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The contribution of connective tissues to muscle morphogenesis : an unexpected role for CXCL12 and CXCL14 chemokines

Sonya Nassari

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The contribution of Connective Tissues to muscle morphogenesis: an unexpected role for CXCL12 and CXCL14 chemokines

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Publically presented and defended on the 2nd of October, 2017

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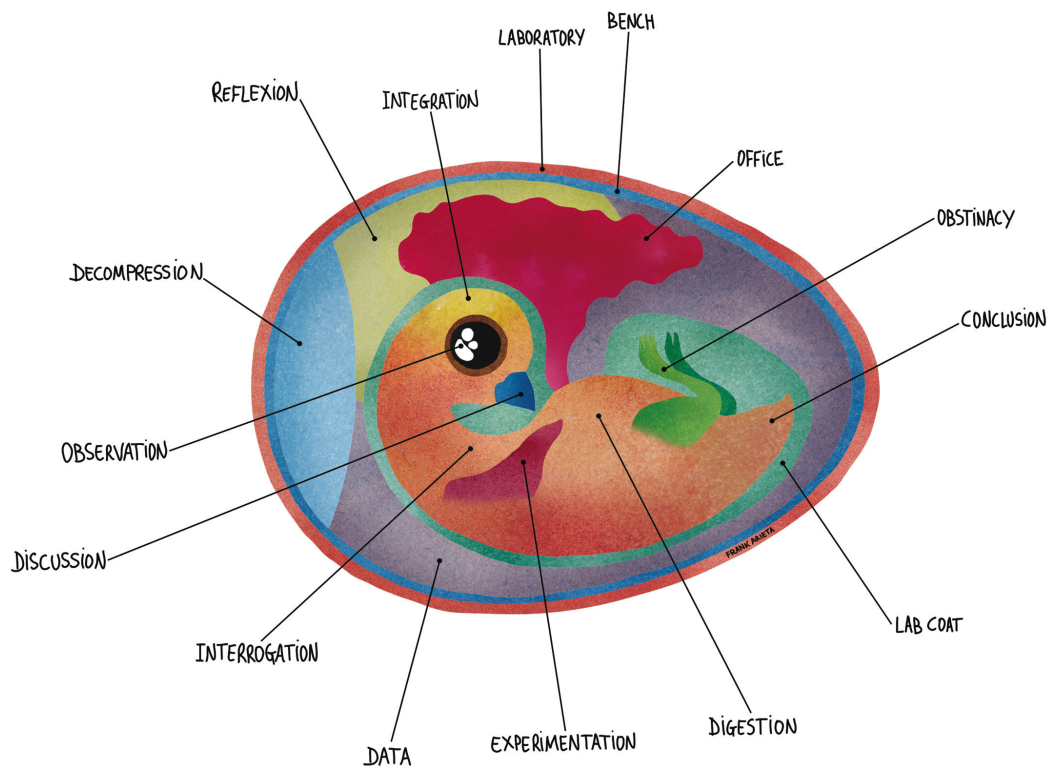
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Declaration

I hereby declare that I have done my doctoral thesis entitled “**The contribution of Connective Tissues to muscle morphogenesis: an unexpected role for CXCL12 and CXCL14 chemokines**” independently with no other sources or aids than cited. I testify that this thesis has not been previously submitted, in whole or in part, for a degree or any other qualification.

Paris, 26th of July 2017

(signature)



TO MY PARENTS AND MY OWL

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Summary

Skeletal muscle development mostly relies on intrinsic capacities of muscle progenitors to proliferate and differentiate. However, extrinsic signals arising from non-myogenic cells also contribute to the establishment of functional skeletal muscles. The aim of this PhD project was to investigate the role of connective-tissue (CT) on the development of skeletal muscle, using the chick embryonic limb as a model. We particularly investigated the influence of the two chemokines CXCL12 and CXCL14, which have been previously shown as expressed in limb mesenchyme giving rise to the different types of CTs during development. The involvement of CXCL12 and CXCL14 in limb CT differentiation was studied, as well as the role of these chemokines in skeletal muscle development mediated by CT.

We first showed that CXCL12 and CXCL14 display distinct restricted expression patterns in limb CT of chick embryos and demonstrated that CXCL12 promotes the expression of *OSR1*, *OSR2* and *COL3A1* genes, three markers of irregular CT, while CXCL14 enhances the expression of a regular CT gene, *SCX*. In addition, the expression of CXCL12, CXCL14 and their putative CT target genes were all negatively regulated by the anti-fibrotic BMP signalling, but also in the absence of musculoskeletal mechanical forces. These results show for the first time the involvement of CXCL12 and CXCL14 chemokines in the differentiation of CTs.

The putative role of both chemokines on CT-mediated myogenesis was then analysed. We observed that CXCR7, one CXCL12 receptor, was expressed both in muscle progenitors and differentiated muscle cells in embryonic chick limbs. Using gain- and loss-of-function approaches in primary cultures of chick limb myoblasts, we revealed that CXCR7 promoted myogenesis by regulating muscle cell fusion, while CXCL12 did not influence muscle differentiation. CXCL14 dramatically inhibits *in vitro* myogenesis. Functional assays performed in chick embryonic forelimbs *in vivo* demonstrate that overexpression of *CXCL12*, *CXCR7* or a dominant-negative form of *CXCR7* all resulted in abnormal and mispatterned muscles in chick limbs. Similarly, *CXCL14* overexpression in chick limb *in vivo* led to profound anomalies in muscle differentiation. All together, our results demonstrate an essential contribution of CXCL12 and CXCL14 chemokines in CT differentiation and in CT-mediated muscle development in embryonic limb.

Résumé

Les muscles se forment au cours du développement embryonnaire, principalement grâce aux capacités de prolifération et différenciation des cellules souches musculaires, néanmoins ces capacités sont insuffisantes pour le développement correct des muscles. La formation des muscles est aussi régulée par des signaux provenant de tissus adjacents, parmi lesquels le tissu conjonctif (TC). Plusieurs facteurs de transcription spécifiquement exprimés dans le TC ont été identifiés comme étant impliqués dans la myogenèse caractérisant ainsi le TC comme une source importante de signaux dans le mécanisme de morphogénèse musculaire. Ces observations soulignent l'importance du rôle du TC dans la formation des muscles, cependant la nature moléculaire des mécanismes médiés par le TC reste à ce jour inconnue.

L'objectif de ce travail de thèse a été d'établir le rôle des chimiokines CXCL12 et CXCL14 dans l'interaction entre le TC et le développement musculaire, en utilisant l'embryon de poulet comme modèle. Dans un premier temps, nous avons défini le patron d'expression de *CXCL12* et *CXCL14* au cours du développement embryonnaire, et avons mis en évidence une corrélation entre la localisation de ces chimiokines et l'expression de gènes spécifiques à différentes sous populations du TC. Afin d'évaluer le rôle potentiel de ces chimiokines dans la différenciation du TC nous avons utilisé des approches de gain de fonction *in vitro* et *in vivo* et avons montré que CXCL12 et CXCL14 activent les facteurs de transcription spécifiques à différentes sous population du TC, démontrant ainsi que CXCL12 et CXCL14 régulent la différenciation du TC au cours du développement du membre. De plus, nous avons établi que la voie de signalisation BMP et les forces mécaniques régulent négativement l'expression des chimiokines CXCL12 et CXCL14. Ces résultats caractérisent pour la première fois l'implication de CXCL12 et CXCL14 dans la différenciation du TC. La deuxième partie de ce travail de thèse a visé à caractériser le rôle paracrine de CXCL12 et CXCL14 sur le développement musculaire. Nous avons pu observer que l'un des récepteur de CXCL12, CXCR7, est exprimé dans les cellules musculaires souches et différenciées, dans les ailes d'embryons de poulets. En utilisant des approches de gains et pertes de fonctions, d'une part *in vitro* dans des cultures primaires de myoblastes de poulets, nous avons montrés que CXCR7 favorise la myogénèse, notamment en régulant la myogénèse, tandis que CXCL12 n'a pas d'impact sur la différenciation musculaire *in vitro*. De plus nous avons pu constater que CXCL14 inhibe la myogénèse *in vitro*. Finalement, *in vivo*, nous avons observé que la surexpression des chimiokines entraîne un développement anormal des muscles tandis que l'expression du récepteur CXCR7 tronqué favorise le développement musculaire,

soulignant l'importance de la signalisation CXCL12/14 dans le processus de morphogénèse musculaire médié par le TC. Ces résultats constituent la première démonstration d'une fonction paracrine du TC dans la morphogénèse musculaire via les chimiokines CXCL12 et CXCL14.

Zusammenfassung

Die Entwicklung der Skelettmuskulatur beruht auf den Fähigkeiten von myogenen Progenitorzellen. Im Laufe der Entwicklung proliferieren diese, differenzieren zu Myoblasten, die schließlich zu Muskelfasern fusionieren. Zur Bildung der embryonalen Muskulatur werden jedoch darüber hinaus extrinsische Signale von nicht-myogenen Zellen, vor allem Zellen des Bindegewebes, benötigt. Das Ziel dieser Arbeit war, die Rolle des Bindegewebes in der embryonalen Muskelentwicklung der Extremitäten des Hühnerembryos als Modell genauer zu untersuchen. Speziell wurde hier der Einfluss zweier Chemokine untersucht, CXCL12 und CXCL14, von denen bereits vorher gezeigt wurde, dass sie im Mesenchym der sich entwickelnden Extremität exprimiert sind. In dieser Arbeit wurde die Rolle dieser Chemokine sowohl für die Differenzierung des Bindegewebes selbst, wie auch deren Einfluss auf die Muskeldifferenzierung analysiert.

Es konnte gezeigt werden, dass sowohl *CXCL12* als auch *CXCL14* distinkte regionale Expressionsmuster im Bindegewebe der Extremität aufweisen. Funktionell erhöht CXCL12 die Expression von *OSR1*, *OSR2* und *COL3A1*, dreier Marker für irreguläres Bindegewebe, während CXCL14 die Expression eines Schlüsselmarkers für reguläres Bindegewebe (Sehne), *SCX*, erhöht. Die Expression von CXCL12 und CXCL14 selbst, wie auch die Expression ihrer putativen Zielgene, wurden demgegenüber durch Stimulation des anti-fibrotisch wirkenden BMP Signalweges negativ reguliert. Zudem bewirkte eine experimentell induzierte Muskelparalyse im Hühnerembryo eine Herunterregulation von *CXCL12* und *CXCL14*, was bedeutet, dass die Expression beider Gene abhängig von mechanischen Signalen ist. Diese Ergebnisse involvieren zu ersten Mal die Chemokine CXCL12 und CXCL14 in die Differenzierung des Bindegewebes.

Im Folgenden wurde die mögliche Rolle beider Chemokine in der Muskelentwicklung analysiert. Es wurde zunächst gezeigt, dass CXCR7, ein Rezeptor für CXCL12, in Muskelvorläufern wie auch differenzierten Muskelzellen in der Extremität des Hühnerembryos exprimiert wird. Durch Funktionsgewinn und -verlust Versuche in primären Myoblasten aus Hühnerembryos konnte gezeigt werden, dass CXCR7 die Muskelbildung durch die Beeinflussung der Zellfusion fördert, während CXCL12 keinen Einfluss hatte. Demgegenüber zeigte CXCL14 eine starke Inhibition des Myogenese *in vitro*. Die Misexpression von CXCL12, CXCR7 sowie dominant-negative Versionen von CXCR7 *in vivo* bewirkten eine abnormale Musterbildung der Extremitäten-Muskulatur. Genauso bewirkte die Misexpression von CXCL14 deutliche Veränderungen der

Muskeldifferenzierung. Zusammenfassend konnte eine Funktion der Chemokine CXCL12 und CXCL14 in der Differenzierung des Bindegewebes sowie im nicht-Zell autonomen Einfluss des Bindegewebes auf die Muskelentwicklung gezeigt werden.

Abbreviations

CT(s): Connective tissue(s)
EOMs: Extraocular muscles
MRF(s): Myogenic regulatory factor(s)
bHLH: Basic Helix-Loop-Helix
PAX: Paired homeobox transcription factors
SIX: Sine oculis homeobox
EYA: Eyes absent homologue
Shh: Sonic hedgehog
BMP: Bone morphogenetic protein
NCC(s): Neural crest cell(s)
FGF: Fibroblast growth factor
FC(s): Muscle founder cell(s)
FCM(s): Fusion competent myoblast(s)
CAM(s): Cell adhesion molecule(s)
HGF/SF: Hepatocyte growth factor / scatter factor
LBX1: Ladybird homeobox 1
Msx1: Msh homeobox one
Sim: Single-minded homolog
ZPA: zone of polarising activity
Ihh: indian hedgehog
TGF β : transforming growth factor β
Mmp(s): metalloprotease(s)
CDH: congenital diaphragmatic hernia
NF- κ B: nuclear factor kappa B

NG2: Neural/glial antigen 2

PIC(s): PW1-positive interstitial cell(s)

PDGFR α : Platelet-derived growth factor receptor

FAP(s): fibro/adipogenic progenitor(s)

GPCR(s): G-protein-coupled receptor(s)

ACKR(s): atypical chemokine receptor(s)

SDF-1: stromal-derived factor 1

WAT: white adipose tissue

MAPK: mitogen-activated protein kinase

ERK: extracellular signal regulated kinase

GKR(s): G-protein-coupled receptor kinase(s)

PKC: protein kinase C

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Introduction

Introduction

During embryonic development, skeletal muscle formation is an important step in the establishment of a functional musculoskeletal system. Muscle development corresponds to sequential waves of myogenesis, which at the end give rise to adult muscles. Myogenesis occurs mainly through the intrinsic proliferation and differentiation capacities of muscle progenitor cells, however it has been shown that signals coming from adjacent tissues also contribute to muscle morphogenesis (Kieny, 1982) among which connective tissues (CTs). The CT-derivatives correspond to bones, cartilage, tendons, ligaments and irregular connective tissue (CT) and constitute with the muscles the musculoskeletal system. CTs exhibit physical interactions with muscles, but more importantly CTs and muscles cooperate together to ensure their own development. Previous works have started to decipher the importance of the different CTs in the establishment of muscles during both embryonic development and adulthood. During embryonic development, defects in the interplay between CTs and muscles trigger severe damages that can lead to critical human pathology such as the Bethlem myopathy (Bönnemann, 2011; Hicks et al., 2014) a congenital muscular dystrophy, or the Holt-Oram syndrome (Hasson et al., 2010) associated to congenital heart and skeletal abnormalities. It is therefore important to elucidate the molecular mechanisms that govern the relations between CTs and muscle morphogenesis during development.

