SF/HGF and Shh in the limb bud mesenchyme to control both CXCR4 and SDF1 signaling, either directly (SDF1, CXCR4) or potentially through other factors such as NF-kB. Downstream of CXCR4 three potentially interesting mechanism can be distinguished. First JAK/STAT signaling which is also controlled by EphA4 which is also expressed in the migrating muscle precursors. Second a positive feedback loop of CXCR4 involving SHIP2, PI-3K and NF-kB. Third we can distinguish a potential mechanism by which CXCR4 regulates c-met by signaling through MAPK and Gab1.

Contents

1 C-X-C ligands and receptors

The family of C-X-C chemokine receptors (CXCR) consist of 7 family members (CXCR1-7) that specifically interact with the chemokine family of C-X-C ligands (CXCL1-20). Structurally, CXCR4 is a 7-transmembrane protein of about 39 KDa. The interaction of CXCR4 with CXCL12, otherwise known as Stromal Derived Factor 1 (SDF1) has been well established [1]. This receptor-ligand pair has been identified to be a player in a wide array of developmental processes regulating adhesion, migration and chemotaxis [2]. In this thesis I will restrict the discussion to the formation of appendicular muscle in which the CXCR4 SDF1 receptor-ligand pair is also involved [3, 4]. Specifically I will show SDF1/CXCR4 to be a regulator of appendicular muscle precursor cell migration. Furthermore I will offer several potential methods by which SDF1/CXCR4 is directly involved in the core regulatory mechanism of this developmental process.

2 Appendicular myogenesis

Vertebrate trunk muscle can be broadly defined in two groups, epaxial and hypaxial muscles. Epaxial muscles, reside dorsally to the horizontal septum, whereas hypaxial muscles are located to its ventral region. Among others hypaxial muscles include all appendicular (limb) muscles. During vertebrate development there are four key processes in a sequential manner regulating the formation of appendicular muscle. In the developing embryo after the initial formation of the somite the dermomyotome forms and specifies a ventrolateral lip (VLL) in the dorsolateral quadrant of the somite [5, 6]. C-met and Pax3 have been identified as factors involved in this process. Already in 1996 it was known for pax3 to regulate C-met and by doing so regulating limb muscle development [7].The tyrosine kinase receptor C-met requires binding to its ligand (HGF) which is expressed in the surrounding cells in order to properly delaminate from the VLL [8, 7, 9, 10]. Myoblasts from this VLL delaminate and migrate to several sites in the body [6, 11]. The final site to which these cell migrate depends, among others, on the localization of the somite on the AP-axis [12, 13]. Among others factors that are thought to regulate myoblast migration are, Lbx1[14], CXCR4[15], Sp5[16], Pitx2[17], Gab1[15] and SDF1[6, 15, 18]. Uppon arrival to the limb these cells differentiate to myocytes and fuse to form multi-nucleated skeletal muscle[19, 10, 18]. Myoblast assume a myogenic fate by expressing several factors known collectively as Myogenic Regulatory Factors (MRFs). These include Myf5, MyoD, Myogenin and MRF4 as core regulatory components [11].

3 SDF1/CXCR4 in limb myoblast migration

As previously mentioned CXCR4 and SDF1 have been associated with the migration of limb myoblasts [6, 15, 18]. CXCR4 is specifically expressed during limb myoblast migration as is shown by co-expression of CXCR4 with Lbx1

and Pax3 in myoblasts, factors well known for their involvement during the migration of myoblasts. MyoD positive populations are distinct from CXCR4 populations thereby implying a role for CXCR4 restricted to limb myoblast migration, without a role during early or later stages of appendicular muscle formation.[15] Since no general role for CXCR4 has been implied during appendicular muscle formation, is seems probable to assume a role for CXCR4 not only during migration but for CXCR4 to be actively involved in migration. SDF1 is a well established ligand for CXCR4[1, 20, 3]. During myogenesis various chemokines and chemokine receptors are expressed. The SDF1/CXCR4 chemokine/chemokine-receptor pair is expressed during in-vitro myogenesis [3]. $CXCR4^{-/-}$mutants show reduced myoblast migration numbers, without affecting myoblast differentiation and proliferation numbers. Cell death numbers are markedly changed as was shown by TUNEL staining. Without CXCR4 myoblasts loose directionality and undergo cell death [15]. SDF1 mutants do not show specific defects in myoblast migration, these defect are obscured due to earliers roles of SDF1 in somite rotation [12]. SDF1 is expressed in the mesenchyme and should therefore be capable of interacting with CXCR4 in the migrating myoblasts [18, 15].