I. Muscle development

Skeletal muscles form a highly complex and heterogeneous tissue that is part of the musculoskeletal system. During development, the correct formation of skeletal muscle is an important step to provide proper functions of the musculoskeletal system, which allows essential functions such as body motion, or more importantly breath ability. The process of generating muscles is defined as “myogenesis”, and occurs mainly during embryonic development.

A. The embryonic origins of skeletal muscles

In vertebrates, all skeletal muscles derive from mesodermal cells (Noden and Francis-West, 2006). However, it has been established that muscles present different embryonic origins depending on their final position in the adult (Figure 1). Lineage tracing experiments performed in the avian model system, such as Di-I labelling and chick-quail grafting, highly

contribute to the establishment of the specific embryonic origins of the different skeletal muscles (Selleck & Stern 1991; Ordahl & Le Douarin 1992; Couly et al. 1992).

Body skeletal muscles

Body skeletal muscles correspond to skeletal muscles of limbs and trunk region. They originate from transient mesodermal structures that arise from segmentation of the paraxial mesoderm, the somites (Chevallier et al. 1976; 1977; Christ et al. 1974; 1977). Somites form sequentially, in a periodic way, along the antero-posterior axis of the embryo. They are disposed by pair on each side of the neural tube and form a layer of epithelial cells. Such simple structures are going to give rise to different cell types, among which the axial muscle cells. Under signals coming from their environment, somites are segmented in two main compartments: the sclerotome and the dermomyotome (Figure 2). Two different domains can be distinguished in the dermomyotome: the dorsomedial and the ventrolateral regions (Figure

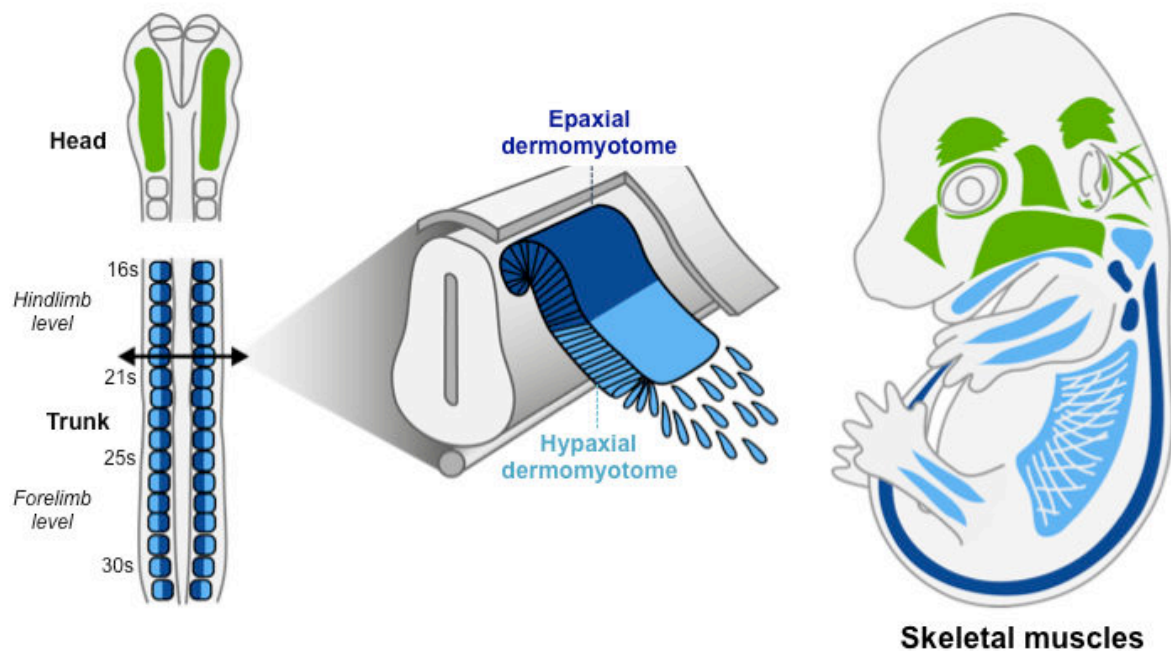


Figure 1: The embryonic origins of skeletal muscles. Skeletal muscles have distinct origins depending on their location in the body. Head muscles derive from the cranial paraxial mesoderm (green), except the tongue and posterior neck muscles, which have the same embryonic origin than axial muscles: the somites. In the trunk, muscles from the back originate from the epaxial lip of the dermomyotome (dark blue), and limb muscles and diaphragm derive from the hypaxial lip of the dermomyotome (light blue). (Adapted from Nassari et al., 2017)

2). Both domains contribute differentially to axial muscle formation. Indeed, the medial part of the dermomyotome gives rise to the epaxial musculature, corresponding to the back and intercostal muscles, while the lateral part of the dermomyotome gives rise to the hypaxial musculature, corresponding to the diaphragm, abdominal and limb muscles.

Craniofacial skeletal muscles

Muscles of the head and the neck form the so-called craniofacial muscles. Whereas trunk and limb muscles originate from axial somitic mesoderm, craniofacial muscles present distinct embryonic origins (Figure 3). Based on these different embryonic origins, head muscles are classified into three distinct groups: 1) the tongue and posterior neck muscles, deriving from anterior somites; 2) the extraocular muscles (EOMs), originating from the prechordal mesoderm and 3) the branchiomeric muscles including muscles of the jaw, anterior neck and

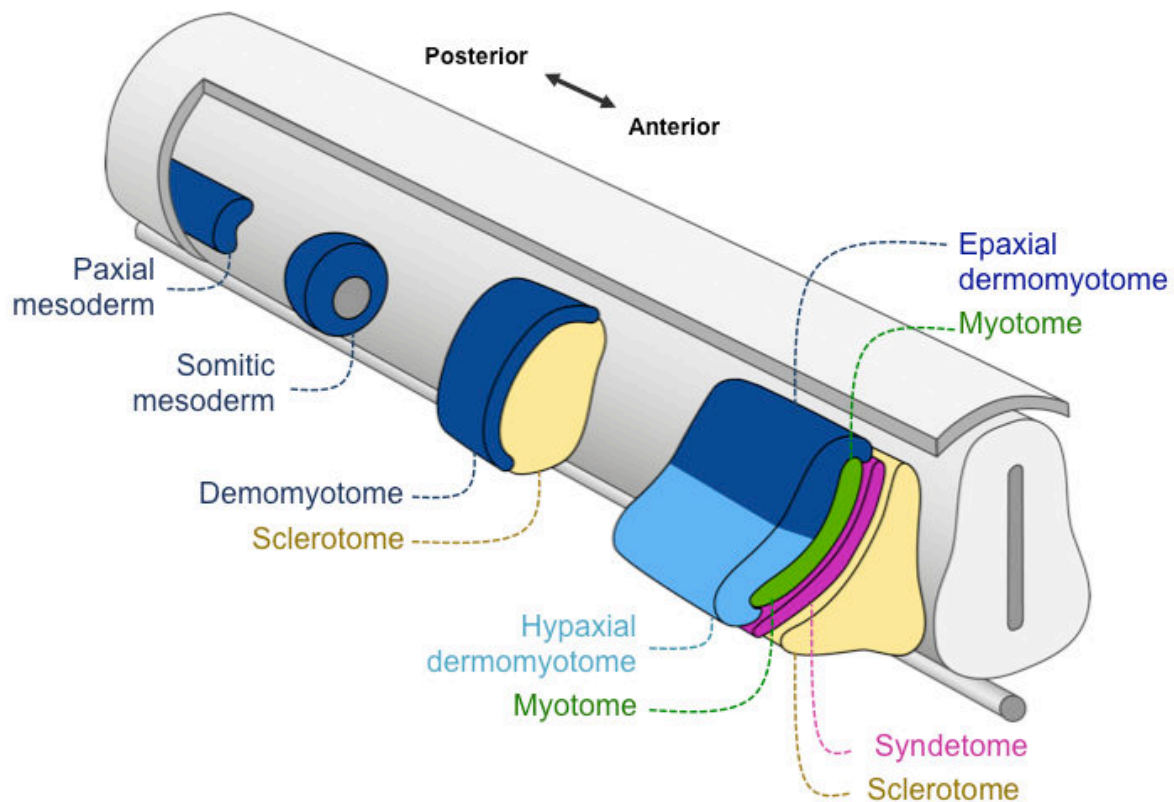


Figure 2: Differentiation of somites. Somites are formed from segmented paraxial mesoderm. Upon different signals, somites undergo a dorso-ventral organization leading to the formation of the sclerotome in the ventral part and the dermomyotome in the dorsal part. Subsequently, the dermomyotome is subdivided in hypaxial and epaxial compartments that will contribute to the formation of the myotome. Finally, a last compartment arising from the sclerotome is formed: the syndetome. The sclerotome compartment give rise to bones, cartilage and irregular connective tissues of the trunk, and syndetome will lead to trunk tendons.

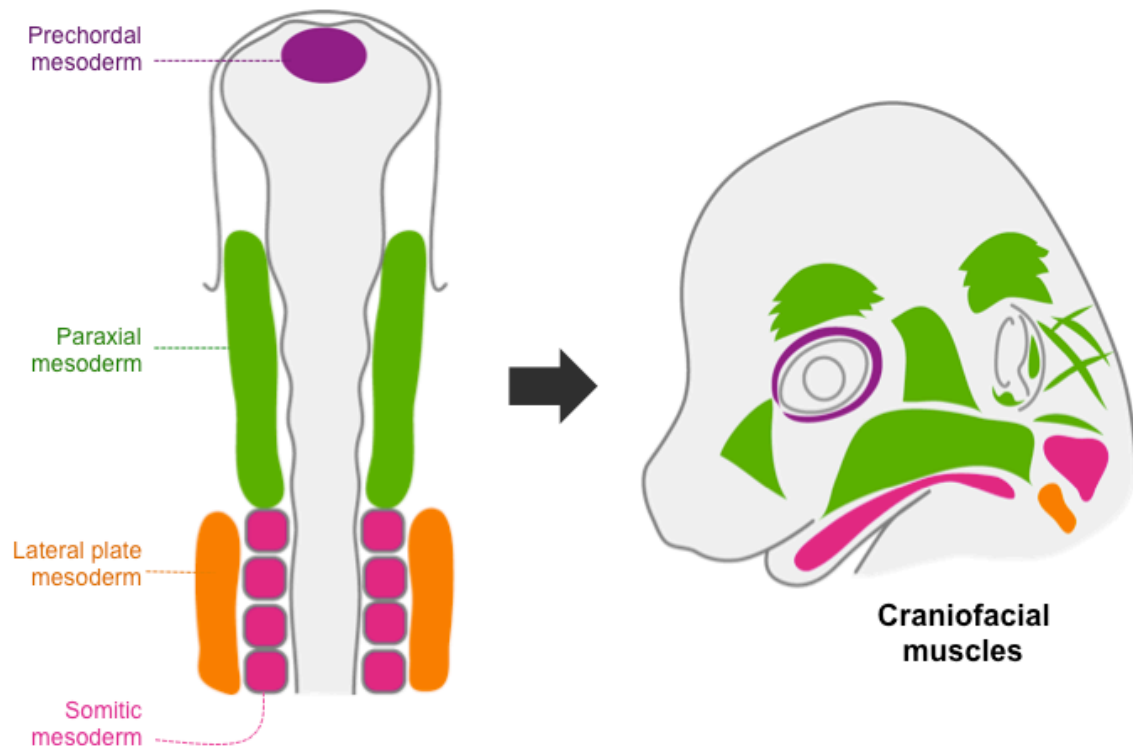


Figure 3: The embryonic origins of craniofacial muscles. Branchiomic muscles originate from the cranial paraxial mesoderm (green), while extraocular muscles derive from the prechordal mesoderm (purple). Posterior neck muscles and tongue arise from somites (pink), and a few anterior neck muscles from the occipital lateral plate (orange). (Adapted from Buckingham et al., 2012)

face, arising from the paraxial mesoderm (Couly et al., 1992; Hacker and Guthrie, 1998; Noden, 1983; Trainor et al., 1994), (Figure 3). In vertebrates, the occipital lateral plate mesoderm is an additional source for the formation of a restricted population of neck muscles (Theis et al., 2010).

B. Muscle specification

The genetic cascade regulating skeletal muscle specification

Despite heterogeneity in the embryonic origins of skeletal muscles, the specification processes of skeletal muscle formation from a founder stem cell in the embryo exhibit common features to all skeletal muscles. Through complex and various cascades of signalling, an undifferentiated cell corresponding to a stem cell (fate is not acquired) switches to a progenitor state (fate being acquired), then to a precursor state (fate acquired), and finally ends up with a differentiated state (functional entity). The transition from an undifferentiated

cell to a fully differentiated muscle cell is intrinsically regulated by the activation of a group of transcription factors called the Myogenic Regulatory Factors (MRFs), (Figure 4). The Myogenic Regulatory Factor (MRF) family includes four different genes: MyoD, Myf5, Mrf4 as determination factors and Myogenin as differentiation factor (Ott et al., 1991; Pownall and Emerson, 1992; Sassoon et al., 1989). They all code for transcription factors that belong to the superfamily of basic Helix-Loop-Helix (bHLH) transcription factors. In vivo genetic manipulations of the MRFs demonstrate the hierarchical relationship and functional redundancy existing between these factors. In mutant mouse for MyoD, Myf5 expression level is increased and muscles are unaffected (Rudnicki et al., 1992), suggesting that Myf5 and MyoD have redundancy roles in myogenesis. In the absence of both MyoD and Myf5, skeletal muscles are absent (Rudnicki et al. 1993). Myod, Myf5 and Mrf4 are characterized as major effectors for early myogenic specification (Rudnicki et al. 1992; 1993; Kassam-Duchossoy et al. 2004). In the absence of Myogenin, a deficit in skeletal muscle differentiation is observed, highlighting an essential role of Myogenin in terminal muscle differentiation (Nabeshima et al., 1993). In fibroblast cells, MRFs have the ability to trigger skeletal muscle specification (Weintraub et al. 1991; Delfini & Duprez 2004), demonstrating that MRFs are crucial actors for the development of muscles. However, the activation of the myogenic program relying on MRF expression requires the activation of upstream specific key regulatory factors that actually differs depending on the anatomical region where the muscles will be formed.

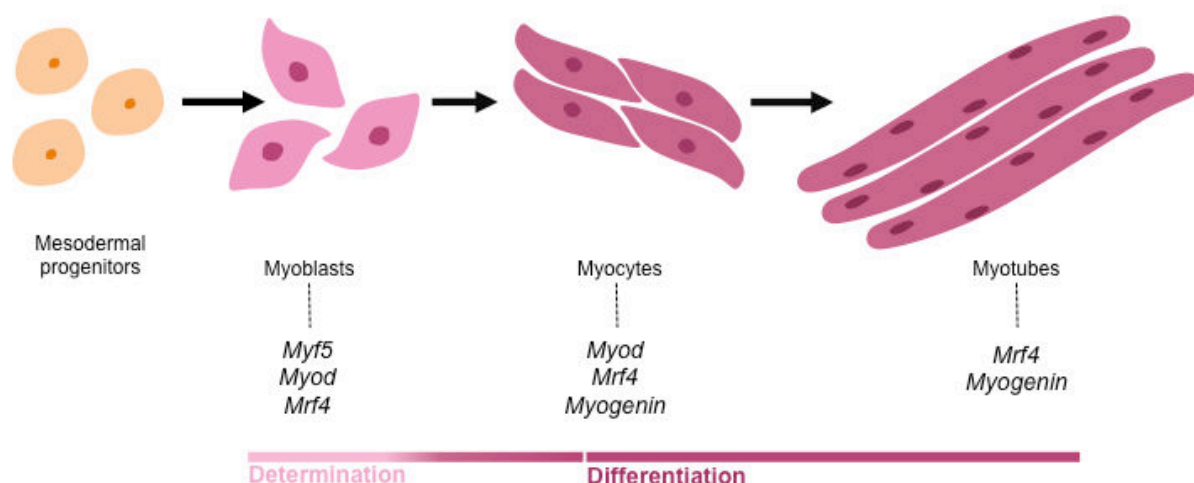


Figure 4: The myogenic regulatory factors (MRFs) regulate myogenic determination and differentiation. Activation of the MRFs in mesodermal progenitors controls myogenic lineage progression. The transcription factors MYOD and MYF5 are crucial for muscle cell determination, while MYOG and MRF4 are required for later differentiating steps of myogenesis.

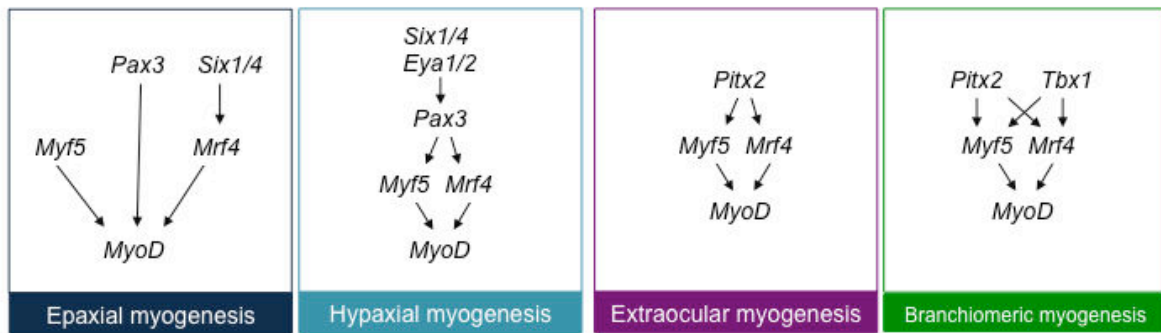


Figure 5: Specific genetic regulatory networks control myogenesis. Distinct gene networks regulate epaxial (dark blue), hypaxial (light blue), extraocular (purple) and branchiomic (green) muscles. In epaxial and hypaxial myogenesis, activation of MRFs is dependent on the PAX3 transcription factor while PAX3 is absent in craniofacial muscles, in which PITX2 and TBX1 are responsible for activation of the MRFs. (Adapted from Buckingham et al., 2012)

- **Axial muscle formation depends on PAX3- and PAX7-expressing progenitors**

The PAX genes that belongs to the Paired homeobox transcription factor family, highly contribute to the formation of different tissues during embryonic development (Wang et al. 2008). During axial skeletal muscle development, PAX3 and PAX7 have been shown to be important upstream regulators coordinating muscle lineage specification. In Pax3/Pax7 deficient mice, all trunk and limb muscles are missing (Kassar-Duchossoy et al., 2005). During embryonic development, both PAX3 and PAX7 are expressed in muscle progenitors cells, however, conversely to MRFs, their expression is not restricted to muscle cells (Buckingham et al., 2003). Initially, PAX3 expression is present in the non-segmented paraxial mesoderm, then in the epithelial somite, and finally restricted first to the dermomyotome compartment, and secondly to its ventrolateral part (Daston et al., 1996; Williams and Ordahl, 1994). Later on, when cells undergo myogenic specification, PAX3 expression is downregulated (Daston et al., 1996; Williams and Ordahl, 1994). The first detection of PAX7 in muscle progenitors is observed in the central domain of the dermomyotome (Relaix et al., 2004). In this domain, both PAX7 and PAX3 are coexpressed. Following activation of the MRFs, PAX7 expression is downregulated. Although PAX3 and PAX7 have redundant functions, they also contribute individually to different steps of myogenesis. PAX3 expression is associated to the primary phase of myogenesis, while PAX7 is mostly related to subsequent step of myogenesis (fetal myogenesis, post-natal growth and muscle homeostasis/regeneration). The first experiments revealing the important role of

PAX3 for muscle specification have been performed in chick embryos (Maroto et al., 1997). Gain-of-

function assays showed that ectopic expression of PAX3 in nascent mesoderm leads to the induction of MyoD and Myf5 (Maroto et al., 1997). In the epaxial domain of the dermomyotome, PAX3 directly regulates MyoD, independently of Myf5 and Mrf4 (Tajbakhsh et al., 1997). In the hypaxial compartment, PAX3 does induce MyoD, but also activates Myf5 (Bajard et al., 2006). In Pax3 mutant, Myf5 expression is lost in the hypaxial compartment while epaxial expression of Myf5 is unchanged, thus demonstrating that PAX3 expression activates Myf5 expression in the hypaxial compartment, but not in the epaxial compartment (Figure 5), (Bajard et al., 2006). The sine oculis homeobox (SIX) transcription factors and their cofactors Eyes absent homologue (EYA) proteins have been identified to act upstream PAX3 (Heanue et al., 1999). In chick embryo, gain-of-function experiments have demonstrated that Six1 and EYA activate PAX3 expression (Heanue et al., 1999). In addition, mouse carrying mutations for either Six1 and Six4 or Eya1 and Eya2 display loss of PAX3 expression in the hypaxial lip of the dermomyotome (Grifone et al. 2005; 2007). Finally, MRF4 expression in the epaxial domain of the dermomyotome is lost in Six1/Six4 double mutant (Grifone et al. 2005). Altogether, these data show that SIX transcription factors and their cofactors EYA proteins are important regulators in the process of axial muscle specification, inducing MRF expression either directly or indirectly through PAX3 activation (Figure 5).

- **Craniofacial muscles formation depends on TBX1- and PITX2-expressing progenitors**

Consistent with the distinct embryological origin of craniofacial muscles, distinct regulatory cascades from that described for limb and trunk myogenesis control muscle formation in the head (Dong et al., 2006; Kelly et al., 2004). Although the genetic cascade of the MRFs, Myf5, MyoD and Myogenin is comparable with the one described in axial muscles (Rudnicki et al. 1993; Hacker & Guthrie 1998), the main factors regulating MRF genes expression in craniofacial muscles (branchiomic muscles and EOMs) are different. In head muscles, PAX3 is not detected (Relaix et al., 2004), suggesting the involvement of distinct activators for the myogenic regulatory cascade than those identified in somites. Indeed, during mouse embryonic development, both Myf5 and Pax3 inactivation leads to a complete lack of muscles in the trunk and limbs, but no defects in branchiomic muscles and EOMs (Tajbakhsh et al.,

1997), clearly demonstrating that Pax3 is not required for head myogenesis. The T-box-containing transcriptional activator Tbx1 and the pituitary homeobox gene Pitx2 have been shown to activate MyoD and Myf5 in the head (Dastjerdi et al., 2007; Diehl et al., 2006; Dong et al., 2006; Grifone et al., 2008; Kelly et al., 2004), (Figure 5). Inactivation of Tbx1 or Pitx2 leads to a similar phenotype resulting in a severe reduction of specific groups of head muscles (Diehl et al., 2006; Gage et al., 1999; Kelly et al., 2004; Kitamura et al., 1999; Zacharias et al., 2011). Indeed, a genetic diversity exists among branchiomic muscles and EOMs, as only Pitx2 is required to induce EOM myogenesis, while both Tbx1 and Pitx2, acting in parallel, activate MRFs to induce branchiomic myogenesis (Figure 5). Pitx2 is expressed in both axial and craniofacial muscles (Shih et al. 2007), however, it is not required for axial myogenesis (Kitamura et al., 1999), in which Pitx2 is acting downstream of PAX3 to induces MyoD expression (L'Honore et al., 2010). In pharyngeal muscles, Pitx2 is necessary to induce Myf5 expression (Shih et al. 2007), through direct binding on its promoters (Zacharias et al., 2011). Pitx2 and Tbx1 regulate each other (Nowotschin 2006; Shih et al. 2007) and both induce branchiomic myogenesis specification by activating Myf5 and MyoD (Kelly et al., 2004; L'Honore et al., 2010; Nowotschin, 2006; Zacharias et al., 2011). Furthermore, two additional upstream regulators of branchiomic myogenesis have been identified: MyoR (musculin) and Capsulin (Tcf21), (Lu et al., 2002). In mutant mice for Capsulin and MyoR, MRF genes are not activated in branchial arch-derived muscles and cell death is observed in branchial arch mesenchyme (Lu et al., 2002), (Figure 5). Such results show that both MyoR and Capsulin are involved in pharyngeal arch-derived muscles, but not in the formation of EOMs. The role of the SIX transcription factors and EYA proteins during craniofacial development remains to be explored. Although it has been demonstrated that mutant embryos for Six1 and double mutants for Six1 and Six4 present craniofacial muscle defects (Laclef et al. 2003; Grifone et al. 2005; Grifone et al. 2007), it is not clear at which level Six factors act in the myogenic regulatory network for head myogenesis.

The extrinsic signals regulating skeletal muscle specification

Extrinsic signals emerging from adjacent tissues highly contribute to the regulation of both axial and head skeletal muscle specification. Axial myogenesis relies on the formation of the dermomyotomal compartment, which arises from the dorso-ventral patterning of somites. The process of somite patterning, which is mediated through signals originating mainly from the neural tube, the notochord and the ectoderm (Brand-Saberi et al., 1993; Buffinger and Stockdale, 1995; Pourquie et al., 1993; Spence et al., 1996), finally leads to the formation of

the sclerotome ventrally and of the dermomyotome dorsally. Sonic Hedgehog (Shh) secreted from the notochord participates in the ventralization of the somite (Chiang et al., 1996; Johnson et al., 1994), while Wnt signalling from the dorsal part of the neural tube and the ectoderm promotes dorsalization of the somite by repressing sclerotomal markers (Capdevila et al., 1998; Fan et al., 1997). Gain-of-function experiments performed in chick embryos have shown that ectopic Shh induces an increase in the expression of the sclerotomal marker *Pax1* (Johnson et al., 1994). In addition, mutant mouse for *Wnt1* presents a loss of the dermomyotome (Ikeya and Takada, 1998). Subsequent to its specification, the dermomyotome is then subdivided in two different domains (Figure 2), which are the epaxial and hypaxial compartments. Again, extrinsic signals orchestrate specification of the two different domains (Figure 6). Classical surgical experiments in chick have shown that the epaxial compartment is specified through signals coming from the neighbouring structures (Rong et al., 1992). In the context of notochord ablation, epaxial myogenesis is prevented

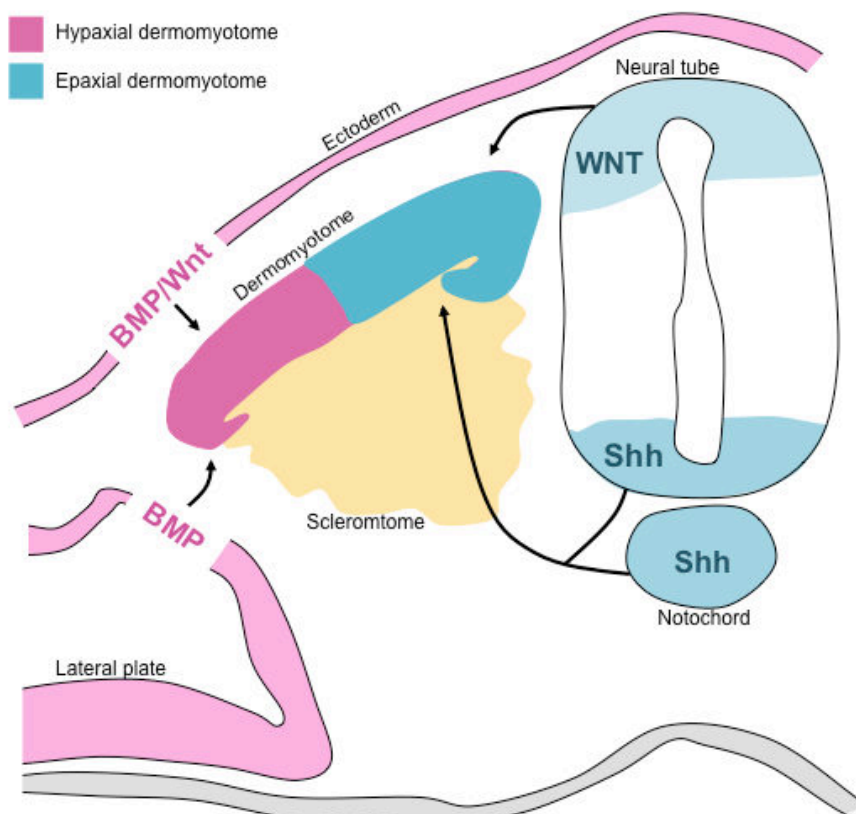


Figure 6: Extrinsic signals specify dermomyotome compartments. BMP and Wnt signalling emanating from the lateral plate and the ectoderm induces formation of the hypaxial region of the dermomyotome (pink), while Sonic Hedgehog (Shh) and Wnt signalling, arising from the neural tube and the notochord, govern specification of the epaxial dermomyotome (blue).

(Borycki et al., 1999; Munsterberg et al., 1995). Shh, which is released from the notochord, is involved in dermomyotome maturation (Figure 6). Indeed, in the epaxial dermomyotome, it has been demonstrated that Shh activates Myf5 expression (Borycki et al., 1999). Additionally, canonical Wnt signalling from the neural tube also participate in the induction of the epaxial compartment (Marcelle et al., 1997), (Figure 6). Finally, formation of the hypaxial compartment is mediated through Wnt and BMP signalling pathways, delivering from the ectoderm and the lateral plate mesoderm (Dietrich et al., 1998; Pourquié et al., 1996; Reshef et al., 1998; Tajbakhsh et al., 1998), (Figure 6).

Extrinsic signals regulating head myogenesis are identical to those acting during axial myogenesis, but display opposite effects. Wnt pathway coming from the dorsal part of the neural tube and the ectoderm acts as a repressor of myogenic specification in the cranial paraxial mesoderm (Tzahor et al. 2003; Tirosh-Finkel et al. 2006; von Scheven et al. 2006).