4 Limb muscle precursor migration

Limb bud muscle migration has been studied for quite some years. This has led to understanding of a core-mechanism involved specifically during migration [21, 9, 6]. The core-mechanism as described by Birchmeier is depicted. In the migrating muscle precursor a core Pax3/c-met/Lbx1 cascade is described. Here pax3 regulates expression of c-met and Lbx1. Several factors are known to regulate Pax3, Msx1 is a negative regulator of Pax3 whereas Mox2 is a positive regulator of Pax3. Dach2, Eya2 and Six1 are all involved in a positive feedback with Pax3 [13, 18, 22, 9].

The limb bud mesenchyme has a core mechanism where FGFs and Shh are positive regulators of SF/HGF. SF/HGF in its turn is a positive regulator of c-met in the migrating muscle precursor cell, furthermore FGFs are known positive regulators of Lbx1. <Fig. 1>

For cells to go from one part of any embryo to another region of the embryo there are three key necessities. The physical process of moving is obviously needed. Furthermore there needs to be a vector to this movement and finally stopping at the intended site of arrival is crucial. When these requirements are met cells can move from one part of the body to another. In biological terms these processes can be described respectively as locomotion, chemotaxis and adhesion. Locomotion is a process that provides physical movement to a cell, chemotaxis provides directionality and adhesion makes sure that cells stay in there intended site. CXCR4 and SDF1 seem to be involved in all three processes [23].

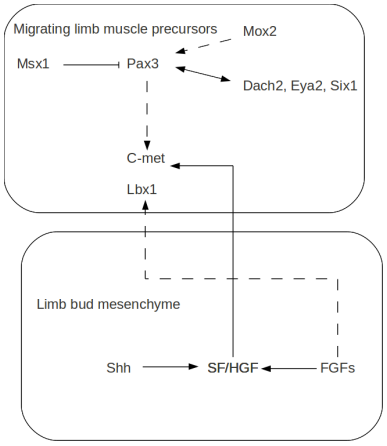

Figure 1: Core regulatory mechanism of the migration of limb muscle precursors. Factors expressed in the limb bud mesenchyme influence the factors in the muscle precursors but not vice versa. Solid lines represent direct interactions, whereas dashed lines represent indirect interactions.

5 SDF1/CXCR4 and the core regulatory mechanism

It is known for quite some time that SDF1/CXCR4 signaling is involved in a variety of cellular and developmental events. The SDF1/CXCR4 signaling cascade has a wide rage of pleiotropic downstream effects and as such is involved in organogenesis, regeneration and tumorigenesis [24]. For example SHIP1, SHIP2, PI3K, Fak JAK/STAT and paxillin all are downstream targets of SDF1/CXCR4 [2]. SHIP1 is well known to be involved in locomotion [25]. Fak and paxillin are involved in the formation of focal adhesions and as such can have a positive effect on cell motility [10, 2]. Also the JAK/STAT signalling pathway is involved in chemotaxis [26].

The core mechanism involved in muscle precursor migration is quite well understood [13, 18, 22, 9]. It is also known that CXCR4 and SDF1 are important factors during limb muscle precursor migration [15]. It is not well understood how SDF1/CXCR4 ties in to the core regulatory mechanism and if it interacts at all.

There is a case to be made for the involvement of SDF1/CXCR4 signaling to be involved in not only migration in general, but also in the migration of limb muscle precursor cells. The SDF1/CXCR4 signalling cascade seems to be involved in migration due to the downstream effectors. These factors have been shown to be downstream interactors of SDF1/CXCR4 signaling in a variety of cells, however is there any evidence that this also happens in migrating muscle precursor cells?