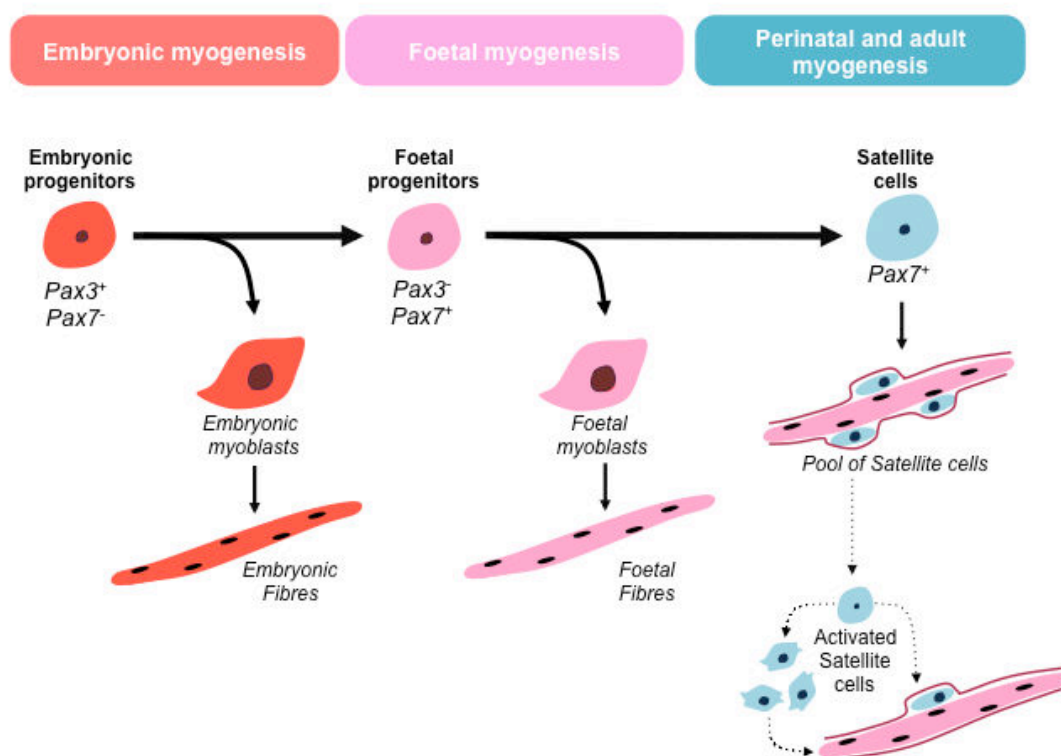


Figure 7: Successive waves of myogenesis contribute to muscle development. Embryonic myogenesis initiates muscle development. This first wave of myogenesis is dependent on the presence of PAX3-positive progenitors. Embryonic progenitors give rise to embryonic myoblasts that build embryonic fibres and establish the pool of foetal progenitors expressing PAX7. During foetal myogenesis, which is the second wave of myogenesis, PAX7-positive foetal progenitors give rise to foetal myoblasts that form foetal fibres and produce the pool of satellite cells expressing PAX7. At adulthood, satellite cells can be activated under specific signals, thus contributing to the formation of new adult myoblasts and new satellite cells through self-renewal.

Ectopic activation of Wnt signalling in cranial paraxial mesoderm inhibits myogenesis both in vitro and in vivo (Tzahor et al., 2003). A similar result has been observed in the context of BMP ectopic activation, thus demonstrating that BMP pathway also regulates negatively craniofacial myogenesis (Tzahor et al. 2003; Tirosh-Finkel 2006; von Scheven et al. 2006). In the contrary, antagonists of WNT and BMP signalling enhance head muscles formation (Tzahor et al., 2003). Ectopic expression of Noggin or Gremlin, two BMP antagonists and of Frizzled, a WNT antagonist, lead to the activation of myogenic markers (Tzahor et al., 2003). During head myogenesis, Noggin, Gremlin and Frizzled signals arise from tissues surrounding cranial muscles, among which cranial neural crest cells (NCCs). Cranial NCCs give rise to all bones, tendons, and connective tissues in the head (Couly et al., 1993; Le Douarin and Ziller, 1993). Cranial NCCs deliver signals that are essential to the establishment of craniofacial muscles (Couly et al., 1992; Noden, 1983; Rinon et al., 2007; Tzahor et al., 2003), however, cranial NCC ablation does not induce a complete loss of head muscles (Tzahor et al. 2003; von Scheven et al. 2006), demonstrating that other signals are involved in craniofacial muscle specification. FGF signalling also participates in the regulation of head myogenesis. It has been shown that FGF pathway arising from branchial arches activates the expression of MyoR, while it induces a decrease in the expression of Myf5 (von Scheven et al. 2006). All together, WNT, BMP and FGF signalling prevent premature head muscle differentiation, while inhibiting WNT and BMP pathways promotes head muscle specification.

C. The different waves of myogenesis rely on different populations of progenitors

The formation of muscles is driven through successive and overlapping phases of myogenesis (Figure 7). The first wave of myogenesis corresponds to the embryonic myogenic phase, during which the basic muscle pattern is established. During embryonic myogenesis, muscle embryonic progenitors express PAX3 and establish the pool of embryonic myoblasts, which generate primary muscle fibres (Figure 7). Primary fibres constitute the scaffold for further muscle development. Embryonic myogenesis is then followed by a second wave, which is called foetal myogenesis, which corresponds to the growth and maturation of scaffold muscles. During this phase, foetal progenitors expressing PAX7 fuse between themselves, and with primary fibres to form secondary fibres, which finally contribute to muscle growth (Messina and Cossu, 2009), (Figure 7). In the limb of mouse and chick embryos, embryonic myogenesis occurs from E11.5 to E14.5 and E2.5 to E6, respectively, and foetal myogenesis is established from E14.5 and E6, respectively (Biressi et al., 2007; Duprez, 2002). Finally, a

third phase of myogenesis, which is called perinatal myogenesis, takes place at the end of embryonic development (Figure 7). During this last myogenic wave, the pool of adult muscle stem cells, the satellite cells expressing PAX7, is established. Satellite cells are essential for subsequent perinatal muscle growth, adult homeostasis and regeneration (Messina and Cossu, 2009), (Figure 7). Although these populations of muscle progenitors exhibit different features (Hutcheson et al., 2009), it has been established that they all derive from the same pool of progenitors. Genetic lineage tracing and ablation experiments performed in mouse embryo shows that a common pool of PAX3-positive progenitors contributes to the establishment of both primary and foetal progenitors (Hutcheson et al., 2009). PAX7-positive cells derive from PAX3-positive cells and downregulate Pax3 to constitute the pool of foetal progenitors (Hutcheson et al., 2009), (Figure 7). Therefore, PAX7 is crucial for foetal myogenesis, but not embryonic one, while PAX3 is essential for embryonic myogenesis, but not foetal one.

D. Muscle cell fusion: an important step for muscle differentiation

Muscle differentiation requires the formation of muscle fibres, which are multinucleated cells resulting from muscle cell fusion events. This mechanism can occur between two myoblasts, between a myoblast and a myotube, as well as between two myotubes. Myoblast fusion takes place during both embryonic and foetal myogenesis, however, it seems that this event is more important during the second phase of myogenesis (Biressi et al., 2007), suggesting that foetal myoblasts display more efficient fusion ability than embryonic myoblasts. In addition, it has been demonstrated that fusion potential of muscle cells differ between different regions in the embryo (Sieiro-Mosti et al., 2014). Although all axial muscle progenitors derive from somites, muscle cell fusion in the limbs arise faster than in the trunk (Sieiro-Mosti et al., 2014). The process of muscle cell fusion is associated to successive cellular mechanisms: migration/recognition between the cells, cellular adhesion, lipid bilayer destabilization of cellular membranes, fusion pore formation and finally cell fusion. Most of the data obtained on the mechanism of muscle cell fusion come from studies performed on muscle cells in vitro (Abmayr and Pavlath, 2012; Pavlath, 2010). However, more recently, molecular actors in muscle fusion processes have been identified in vivo in the drosophila model. In drosophila, two different populations of muscle cell progenitors can be distinguished: the muscle founder cells (FCs), and the fusion competent myoblasts (FCMs). FCs attract FCMs and both cell types fuse together to form multinucleated cells. The first event for fusion mechanism is the recognition of the two partner cells and the adhesion of membranes that ultimately trigger

fusion. In *Drosophila*, different immunoglobulin-domain-containing cell adhesion molecules (CAMs) have been identified as mediating recognition and adhesion steps between FCs and FCMs (Kim et al., 2015). The different CAMs shown to be involved in muscle cell fusion in *Drosophila* are conserved in mammals (Figure 8). During embryonic development, loss-of-function of Nephrin, the vertebrate homolog of the *Sns* *Drosophila* CAM, leads to incompletely fused myoblasts in both zebrafish and mouse (Sohn et al., 2009). *In vitro*, primary myoblasts from Nephrin mutant mice show a severe decrease in their ability to fuse (Sohn et al., 2009). In zebrafish, *Jamb* and *Jamc*, two Ig domain-containing transmembrane proteins interacting together, have been identified to be expressed in the myotome (Powell and Wright, 2011). *Jamb* or *Jamc* loss-of-function leads to a defect in myoblast fusion characterized by the abnormal high number of mononucleated cells (Powell and Wright, 2011). *Kirrel*, the zebrafish homolog of the *Drosophila* CAM *Duf* has also been identified to control fusion ability in muscle cells (Srinivas et al., 2007).

Recently, a new membrane-associated factor has been characterized for its contribution to muscle cell fusion: *Myomaker* (*Tmem8c*), (Millay et al., 2013), (Figure 8). *Myomaker* is the

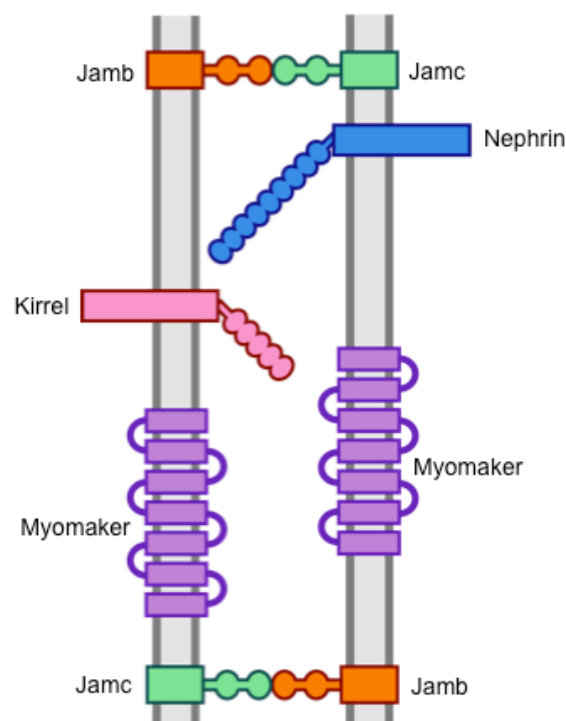


Figure 8: Different fusion partners contribute to muscle fusion steps. Myogenic differentiation relies in part on fusion events occurring between muscle cells. Such events require the expression of specific fusion partners in muscle cells. Some of them are described on the scheme. (Adapted from Kim et al., 2015)

first molecule involved in the process of muscle fusion that is specifically expressed in muscle cells during development (Millay et al., 2013). Mutant mice for Myomaker display a strong defect in myoblast fusion. Indeed, skeletal muscles deficient for Myomaker contain only mononucleated cells. Furthermore, the expression of Myomaker in fibroblasts (fusion incompetent cell) induces fusion between fibroblasts and myoblasts (Millay et al. 2013; 2016).

Finally, the extracellular matrix receptor $\beta 1$ integrin also regulates myoblast fusion. Loss of $\beta 1$ integrins in mouse embryos leads to shortened muscle fibres, with an accumulation of mononucleated cells (Schwander et al., 2003). Similarly, in vitro, $\beta 1$ integrin-deficient muscle cells fail to fuse (Schwander et al., 2003). The integrin-associated tetraspanin CD9 interacts with $\beta 1$ integrin to regulate muscle fusion and in $\beta 1$ integrin-deficient myoblasts, CD9 is no longer expressed at the cell surface (Schwander et al., 2003). Consistent with these results, it has been shown that CD9 tetraspanin has a promoting effect on muscle cell fusion (Tachibana and Hemler, 1999).

E. Limb muscle development

The different limb muscles all originate from somites localized at the axial levels of limb buds. In the chick embryo, somites 16 to 21 and 26 to 33 give rise to forelimb and hindlimb muscles, respectively (Chevallier et al. 1977; Christ et al. 1977; Zhi et al. 1996; Lance-Jones 1988). Only the hypaxial compartment of the dermomyotome contributes to limb muscle formation. Muscle progenitors first delaminate from the epithelium of the hypaxial dermomyotome, and then migrate toward the limb bud. Once they reached their final position, muscle progenitors enter the myogenic program and start to proliferate. At this time, muscle cells aggregate into two distinct ventral and dorsal muscles masses (Chevallier et al., 1976; Schramm and Solursh, 1990). Finally, muscle masses undergo a splitting process to form the individual limb muscles (Tozer et al., 2007). In chick embryo, twelve and fifteen main muscles can be distinguished in forelimbs and hindlimbs, respectively (Murray and Wilson, 1997; Robson et al., 1994; Zhi et al., 1996), (Figure 9).