A recent study suggests interaction between the FGF and SDF pathway

[27]. This study showed that during zebrafish fin regeneration FGFs can act as positive regulators of SDF1 expression. Not only did they show FGFs as a postive regulator of SDF1, also CXCR4a/b and CXCR7 are regulated positively by FGFs. It is interesting to note that fgf20 expression is repressed by SDF1 leading to a negative feedback cycle that could help keeping FGF levels in check. This mechanism was shown to be active in fin regeneration in zebrafish. Altough fin regeneration and limb muscle migration are not identical processes, they do give an interesting understanding how SDF1/CXCR4 are regulated during limb muscle precursor cell migration and it provides a mechanism how the core migratory mechanism could potentially regulate SDF1/CXCR4 signaling.

Regarding CXCR4 regulation several factors have been proposed. HIF1a is induced by hypoxia and a potential positive regulator of CXCR4 expression [28]. Also NF-kB has been suggested as a regulator of CXCR4 [29, 30, 31]. Both factors are also discussed in a review regarding regulators of SDF1/CXCR4 in general [24]. There is currently no evidence that suggests a potential role for HIF1a to be involved in the migration of limb muscle precursor cells. The migrating limb muscle precursor cells are part of the skeletal muscle and NF-kB is expressed in skeletal muscle [30]. Not only is it expressed in skeletal muscle, it is used by HGF as a positive regulator of CXCR4 in glioma cell migration [29]. All the known factors in this mechanism are present during limb muscle precursor migration, HGF is expressed in the limb bud mesenchyme, CXCR4 is expressed in the limb muscle precursor cells and NF-kB in all skeletal muscle cells. This would provide a second mechanism whereby the factors secreted by the mesenchyme can regulate CXCR4 expression.

6 Downstream targets of SDF1/CXCR4

Understanding downstream targets of SDF1/CXCR4 in the context of the migrating limb muscle precursor cells can be difficult due to the pleiotropic nature of the downstream targets of SDF1/CXCR4 [2, 24]. Kucia distinguishes five downstream processes of SDF1/CXCR4 in regard to locomotion, chemotaxis and adhesion. These are, Adhesion (Fak, paxillin, p130), PI-3K (PI-3K, AKT, IkB, NF-kB), MEK (MEK, MAPK, p42/44, ELK-1), JAK (JAK, Tyk, STAT) and Phosphatases (SHIP1, SHIP2, CD45) [2].

Fak, paxillin and p130 do not seem to be involved in limb muscle migration, they are however known as a regulators in vascular smooth muscle cell migration [26].

As a part of the PI-3K cascade, NF-kB is of particular interest. It has already been mentioned as a factor through which HGF promotes CXCR4. If NF-kB is indeed a valid downstream target of CXCR4, this would make for an interesting positive feedback loop. A mouse model in which subunits of PI-3K where knocked out in skeletal muscle resulted in impaired muscle growth [32]. These results could imply a role for PI3K in the migration of limb muscle precursor cells, and thus a potential positive feedback loop through NF-kB.

MEK activity is needed for inhibition of skeletal muscle differentiation [33].

As such it is expressed in muscle precursor cells and could therefore also be involved in SDF1/CXCR4 signaling in migrating limb muscle precursor cells. Furthermore MAPK has been reported to control the recruitment of Gab1 to the plasma membrane by phosphorylation of Ser551 on Gab1 [34]. CXCR4 and Gab1 show a genetic interaction in regulation migration of muscle precursor cells [15]. This regulation will be most likely through c-met since both a genetic and physical interactions between Gab1 and c-met have been shown. $Gab1^{-/-}$mice and $c\text{-}met^{-/-}$ show specific defects in the formation of the limb musculature. This effect is more severe in the $Gab1^{-/-}$; $c\text{-}met^{-/-}$double mutant [35]. This ties CXCR4 activity to c-met through MAPK and Gab1.

JAK activity has been reported in skeletal muscle in general and in limb skeletal muscle on rat E20 [36]. Interestingly not only JAK was found to be present, and JAK was also found to be regulated by EphA4. Interestingly EphA4 has been mentioned by Vasyutina as a regulator of limb muscle migration.[18]That SDF1 can activate the JAK/STAT through CXCR4 has been well established [37]. If JAK/STAT signaling is indeed activated in migrating limb muscle precursor cells this could be through either EphA4 or CXCR4, both are expressed in the muscle precursor cells.

As opposed to SHIP1, SHIP 2 is specifically expressed in the heart, skeletal muscle and placenta [38]. Interestingly, SHIP2 has been implicated as a postive regulator of both CXCR4 and the Akt pathway in in-vitro metastasis experiments [39]. CXCR4 has also been suggested to the regulator of SHIP2. If this mechanism holds up in migrating muscle precursor cells, not only would CXCR4 regulate Akt through SHIP2 and PI-3K it would also implicate yet another positive feedback mechanism regulating CXCR4.

If, when and how these potential downstream targets are actually activated during migration of limb muscle precursors remains unclear.

7 Conclusion

That there is a role for SDF1/CXCR4 signaling in the migration of limb muscle precursor cells has been well established. Also the core mechanism that is active during migration of limb muscle precursor cells has been established. There is a distinction made between factors expressed in the migrating muscle precursor cells and the limb bud mesenchyme. In the muscle precursor cells Pax3 expression and its downstream effectors, c-met and Lbmx1 are key. Several factors are known to regulate Pax3 such as Mox2, Dach2 and Eya2 which are also expressed in these migrating cells. CXCR4 is also expressed in these cells, however how and if this ties in to the core regulatory mechanism is not that clear. The core mechanism that is active in the mesenchyme is dependent on FGFs and Shh regulating SF/HGF expression. Both FGFs and SF/HGF can provide long range guidance cues to migrating cells. Indeed SF/HGF in the mesenchyme has been proposed to regulate c-met in migrating muscle precursor cells. FGFs have been proposed to control the migration of muscle precursor cells by indirectly interacting with Lbx1.

Much less is understood of how CXCR4 and SDF1 fit in to this model. Several papers describe roles for FGFs in regulating SDF1 and CXCR4 and for SF/HGF to regulate CXCR4 through NF-kB. Although expression of these factors has been established the underlying mechanism that is used has not been formally resolved. However since the regulation of CXCR4 and SDF1 by FGFs has been studied in fin regeneration this makes for a rather similar model and as such makes it probable for this mechanism to also be active during the migration of limb muscle precursors. The interaction of SF/HGF with CXCR4 by means of NF-kB has been shown in migrating glioma cells. Altough glioma cells are developmentally distinct from muscle precursor cells, another body of evidence pointing to a role of CXCR4 in regulating PI-3K and further downstream NF-kB would provide for a positive feedback loop of CXCR4 and as such warrants further investigation. MAPK has been mentioned as one of the many potential downstream targets of CXCR4, however due to the potential interaction of MAPK with Gab1 and the genetic interaction of CXCR4 and Gab1 in migration muscle precursor provides a pretty clear picture of CXCR4, MAPK and Gab1 signal transduction. Furthermore since Gab1 has been shown to interact directly and genetically with c-met it is possible to tie CXCR4 signaling in with c-met which is a well known core factor in the migration of limb muscle precursor cells. <Fig. 2>

Due to the pleiotropic downstream nature of SDF1/CXCR4 signaling it is difficult to pin-point what downstream targets are actually regulated in limb muscle precursors. Two main potential mechanisms can be deduced, which are not mutually exclusive. There is a body of evidence for EphA4 to be involved in migration of muscle precursor cells to the limb as is CXCR4, both have been implicated in regulating JAK/STAT signaling which could potentially be a key player as it has been a well established factor in regulating cellular migration [40, 41]. The previously mentioned regulation of CXCR4 by SF/HGF through NF-kB can have a potentially interesting role in setting up a powerful positive feedback loop where CXCR4 can positively regulate SHIP2 and PI-3K and through NF-kB can signal back to CXCR4. The most promising reports review an interaction of CXCR4 and c-met through MAPK and Gab1. This would provide a clear integration of the SDF1/CXCR4 signaling cascade into the core-regulatory principle in migrating limb muscle precursors.