Delamination and migration

In contrast to the trunk muscles, muscle cells originating from the hypaxial region of the dermomyotome require migration ability to reach their final destination. The transcription factor PAX3 has an important role in the establishment of limb muscles. PAX3 is highly expressed in the hypaxial lip of the dermomyotome at the limb levels. Furthermore, deficient

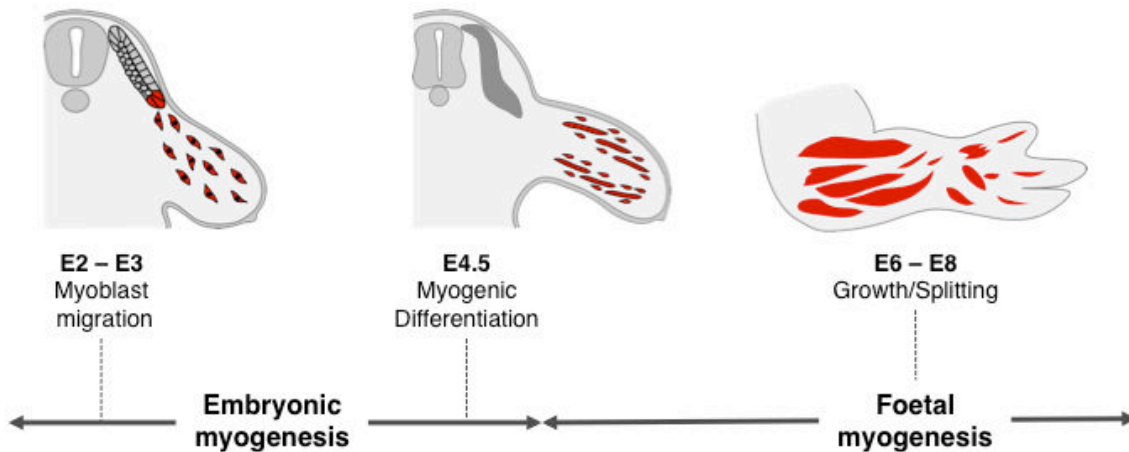


Figure 9: Limb myogenesis during chick embryonic development. In chick embryos, limb myogenesis start at E2. At this stage, muscle progenitors delaminate from the hypaxial lip of the dermomyotome and migrate toward the limb bud. From E3, muscle progenitors undergo proliferation and aggregate to form the dorsal and ventral muscle masses. At this stage, activation of the MRFs is achieved. At E4.5, the first differentiation events occur. Finally, from E6 to E8, ventral and dorsal muscles masses split to form the different limb muscles.

mice for PAX3 presents a loss of all limb muscles, demonstrating that cell delamination from the hypaxial lip of the dermomyotome is impaired (Tajbakhsh et al., 1997). Similarly to PAX3 mutant, mutant mouse for the c-met tyrosine kinase gene or its ligand, the Hepatocyte Growth Factor (HGF) also named Scatter Factor (SF), leads to a complete absence of limb muscles (Bladt et al., 1995; Dietrich et al., 1999). PAX3 expression induces c-met expression in muscle cell progenitors. In turn, c-met expression regulates delamination of PAX3-positive cells from the hypaxial dermomyotome that undergo an epithelial to mesenchymal transition and migrate into the limb mesenchyme (Buckingham et al., 2003).

Oriented migration of muscle progenitors expressing c-met depends on the interaction between the tyrosine kinase receptor and its ligand HGF/SF (Bladt et al., 1995). HGF/SF is expressed in the limb mesenchyme and provides a signal highlighting the migratory route for myogenic progenitors into the limb bud. LBX1 that belongs to the homeobox gene family related to drosophila Lady Bird genes, is also detected in the hypaxial lip of the dermomyotome at the limb level and in migrating muscle precursor cells (Brohmann et al., 2000; Jagla et al., 1995). Mutant mouse for LBX1 presents a defect in muscle precursor cell migration. Although muscle progenitors delaminate normally, they do not migrate correctly, leading to a complete lack of dorsal muscles and reduced ventral muscles in the forelimbs (Brohmann et al., 2000). These data show that in the absence of LBX1, muscle progenitor cell migration is less efficient, demonstrating that LBX1 is essential for muscle precursors to

correctly integrate the different guidance cues and maintain their migratory potential. Expression profiling analysis in LBX1-positive muscle progenitor cells evidenced CXCR4 as a novel actor regulating muscle cell migration (Vasyutina et al., 2005). CXCR4 is expressed in limb migrating muscle cells, while its ligand CXCL12 is expressed in limb mesenchyme. Deficient mice for CXCR4 present a reduced number of muscle progenitors reaching the distal part of the limbs (Vasyutina et al., 2005). In addition, an ectopic source of CXCL12 attracts migrating CXCR4-positive muscle progenitors, and thus impairs their correct migration route (Vasyutina et al., 2005), demonstrating that CXCR4/CXCL12 axis contributes to the regulation of muscle cell migration during limb muscle formation. In addition to attractive guidance mechanisms, it has also been shown that repulsive guidance processes occur during muscle cell migration. During migration of limb muscle precursors, the tyrosine kinase receptor EphA4 is expressed in muscle progenitors, while its ligand ephrinA5 is expressed limb mesenchyme (Swartz, 2001). In chick embryos, gain-and-loss of function experiments of EphA4/ephrinA5 signalling demonstrate that ephrinA5 acts as a repulsive signal on EphA4-positive muscle progenitors, thus preventing their aberrant migration in inappropriate locations (Swartz, 2001). Taken together, these results show that the combination of different signals, mainly emanating from the limb mesenchyme, allows the guidance of migrating muscle progenitor cells to their correct position in limbs (Figure 10).

Dorsal and ventral muscle masse formation

In migrating muscle precursor cells, the myogenic program is not active. The first activation of MRFs takes place after muscle cells have reached their final destination (Figure 10). They start to express first Myf5 and then MyoD (Delfini and Duprez, 2000; Ontell et al., 1995). In the limb the homeobox transcription factor Mox2 is involved in the activation of Myf5. Indeed, in Mox2 deficient mice, Myf5 expression is downregulated, while MyoD is still present (Mankoo et al., 1999). Furthermore, expression of MRFs in limbs is associated with a decrease in PAX3 expression (Mankoo et al., 1999), as well as that of the Msx1 homeobox factor. Msx1 expression is observed in migrating muscle cells and has been shown to prevent muscle differentiation through maintenance of migrating progenitor cells in a proliferative state (Houzelstein, 1999; Odelberg et al., 2000). Similarly, the bHLH transcription factor Sim2 prevents limb myogenic differentiation by repressing MyoD through direct binding on MyoD promoter (Havis et al., 2012). Finally, proliferative limb muscle precursors activating the myogenic program undergo differentiation into myotubes to progressively form dorsal and ventral limb muscle masses. In the chick embryo, dorsal and ventral muscle masses are

formed at E3. Shh, which is expressed in the zone of polarizing activity (ZPA) of the developing limb bud, also regulates the correct development of dorsal and ventral muscle masses as an ectopic expression of Shh induces the transformation of anterior muscles into muscles with a posterior identity (Duprez et al., 1999).

Muscle masses splitting

Ultimately, the dorsal and ventral muscle masses undergo splitting. They progressively cleave into different muscle masses to finally form all individual limb muscles (Figure 10). The process of muscle splitting arises from E6 to E8 in the chick embryo (Robson et al., 1994). The limb vasculature plays an important role in this process (Tozer et al. 2007). Blocking blood vessel formation leads to a defect in muscle splitting. Instead of being splitted, muscles are fused in domains where blood vessels did not develop. Conversely, ectopic formation of blood vessels induces ectopic muscle cleavage or inhibits muscle formation (Tozer et al. 2007). The limb vasculature actually delineates the cleavage pattern of muscles (Figure 10). The role of the innervation during limb muscle splitting has also been assessed (Edom-Vovard et al., 2002; Rong et al., 1992; Shellswell, 1977), but neural tube ablation in the chick embryo has been shown to give rise to normal limb muscle-patterning (Edom-Vovard et al., 2002; Rong et al., 1992), indicating that innervation is not implicated in limb muscle splitting. However, nerves are important for further maintenance and development of muscles.

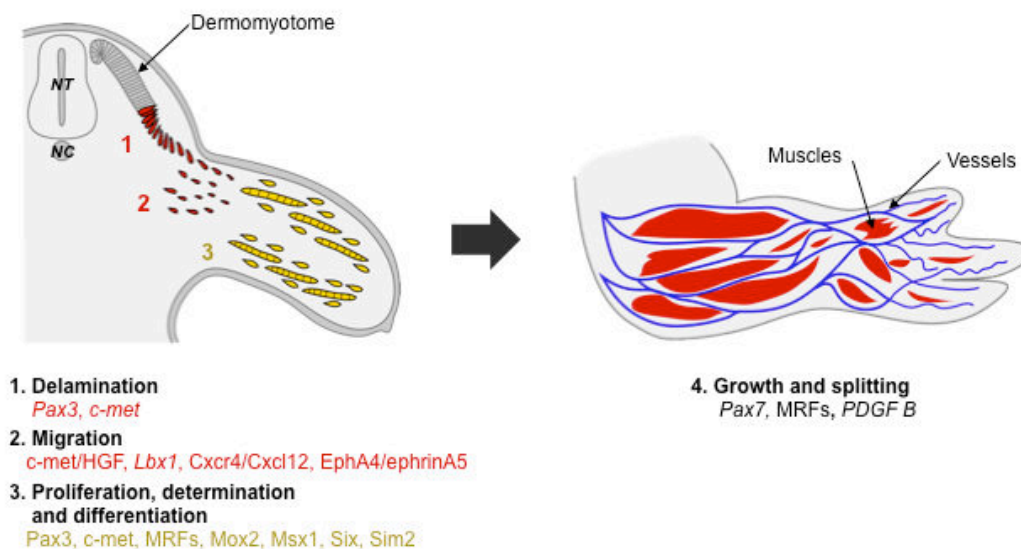


Figure 10: The different mechanisms controlling limb muscle formation. Limb muscle formation is mediated through successive steps that require specific orchestration of many different actors. Schematic representation of the different players intervening during the distinct phases of limb muscle development. NT, neural tube; NC, notochord. (Adapted from Buckingham et al., 2003)

II. Connective tissue development

In the body, connective tissue (CT) function is to support and connect organs together. CT is composed of fibroblasts and extracellular matrix, forming an amorphous gel-like and matrix fibers. The amorphous gel-like contains mainly glycoproteins and proteoglycans, while the fibrous network is made of collagen and elastic fibers (Omelyanenko and Slutsky, 2013). Two main types of supportive CTs can be distinguished: the specialized CT and the dense CT. The specialized CT comprises bones and cartilage elements. The dense CT can be divided into two subtypes: the dense regular CT and the dense irregular CT, which refer respectively to tendon/ligament structures and to CT embedding organs (Table 1). The different CTs mainly differ by the composition and organization of the extracellular matrix. In dense regular CT, collagen produced by fibroblasts is organized in fibers with the same orientation, while in the dense irregular CT, fibroblasts produce collagen fibers without specific organization.

During embryonic development, the different forms of CT (bones, cartilage, tendons, ligaments and irregular CT) differentiate from mesenchymal cells derived from mesodermal and mesectodermal (neural crest cells) origins (Wachtler et al., 1981). Neural crest cells give rise to head CTs, while CTs of the body originate from paraxial or lateral plate mesoderm (Figure 11). Specific key transcription factors or signalling molecules control the specification and differentiation processes of the different types of CTs, irrespective to their embryological origins (Figure 12).

Connective Tissues			
Proper			Specialized
Soft	Dense		<p>Bone</p> <p>Cartilage</p>
<p>Adipose tissue <i>Brown, white, beige</i></p> <p>Areolar tissue <i>Scutaneous, around vessels & nerves</i></p> <p>Reticular tissue <i>into liver, pancreas, spleen, nodes, bone marrow</i></p>	Regular	Irregular	
	<p>Tendon <i>Direct tendons, wrap-around tendons</i></p> <p>Ligament <i>Intra/extra articular, synovial joints</i></p>	<p>Capsules of organs <i>(Periosteum, epimysium)</i></p> <p>Muscle connective tissue <i>(Endomysium, perimysium)</i></p> <p>Dermis</p>	

Table 1: Classification of the different type of connective tissues

A. Specialized connective tissue (bone and cartilage)

The embryonic origins of cartilage and bone are multiple. Indeed, three distinct embryonic structures, somites, neural crest cells and lateral plate mesoderm are at the origin of the elements of the trunk, head and limb skeleton (Christ and Wilting, 1992; Noden and Trainor, 2005; Wachtler et al., 1981) (Figure 11). The development of cartilage and bone elements, which form the skeleton, starts by the condensation of mesenchymal cells at the future sites of bones. Following condensation, mesenchymal precursors differentiate in chondrocyte or osteoblast, forming respectively cartilage or bone. The process of ossification is named osteogenesis and occurs through two different mechanisms. Intramembranous ossification corresponds to a direct transition from condensed mesenchymal cells into osteoblasts (as described above) while the second step of bone formation is called endochondral ossification, characterized by the replacement of cartilage by bone. In this case, chondrogenesis is the first step of a mechanism that ultimately gives rise to bones. Intramembranous ossification occurs in bones of the skull, while endochondral ossification forms the other bones (reviewed in Karsenty & Wagner, 2002).

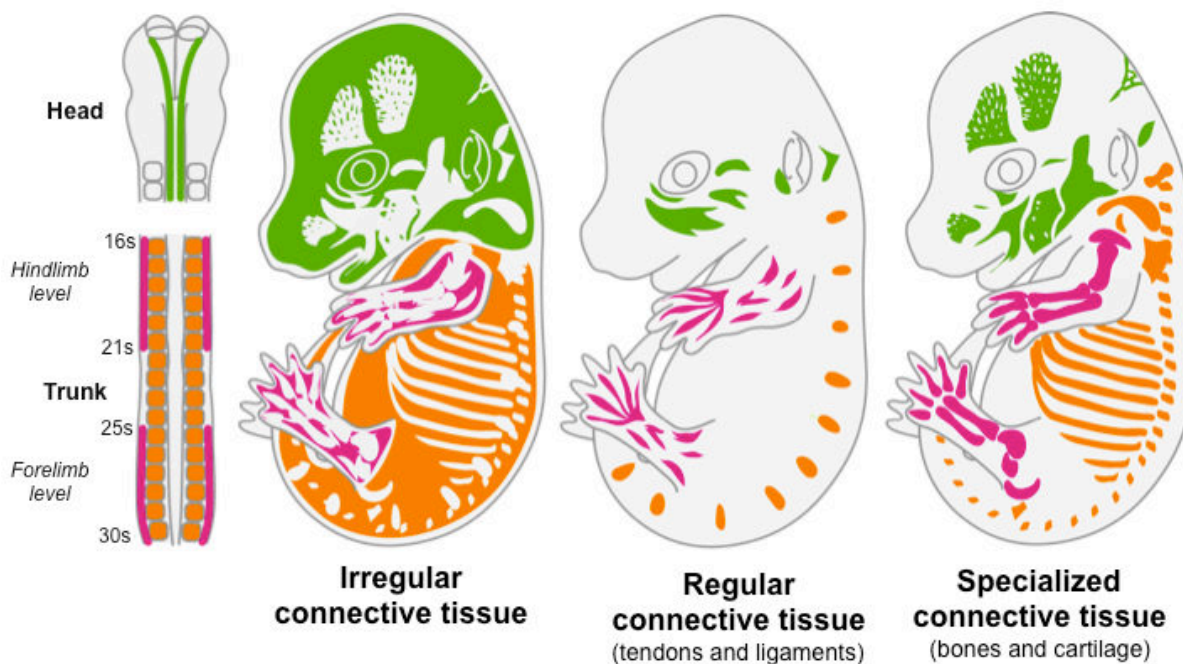


Figure 11: The embryonic origins of connective tissues. Connective tissues (CTs) have distinct embryonic origins depending of the anatomical regions. CTs from the head arise from the neural crest cells (green), while CTs in the trunk derive from the somites (orange) and limb CTs originate from the lateral plate mesoderm (purple). (Adapted from Nassari et al., 2017)

Molecular mechanisms leading to cartilage and bone specification are well understood

(Figure 12). Cartilage specification has been shown to involve the members of the SOX (SRY-related HMG-box) transcription factor family (Lefebvre et al., 1998). During mouse embryonic development, *Sox9* exhibits a similar expression pattern to *Col2a1*, the main collagen forming the cartilage extracellular matrix (Zhao et al., 1997). In mouse mutant embryos for *Sox9*, cartilage elements are missing, demonstrating the major role for *Sox9* in mesenchymal cell condensation and differentiation towards a cartilage fate (Akiyama et al. 2002; Bi et al. 1999). It has also been shown that *Sox9* is required for the expression of two other *Sox* genes, *Sox5* and *Sox6* that are co-expressed with *Sox9* in chondrocytes (committed cartilage cells), (Lefebvre et al., 1998). Both *Sox5* and *Sox6* mutant mice show skeletal abnormalities, but *Sox9* expression remains unchanged, showing that *Sox9* acts upstream of *Sox5* and *Sox6* (Smits et al., 2001). *Runx2* (Runt-related transcription factor 2) is specific to bone progenitor cell lineage and appears as an important actor for osteogenesis as osteogenesis does not occur in mutant mice for *Runx2* (Ducy et al., 1999; Komori et al., 1997). Cartilage elements are present in *Runx2*^{-/-} mouse, but all bones are missing, demonstrating the major role of *Runx2* in bone specification (Komori et al., 1997). Conversely to *Sox9*, which is required for cartilage specification and differentiation (Akiyama et al. 2002), *Runx2* is not required for bone differentiation (Takarada et al., 2013). *Runx2* expression has to be downregulated after osteogenic cell commitment to allow committed bone cells to mature and differentiate (Adhami et al., 2014; Takarada et al., 2013; Yoshida et al., 2004). *Osterix* (*Osx*) is also a key transcription factor in the formation of bones (Nakashima et al., 2002). It is specifically expressed in all bones (Nakashima et al., 2002) and required for the differentiation of bone progenitors. No bone is observed in mutant mouse for *Osx*, however *Runx2* expression is maintained (Nakashima et al., 2002). Conversely, *Osx* is not expressed in *Runx2* mutant mice (Nakashima et al., 2002), indicating that *Runx2* and *Osx* are involved in bone specification and differentiation, respectively.

Beside the specific transcription factors required for skeletal development, signalling pathways have also been demonstrated to be involved in this process. Wnt signalling controls skeleton differentiation in a cell-autonomous manner, which favors osteoblast differentiation at the expense of chondrocytes (Day et al., 2005; Hill et al., 2005). Conditional inactivation of β -catenin in mesenchyme leads to the formation of ectopic chondrocytes while osteoblast differentiation is blocked (Day et al., 2005). In vitro, the expression of *Sox9* and *Runx2* is controlled by β -catenin (Day et al., 2005). *FgfR3* and *FgfR1* inactivation in mouse leads to achondroplasia and hypochondroplasia, demonstrating the role of FGF signalling in skeletal

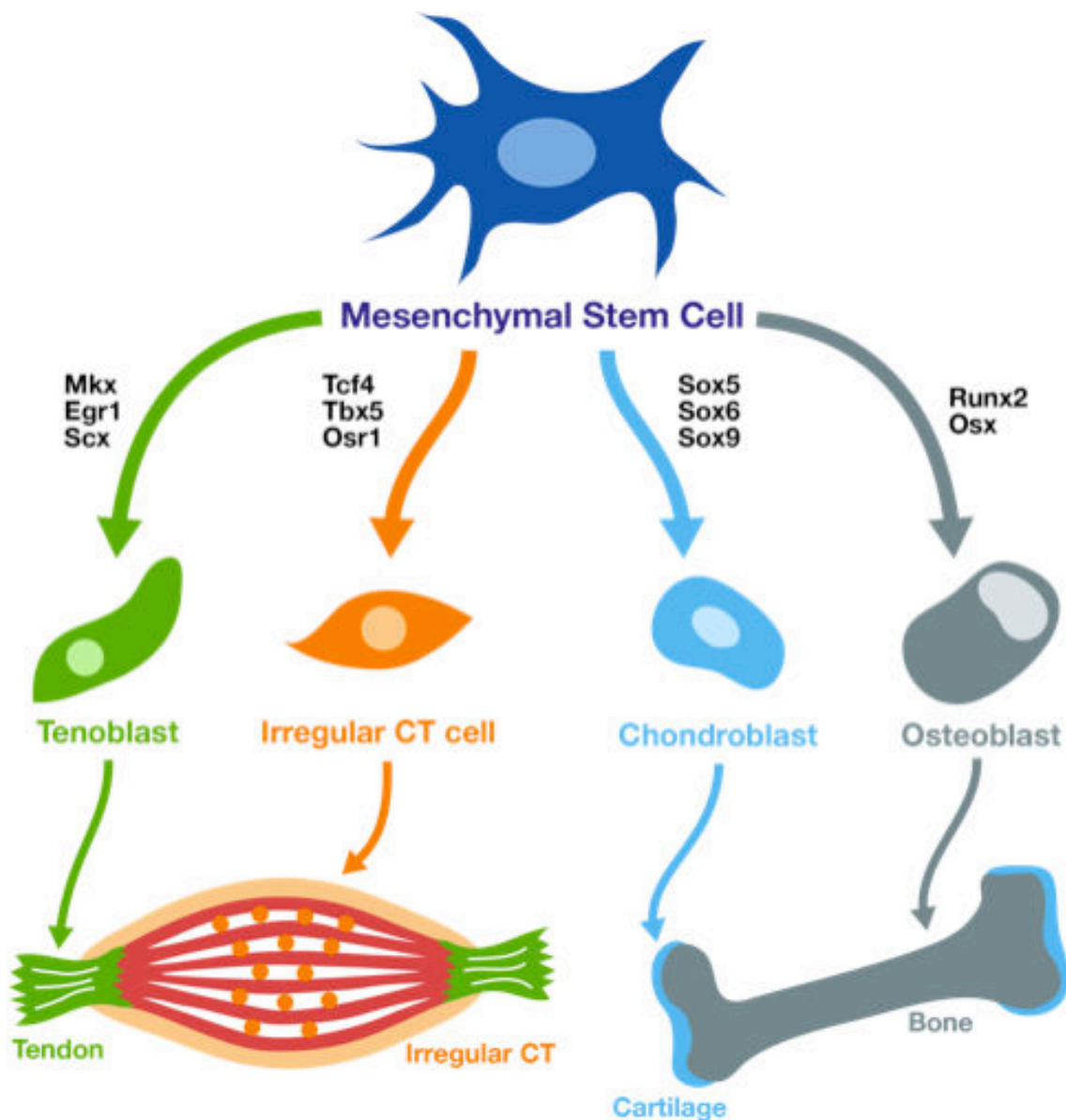


Figure 12: The key regulators driving connective tissue specification. Upon the expression of specific transcription factors, a mesenchymal cell is able to specify and give rise to the different types of connective tissues (CTs) including bone, cartilage, tendon and irregular CT. The activation of *Sox5/6/9*, *Runx2/Osx*, *Scx/Mxk/Egr1* and *Tcf4/Tbx5/Osr1* genes promotes cartilage, bone, tendon and irregular CT, respectively. (Adapted from Nassari et al., 2017)

development (Deng et al., 1996; Jacob et al., 2006). Both mutant mice exhibit an expansion of the hypertrophic chondrocyte zone, suggesting that FGF signalling is a negative regulator of chondrocyte proliferation (Deng et al., 1996; Jacob et al., 2006). Inactivation of *Ihh* (Indian Hedgehog), one member of the Hedgehog family decreases chondrocyte proliferation, leading to a defect in osteoblast formation (Vortkamp et al. 1996). *Ihh* interacts with the Parathyroid hormone, which controls the balance between cell proliferation and differentiation

(Kronenberg, 2006). In addition, RUNX2 induces *Ihh* expression which in turn inhibits *Runx2* expression by a feedback loop mechanism (Yoshida et al., 2004). Finally, BMPs (Bone Morphogenetic Proteins) regulate *IHH* expression in chick embryos (Zhang et al., 2003) and have been shown to regulate chondrocyte differentiation (Kobayashi et al. 2005; reviewed in Li & X. Cao 2006).

B. Dense regular connective tissue (tendon)

As specialized CTs (bone and cartilage), tendons emerge from distinct embryological origins depending on their location in the body (Figure 11). Tendons of the trunk originate from the somites, from a subregion of the sclerotome named the syndetome (Brent et al., 2003), while tendons of the craniofacial region derive from neural crest cells (Crane & Trainor 2006; Grenier et al. 2009) and limb tendons are formed from the lateral plate mesoderm (Kieny and Chevallier, 1979). Tendons attach muscles to bones by connecting muscle at the myotendinous junction and bone at the enthesis, while ligaments connect bone to bone. The role of tendons is to transmit forces generated by muscle contractions to bones, in order to allow joint movements and maintain articular stability. The extracellular matrix of the tendon is characterized by type I collagen fibers, organized in parallel to the tendon axis. This organization allows the mechanical properties of the tendons (Benjamin and Ralphs, 2000). Ligaments are essential components of the skeletal joints. Ligament elasticity defines the range of motion of the joints, supports joint stability and protects joints and bones by their stretching capacities. Collagen organization is similar in tendons and ligaments (Benjamin and Ralphs, 2000) but it has been shown that both cell types in adult differ in the level of expression of individual genes (Richard et al., 2009). However, development mechanisms have been more studied in tendon than in ligament (Tozer and Duprez, 2005). It is noteworthy that tendon and ligament exhibit the same gene expression profile during embryogenesis (Richard et al., 2009).

Conversely to cartilage and bone, the master gene(s) involved in tendon specification during development is(are) still unknown. To date, the unique early tendon marker known in vertebrates is *Scx* (Scleraxis). *Scx* is specifically expressed in tendon progenitors and differentiated cells (Schweitzer et al. 2001). Mutant mice for *Scx* exhibit tendon defects, leading to a severe impairment of limb and tail force-transmitting tendons, while anchoring tendons are less affected (Murchison et al., 2007). However, tendon progenitors are still present in *Scx* mutant, indicating that *Scx* is not the only gene implicated in tendon formation during development. Two additional transcription factors are involved in tendon formation,

the homeobox transcription factor *Mkx* (Mohawk), (Ito et al. 2010; Liu et al. 2010) and the zinc finger transcription factor *Egr1* (Early Growth Response 1), (Lejard et al., 2011), (Figure 12). Both *Mkx* and *Egr1* mutant mice exhibit tendon defects associated with a decrease in *Colla1* expression and in the number of type I collagen fibers in tendons (Ito et al. 2010; Liu et al. 2010; Lejard et al. 2011; Guerquin et al. 2013). However, both *Mkx* and *Egr1* are expressed later than *Scx* during development and are also expressed in many other lineages (Anderson et al., 2006; Rackley et al., 1995).

TGF β (Transforming growth factor) and FGF (Fibroblast growth factor) signalling pathways have been shown to regulate tendon specification and differentiation at various body locations (recently reviewed in Gaut & Duprez, 2016). As mentioned above, axial tendon progenitors arise from the syndetome, a subcompartment of the sclerotome. The syndetome is localized at the interface between the sclerotome and the myotome and formed by *Scx*-expressing cells. In chick embryos, dermomyotome removal leads to the absence of tendons, showing that axial tendons do not develop in the absence of axial muscles (Brent et al., 2003). *SCX* expression at the axial level is induced in response to FGF signalling coming from the myotome, which concomitantly downregulates *PAX1* expression in the sclerotome (Brent et al., 2003). In contrast to axial tendons, head and limb tendons form independently from muscle. *Scxa/SCX/Scx* expression is induced in limb and head of zebrafish, chick and mouse embryos in the absence of limb or craniofacial muscles (Schweitzer et al. 2001; Edom-Vovard et al. 2002; Grenier et al. 2009; Chen & Galloway 2014). Ectoderm removal in chick limbs results in the absence of *SCX* expression, demonstrating that *SCX* induction is mediated via ectodermal signals (Schweitzer et al. 2001). In the limb mesenchyme, BMP signalling represses *SCX* expression and overexpression of the BMP antagonist NOGGIN induces ectopic *SCX* expression. These results suggest that tendon specification in chick limbs results from a balance between an unidentified factor coming from the ectoderm and BMP signalling from the mesenchyme (Schweitzer et al. 2001). In addition, TGF β signalling is required and sufficient for *Scx/SCX* expression in chick and mouse embryos (Pryce et al. 2009; Havis et al. 2014; 2016), while FGF signalling is required and sufficient for *SCX* expression in chick limb cells but not in mouse limb cells (Pryce et al. 2009; Havis et al. 2014; 2016).

Although muscles are dispensable for specification of head and limb tendons, they are required for *Scxa/SCX/Scx* late expression in tendons and for full tendon differentiation. In the absence of muscles, tendons degenerate in chick, mouse and zebrafish embryos (Kardon 1998; Edom-Vovard et al. 2002; Grenier et al. 2009; Chen & Galloway 2014). Moreover, ectopic expression of tendon-associated genes in chick limbs is observed after overexpression

of FGF4, which is normally expressed at the tips of muscle fibers (Edom-Vovard et al., 2002; Eloy-Trinquet et al., 2009). Finally, chick embryo paralysis decreases *SCX* expression in limb tendons, an effect that can be prevented by application of FGF4 or TGF β ligands, demonstrating that FGF and TGF β signalling pathways act downstream of mechanical forces to regulate tendon differentiation (Havis et al. 2016) .

C. Dense irregular connective tissue

The irregular CT forms the scaffold of organs, with scattered cells embedded in high extracellular matrix content. It is present all around and inside organs. First studies on the differentiation of irregular CT have focused on the extracellular matrix composition. During development, Type I and type III collagen are both expressed in dense regular and irregular CTs, however type I collagen tends to replace type III collagen in adult tendons, while both type III and type VI collagen are expressed in mature irregular CT (Gara et al., 2011; Kieny and Mauger, 1984; Stricker et al., 2012; Zhang et al., 2005). The mechanisms driving irregular CT specification have been poorly investigated for a long time due to the lack of specific early molecular markers. More recently, the identification of transcription factors expressed in irregular CT has allowed to progress in the understanding of irregular CT formation and function (Figure 12).