8 Future directions

Studying what regulatory mechanisms are involved in a specific developmental process can be challenging. Using the right model to study this specific process is essential. Using myoblast cultures to get a rough understanding what factors are involved in muscle development would be acceptable, however using the same approach toward understanding the signaling cascades involved in muscle precursor cells that migrate to the limb would be asinine. Not only is a specific model required, to actually do experiments on this model there is a requirement for knock-out and fluorescent mutants of candidate genes. Ideally these

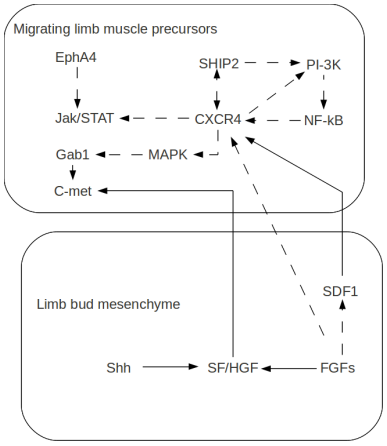

Figure 2: Schematic representation of the potential SDF1/CXCR4 interactions that can relate to limb muscle precursor migration. Especially the positive feedback loop of CXCR4 with SHIP2, PI-3K and NF-kB and the CXCR4, MAPK, Gab1, C-met cascade that ties CXCR4 back into the core-regulatory mechanism of migrating limb muscle precursor cells. Solid lines represent direct interactions, whereas dashed lines represent indirect interactions.

knock-outs have to be inducible specifically during muscle precursor migration and restricted to the developing limb. Without such an approach, it could be possible for earlier processes to have been disturbed by simply knocking out a gene. This could subsequently lead to disturbances in the process that is being studied, without the candidate gene actually playing a role during this specific process. Since this is a developmental process that is all about cell migration, actually showing how these cells migrate creates the potential of detecting small behavioral differences of migrating cells that could have been easily missed when solely relying on fixed tissue samples.

The combination of developmental process and preferred method of studying this process leads to the requirement of zebrafish as a model. Zebrafish is a model that are already being used to study muscle formation and complex methods of cell migration in vivo[12, 42]. Zebrafish have been used in a wide range of research. Notable the migration and development of the posterior lateral line (pLL) has been studied as a model of collective cell migration and organogenesis [42, 43, 20]. Elucidating the finer details, such as the organizational dynamics within the migrating pLL placate can only be studied using fluorescent imaging techniques since these are dynamic processes by nature and have to be studied as such. Zebrafish have been used to study pharmacological inhibitors, combining the scalability of small invertebrates with the ease of pharmacological inhibitor administration makes zebrafish a power tool to study vertebrate development [44]. Fluorescent transgenic zebrafish for specific genes

are already being used extensively and should pose no problem. The various methods available to create knock-out mutants in zebrafish is limited in comparison to mice. In mice, for example, it is possible to create organ or cell type specific knock-outs. Although complex it is even possible to combine this with methods to restrict the knock-out to a specific moment. In zebrafish this is not easily done. Creating zebrafish that express dominant negative mutants and employing pharmacological inhibitors is possible. Implantation of beads soaked in SF/HGF or FGF can provide further methods in studying this developmental process. Combining this with the potential of creating fluorescent mutants and imaging the entire developmental process of interest makes for the ideal developmental model.

Using a pax3-GFP transgenic zebrafish it would be possible to track limb muscle precursor cell migration in real-time. Using a candidate approach other proteins can be distinguished in these cells. Either isolating pax3 positive cells from during migration followed by mass-spec analysis for the presence of candidates or co-localization studies can be performed to ultimately show expression of candidates. Showing that indeed a candidate is expressed is not sufficient to proof that in this context there is indeed an interaction. In order to show functionality of these candidates in muscle precursor cell migration to the limb the system needs to be broken. This can be done by knock-out, dominant negative constructs and pharmacological inhibitors. The use of pharmacological inhibitors can be an interesting approach to study all candidates mentioned in this thesis. Pharmacological inhibitors can be added on the latest possible time and thus do not influence earlier developmental processes. Furthermore it is relatively easy to use different dosages in order to study dose dependent effects. Combining different pharmacological inhibitors can be used to study potential genetic interactions between candidates. In order to do this a synergistic effect has to be demonstrated, thus the combined effect of the pharmacological inhibitors has to be greater then the sum of its parts. Again zebrafish are an ideal model for this, not only is it easy to add pharmacological inhibitors to large amounts of embryos, it is also possible to track migration of the limb muscle precursor cells and could therefore offer a quantitative power specifically for the effect of candidates during migration.

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