TCF4, belonging to the TCF/LEF family, is the first transcription factor that has been identified in irregular CT fibroblasts. In limbs of both mouse and chick embryos, *Tcf4*-expressing cells discriminate the lateral plate-derived mesodermal population from myogenic cells (Kardon et al. 2003; Mathew et al. 2011). During muscle differentiation in chick limbs, *TCF4* expression is progressively restricted to muscle CT (Kardon et al., 2003) and colocalizes with type I collagen. This expression is maintained in the adult (Mathew et al. 2011). *TCF4* expression is repressed by BMP signalling (Bonafede et al., 2006) and activated by Wnt pathway (Kardon et al., 2003) in developing chick limbs. *TCF4* is also expressed in avian jaw muscle CT and its expression has been shown to be controlled by the mesenchymal neural crest (Lin et al. 2014).

Fibroblasts constituting the irregular CT also express the T-box transcription factor *Tbx5*. At early stage of limb development in mouse (E11.5), *Tbx5* is broadly expressed in lateral plate mesodermal cells in domains overlapping with bone, tendon and muscle progenitors (Hasson et al. 2007). Mutant mice for *Tbx5* leads to the disorganization of muscle CT during embryonic development (Hasson et al., 2010), a phenotype that could be related to alterations of muscle CT markers, as *Tcf4* and *Osr* genes (see below), (Hasson et al., 2010). *Tbx5*

increases the expression of N-cadherin and β -Catenin in muscle CT. It seems noteworthy that *Tbx5* mainly affects cell adhesion mechanisms independently of *Tcf4*, as the expression of Wnt signalling targets are not modified in *Tbx5* mutant.

Two orthologs of the Odd-Skipped genes, *Osr1* and *Osr2*, have been described in the irregular CT in chick and mouse embryos (Stricker et al. 2006; 2012). Both genes are expressed in different organs such as kidney, eye, branchial arches and dermis (Lan et al., 2001; So and Danielian, 1999; Stricker et al., 2006). In the developing limb of mouse and chick embryos, *Osr1* is expressed in irregular CT, exhibiting a partial overlap with *Tcf4* expression (Stricker et al., 2006). *Osr2* is also widely expressed in irregular CT, but with a stronger expression in muscle CT (Stricker et al. 2006; 2012). Both genes are also observed in the mesenchyme of branchial arches in chick (Stricker et al., 2006) and mouse (Liu et al., 2013) embryos. Overexpression of OSR1 or OSR2 in chick mesenchymal limb cells induces the expression of irregular CT markers such as *COL3A1* and *COL6A1* and down-regulates the expression of cartilage (specialized CT) and tendon (dense regular CT) markers, (Stricker et al., 2012). Conversely, *OSR1* or *OSR2* inactivation down-regulates *COL3A1* and *COL6A1* expression, while increasing cartilage formation in chick limb mesenchymal cells (Stricker et al., 2012). Similarly, conditional inactivation of *Osr1* in cranial neural crest cells results in the formation of an ectopic cartilage in the developing mouse tongue (Liu et al., 2013). OSR1 has been shown to bind *Sox9* promoter and repress *Sox9* expression, demonstrating that OSR1 inhibits chondrogenesis in the mammalian tongue through *Sox9* repression (Liu et al., 2013).

D. Fibrosis: dense irregular CT formation out of control

Proper formation of the irregular CT is a crucial step in development that is characterized by an activated state of fibroblastic cells. The term “fibroblast” corresponds to a population of cells capable to secrete non-rigid collagenous extracellular matrix. During embryonic development, activated fibroblasts, also named mesenchymal or stromal cells, greatly contribute to tissue/organ remodelling. Their activated state is characterized by their ability to proliferate and migrate, secrete growth factors and produce extracellular matrix (Table 2). Conversely, inactivated fibroblasts correspond to quiescent/resting fibroblasts (Table 2). In

Inactive Fibroblasts	Active Fibroblasts
G0/G1 arrest Non migratory No ECM production α -SMA ⁻	Proliferative Migratory ECM production α -SMA ⁺

Table 2: Specific features characterizing inactive and active fibroblasts

the embryo, only few inactivated fibroblasts are present and it has been proposed that they constitute the pool of adult tissue resident mesenchymal stem cells (Yusuf et al., 2013).

In the adult, resident fibroblasts are present in their inactivated form. Following acute or chronic stimuli such as infection, allergic/autoimmune reaction or mechanical injury, fibroblasts are activated and participate to different fibrotic responses that can be beneficial or pathological. During the wound healing process that takes place after mechanical injury, activated fibroblasts contribute to the maintenance of the structural integrity of the irregular CT, as well as the replacement of certain damaged cells (Kendall and Feghali-Bostwick, 2014; Wynn, 2007). Indeed, it has been demonstrated that activated fibroblasts are able to give rise to other mesenchymal lineages (chondrogenic, adipogenic, osteogenic, myogenic and endothelial cells), (Kalluri, 2016) (Figure 13). Under stimuli, activated fibroblasts move to the wound region and undergo massive proliferation associated with myofibroblastic

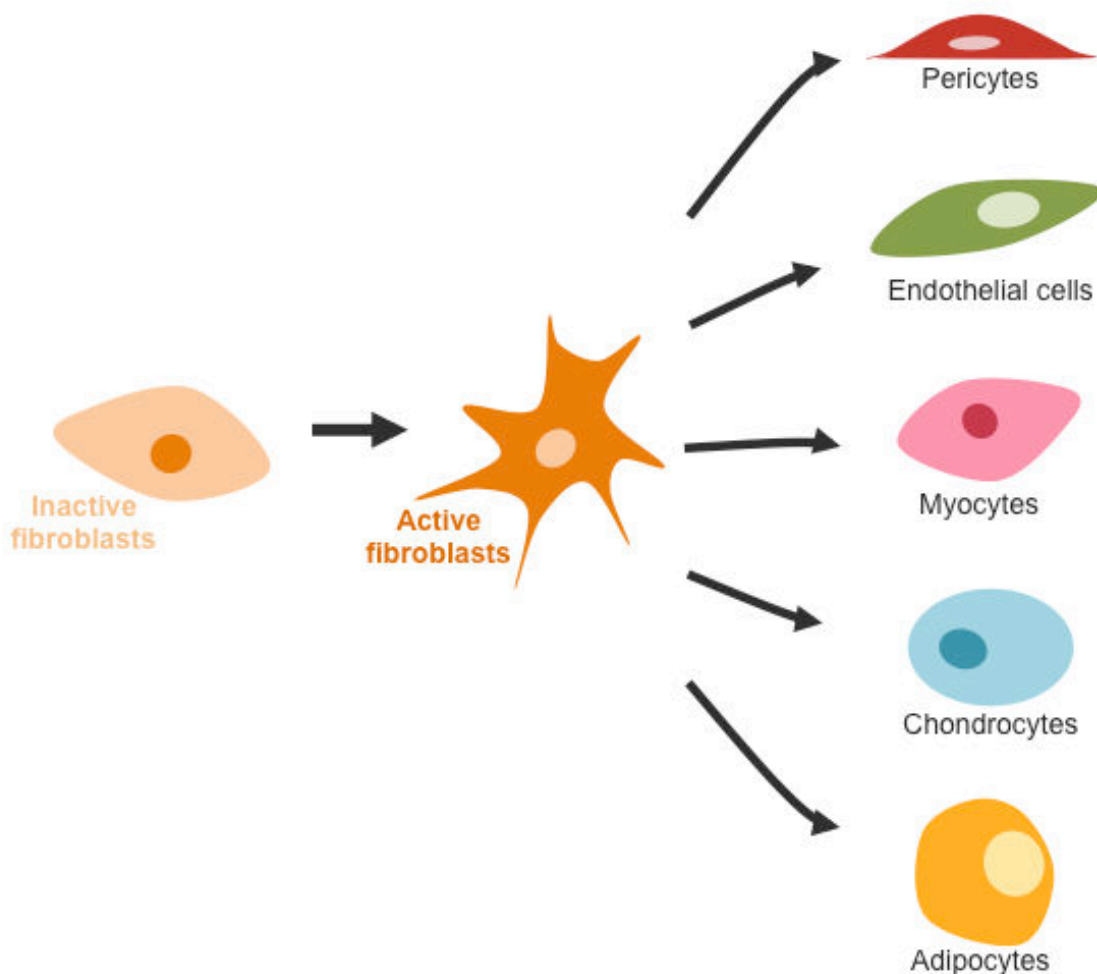


Figure 13: The plasticity of activated fibroblasts. Active fibroblasts are able to give rise to different mesodermal-derived cell types including pericytes, endothelial cells, myoblasts, chondrocytes and adipocytes.

differentiation to contribute to the wound healing response (Bochaton-Piallat et al., 2016). The main characteristic of myofibroblast differentiation is the acquisition of contractile properties, partly mediated through the de novo expression of the *alpha-smooth muscle actin* (α SMA) gene (Bochaton-Piallat et al., 2016; Strutz and Zeisberg, 2006). Although most myofibroblasts originate from fibroblasts, other cell types can also contribute to the formation of the myofibroblast pool, among which endothelial and epithelial cells, pericytes and fibrocytes, a specialized inflammatory cell type derived from the bone marrow (Kendall and Feghali-Bostwick, 2014) (Figure 14). At the end of the scarring mechanism, myofibroblasts undergo cell death but misregulation of their survival can result in a dramatic excess of collagen deposition, associated with a loss of collagen degradation, thus leading to the development of a hypertrophic scar. This phenomenon is called fibrosis, and can ultimately lead to organ/tissue dysfunction. Fibrosis is associated with many pathologies, affecting different organs, such as skin (systemic sclerosis), heart (aortic stenosis, hypertension), liver (alcohol-induced and non-alcoholic steatohepatitis) and lung (idiopathic pulmonary fibrosis, cystic fibrosis) (Bonnans et al., 2014; Clarke et al., 2013; Wynn, 2007). In skeletal muscles, fibrosis is associated to muscular dystrophies, corresponding to a heterogenous group of muscle diseases characterized by tissue inflammation and wasting of skeletal muscles. Furthermore, all metastatic cancers are associated with fibrosis. Myofibroblasts are activated through secreted factors released from the tumor, leading to the reorganisation of the stromal environment, which allows the migration of tumor cells (Kalluri, 2016).

Different signals have been shown to play a key role in the development of fibrosis. TGF β is characterized as the most important profibrotic factor (Meng et al., 2016). Activation of TGF β triggers proliferation and activation of fibroblasts (Meng et al., 2016). It has been shown in dermal fibroblasts that TGF β signalling induces the expression of extracellular matrix-related genes *Coll1a1*, *Coll1a2*, *Col3a1*, *Col5a2*, *Col6a1* and *Col6a3* (Verrecchia et al., 2001). Conversely, BMP pathway has an anti-fibrotic effect. BMP treatment inhibits TGF β -induced fibrosis and triggers a decrease in the expression of α SMA (Yang et al., 2011). In addition to classical signalling pathways, mechanical forces are also important regulators of fibrosis. They enhance TGF β activation and myofibroblast differentiation, thus promoting a pro-fibrotic response.

Cytokines have also been characterized to promote fibrosis (Borthwick et al., 2013). For instance, it has been shown that IL-13 is increased in fibrotic intestinal muscle of Crohn's disease patients and mediates inhibition of metalloprotease synthesis, leading to an increase in extracellular matrix deposition that ultimately promotes fibrosis (Bailey et al., 2012). Profibrotic effects of IL-13 have been also shown in pulmonary fibrosis (Murray et al., 2008). Furthermore, it has been demonstrated that IL-13 enhances myofibroblastic differentiation and TGF β signalling (Bonnans et al., 2014). Similarly, the CXCL12 chemokine promotes myofibroblast differentiation in gut fibrosis, demonstrating the fibrotic effect of this chemokine (Gharaee-Kermani et al. 2012; Rodriguez-Nieves et al. 2016; Olsen et al. 2016). However, this mechanism is independent of TGF β signalling (Gharaee-Kermani et al. 2012; Rodriguez-Nieves et al. 2016). CXCL12 positively regulates the expression of *Ctgf*

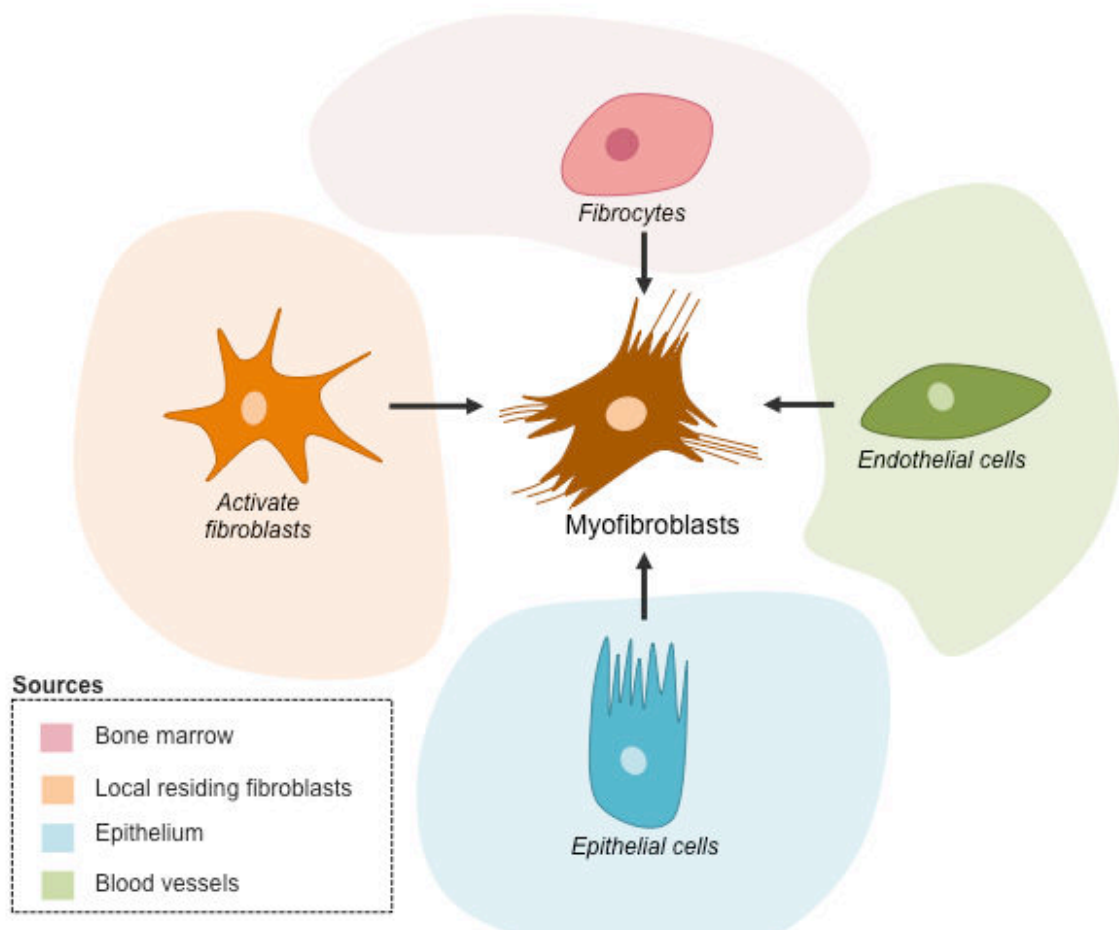


Figure 14: Various cell types contribute to myofibroblast formation. Myofibroblasts are specific cells characterizing fibrotic tissues. They arise from the transdifferentiation of different cell types including active fibroblasts, circulating fibrocytes, epithelial cells or endothelial cells. Active fibroblasts originate from local resident fibroblasts, while fibrocytes arise from the bone marrow, and endothelial and epithelial cells derive from blood vessels and epithelia, respectively.

(Connective tissue growth factor) in lung fibroblasts, a main actor in the fibrosis process (Lin et al. 2014). CXCL12 additionally contributes to the recruitment of bone marrow-derived fibrocytes to the site of inflammation, participating to the formation of the myofibroblast pool. Finally, both *Cxcl12* and *Cxcl14* expression is increased in pulmonary fibroblasts of patients with idiopathic pulmonary fibrosis (Emblom-Callahan et al., 2010; Jia et al., 2017).

III. Connective-tissue-mediated muscle morphogenesis

Classical experiments in avian embryos have demonstrated that muscle differentiation and patterning partly derived from surrounding tissues (Lance-Jones, 1988; Ordahl & Le Douarin, 1992; Kardon, 1998). Over the last years, the identification of specific molecular markers for the different types of CT allowed to progress in the identification of the molecular mechanisms underlying the interactions between CT and muscle development. It has been shown that, depending on their embryological origin and their location in the body, the different types of CT influence spatially and temporally muscle morphogenesis.

A. Specialized-connective-tissue-mediated myogenesis (bone and cartilage)

In the limb, muscles and specialized CTs (bone and cartilage) do not exhibit direct physical interactions, as they are linked together via tendons. During limb development, mechanisms that regulate skeleton and muscle formation can be dissociated (Hasson et al., 2010; Li et al., 2010). Indeed, mutation in the LIM-homeodomain transcription factor *Lmx1b* in skeletal progenitors using the *Sox9-Cre*, which leads to skeletogenesis defects, has no effect on muscle development (Li et al., 2010). Similarly, profound skeletal defects are observed after inactivation of the BMP antagonist, *Noggin*, which is expressed in condensing cartilage and immature chondrocytes, but the early stages of myogenic differentiation are not affected (Tylzanowski et al., 2006). However, skeleton-derived signals are required for proper myogenesis. Indeed, terminal muscle differentiation is impaired in *Noggin* null-mutant mice although no defect at the onset of myogenesis is observed (Tylzanowski et al., 2006; Costamagna et al., 2016). The Indian hedgehog (Ihh) secreted factor, which belongs to the Hedgehog family, is released by developing chondrocytes (Vortkamp et al. 1996) and muscles are affected in the absence of Ihh (Bren-Mattison et al., 2011). As for *Noggin* null-mutant, muscle impairment is observed during late myogenesis, resulting in reduced muscle masses. Finally, in vitro experiments show that C2C12 myoblasts can be converted towards the osteogenic lineage when exposed to BMPs (Precursor et al., 2000).

Axial muscles arise from the myotomal compartment of the somite, which is formed by cell delamination occurring first at the dorsomedial lip of the dermomyotome and later at its caudal and rostral lips. This process is partly controlled by the sclerotome, another somitic compartment. During chick embryonic development, the medial part of the somites contains the pioneer myoblasts, which express the ROBO2 receptor, while its ligand SLIT1 is expressed in the caudal domain of the nascent sclerotome (Halperin-Barlev & Kalcheim,

2011). Loss-of-function of ROBO2 or SLIT1 lead to the disruption of the caudo/rostral migration of pioneer myoblasts and of myofibre formation, showing that myotome morphogenesis is controlled by sclerotome (skeletal precursors)-derived signals (Halperin-Barlev & Kalcheim, 2011). However, since the sclerotome give rise to both skeleton and tendon progenitors (syndetome), these data cannot discriminate between the effects of bone or tendon progenitors on muscle morphogenesis.

Skeletal elements in the head derive from the cranial neural crest cells (Couly et al., 1992). In the *HoxA1/HoxB1* double-mutant mouse, cranial neural crest cells fail to form and migrate into the second branchial arch. In these conditions, cranial myogenesis was initiated although neural crest cells at the origin of skeletal progenitors were missing. However, muscle patterning was affected, as shown by the expansion in *Tcf21* (Capsulin) and *Tbx1* expression. Similarly, in chick embryo, early steps of head myogenesis are not impaired by ablation of cranial neural crest cells at the origin of skeletal progenitors but expression of myogenic genes is expanded to the entire arch mesenchyme, suggesting that the molecular interactions between cranial skeleton and muscles are conserved between chick and mouse embryos (Tzahor et al., 2003; Rinon et al., 2007). Such interactions are mediated at least by BMP and Wnt signalling (Tzahor et al., 2003; Rinon et al., 2007). However, neural crest cells give rise to skeleton, tendon and CT progenitors in the head. Again, as for axial myogenesis, these experiments are not able to discriminate between prospective bone, tendon or muscle CT in the control of cranial myogenesis.

B. Dense regular connective tissue (tendon) as an important source of signals during muscle development

Muscle and dense regular CT (tendon) displays interactions during their development. In chick, mouse and zebrafish embryos, tendon requires muscle to fully develop (reviewed in Gaut & Duprez, 2016). Conversely, the influence of tendon on muscle development is less clear in vertebrates. During limb muscle development, muscle masses differentiate between tendon primordia. In chick embryo, ectopic muscles form in the absence of tendons at the place where the latest normally develop, demonstrating the role of tendon in delimiting regions of muscle growth and differentiation (Kardon 1998). In *Drosophila*, the role of tendon cells on muscle development has been studied more extensively. Tendon precursor cells in *Drosophila* are ectodermal cells, that constitute the apodeme and express the Early growth response (EGR)-like transcription factor *Stripe* (Frommer et al., 1996). The localization of myoblasts is altered when apodeme formation is disrupted during the early steps of leg

development (Soler et al., 2016). A correct migration of myogenic cells toward tendon cells is required for the myotendinous junctions to form. This migration step is mediated through guidance cues delivered by tendon cells. In tendons, *Stripe* activates the expression of the *Slit* gene (Vолоhonsky et al., 2007), coding for a secreted protein involved in axonal guidance. Tendon cells express *Slit*, while its receptor *Robo* (Roundabout) is expressed in muscle (Kramer et al., 2001). *Slit* mutants exhibit defects in muscle patterning (Ordan et al., 2015), showing that proper muscle development requires signals coming from tendon. Tendon and muscle interactions via *Slit/Robo* is necessary for the migration arrest of muscle progenitors in *Drosophila* (Wayburn and Volk, 2009). The formation of the myotendinous junction in *Drosophila* also requires the transmembrane protein KON-TIKI, enriched at the tips of myotubes. This protein directs muscle progenitor migration and the subsequent recognition between muscle and tendon cells (Schnorrer et al., 2007). These results show that signals coming from tendon cells are required for muscle morphogenesis. Such signals remain to be elucidated during development of the vertebrate musculoskeletal system. In zebrafish, *Tsp4b* (thrombospondin-4) appears critical to orchestrate the assembly of tendon extracellular matrix necessary for muscle attachment at the myotendinous junction (Subramanian and Schilling, 2014). The vertebrate orthologs of *Stripe*, *Egr1/2* have been shown to be involved in vertebrate tendon differentiation (Lejard et al., 2011; Guerquin et al., 2013), but no obvious defect in muscle formation is observed in *Egr1* mutant mouse. Inactivation of *Tsp4* in mice shows that thrombospondin-4 controls the formation of extracellular matrix in both tendons and muscles and is necessary for the correct organization of collagen fibrils in tendon (Frolova et al., 2014). However, the absence of *Tps4* also directly affects skeletal muscle structure, by controlling the expression of heparan-sulfate proteoglycans in muscle (Frolova et al., 2014). Finally, some late events of vertebrate muscle morphogenesis also require tendons. Myofibre translocation necessary to give the final position of the flexor digitorum superficialis muscle in the mouse forelimb is largely impaired in *Scx* mutant, showing that tendon is involved in the patterning and final position of embryonic muscles (Huang et al., 2013).

C. Dense irregular connective tissue establish a pre-pattern for muscle differentiation

Most of our knowledge concerning the relationships between irregular CT and muscle during development comes from the limb model. As previously described, the different steps of limb myogenesis are the following. Somitic-PAX3-positive cells migrate towards the limb bud

through limb mesenchyme, proliferate and form dorsal and ventral muscle masses. Muscle differentiation is then initiated, followed by muscle mass growth and splitting (reviewed in Duprez, 2002; Deries & Thorsteinsdóttir, 2016).

Delamination and migration of muscle progenitors

Delamination and migration of muscle progenitor cells from the ventrolateral lip of the dermomyotome are mediated via the tyrosine kinase receptor c-Met and its ligand, the Scatter Factor/Hepatocyte Growth Factor (SF/HGF), (Heymann et al., 1996; Brand-Saber et al., 1996; Dietrich et al., 1999). Cells from the ventrolateral dermomyotome express *c-Met*, while SF/HGF is released by irregular CT progenitors in the limb mesenchyme. In *Hgf* or *c-Met* mutant mice, limb muscles are missing (Bladt et al. 1995). Dermomyotome development proceeds normally and myogenic precursors are specified but they remain aggregated and fail to migrate towards the limb buds (Dietrich et al., 1999). Migration of myogenic progenitors from occipital and cervical somites, giving rise to the tongue, diaphragm and shoulder muscles is also controlled by SF/HGF (Dietrich et al., 1999). SF/HGF is not the only factor expressed in irregular CT and involved in the guidance of muscle progenitors to reach the limb target. The CXCL12 chemokine is expressed in restricted regions of irregular CT in the limb bud and attracts muscle progenitors, which express the CXCR4 chemokine receptor (Vasyutina et al., 2005). Ectopic expression of CXCL12 in limb mesenchyme of chick embryos, or inactivation of *Cxcr4* in mouse embryos both give rise to aberrant migration of muscle progenitors (Vasyutina et al., 2005), demonstrating a chemoattractive role of CXCL12 positive-CT cells for *Cxcr4* expressing muscle precursors. During their migration towards the limb, muscle progenitors also express the *EPHA4* receptor, while its ligand *EPHRINA5* is expressed in limb irregular CT (Swartz, 2001). *EPHRINA5* acts as a repulsive signal for muscle cells expressing *EPHA4* (Swartz, 2001), demonstrating that both chemoattractive and repulsive signals from irregular CT act on muscle progenitors to restrict and define their migration pathways. Finally, during their migration, muscle progenitor cells stay in an undifferentiated state and this step is regulated through secreted signals produced by the limb mesenchyme. Previous studies suggest that BMPs and FGFs secreted by limb irregular CT might be important to prevent differentiation of migrating cells by respectively inhibiting and promoting the expression of SF/HGF (Pourquié et al., 1996; Heymann et al., 1996; Scaal et al., 1999). In chick embryo, the timing of *Myod1* and *Myf5* expression in myogenic cells is controlled by FGF18 and retinoic acid, secreted by the limb mesenchyme (Mok et al., 2014).

Muscle differentiation and patterning

During the whole processes of limb muscle morphogenesis, irregular CT and muscles (progenitors or differentiated cells) are in close association. TCF4 has been identified as a putative actor in the process of irregular CT-mediated muscle morphogenesis (Kardon et al., 2003). TCF4 is expressed in the limb mesenchyme in close association with muscles during their differentiation and patterning. In the absence of limb muscles, TCF4 expression pattern is unchanged, suggesting that TCF4 expression may serve as a pre-pattern for limb musculature. Gain- and loss-of-functions of *TCF4* performed in lateral plate-derived limb mesodermal cells leads to muscle mispatterning, demonstrating that TCF4 in irregular CT is important to establish the correct location of limb muscles (Kardon et al., 2003). *Tcf4* deletion in mice also resulted in aponeurosis defects (Mathew et al. 2011). However, TCF4 is also expressed at a low level in myogenic cells and is involved in the intrinsic regulation of muscle fiber type differentiation in mice (Mathew et al. 2011).

Recently, irregular CT has been shown to be involved in a common and often lethal muscle diaphragm defect, called congenital diaphragmatic hernia (CDH). The pleuroperitoneal folds, which are transient embryonic structures, give rise to the irregular CT of the diaphragm (Merrell et al. 2015). Muscle progenitor cells migrating from the cervical somites invade the *Tcf4*-positive pleuroperitoneal cells, which guide muscle cells to organize the diaphragm morphogenesis. *Tcf4*-positive CT cells also express *Gata4*, known to be mutated in CDH, and *Gata4* inactivation in diaphragm CT leads to hernias similar to those observed in CDH, demonstrating that this congenital muscular disease is related to a defect in muscle irregular CT (Merrell et al., 2015).

Human Holt-Oram syndrome is characterized by limb and heart musculoskeletal defects and irregular CT disorganization. This syndrome is attributed to a mutation in the *TBX5* gene, which is expressed in irregular CT during limb development (Hasson et al., 2010). In *Tbx5* mutants, a defect in the organization of irregular CT is observed during embryonic development (Hasson et al., 2010). In these mutants, ectopic splitting of nascent muscle bundles is observed, while the early steps of limb myogenesis are not affected. *Tbx5* inactivation also affects muscle irregular CT and induces a decrease in *Tcf4* expression, but also in β -catenin and N-cadherin in muscle irregular CT cells (Hasson et al., 2010). β -catenin deletion in the limb mesenchyme also leads to ectopic muscle splitting, demonstrating that the N-cadherin/ β -catenin complex in muscle CT is critical for muscle patterning (Hasson et al., 2010). Finally, *Tbx5* deletion also downregulates the expression of CXCL12 and SF/HGF, two factors secreted by the mesenchyme and important for limb myogenesis (Hasson et al.,

2010). It is noteworthy that *Cxcl12* is a target of *Tbx5* in human synovial fibroblasts (Karouzakis et al., 2014). Recently, it has been shown that the conditional deletion of *Tbx3*, another T-box gene, in the lateral plate mesoderm leads to defects in myofiber formation in a subset of limb muscles in mice (Colasanto et al., 2016). These muscle defects are correlated with *Tbx3* expression in a subset of limb bones, tendons and muscle CT. Similar muscle defects are observed in the human Ulnar-mammary syndrome in which *TBX3* mutations have been identified (Colasanto et al., 2016). *Hoxa11* gene is not only expressed in limb skeletal elements, but also in mouse muscle CT and in addition to the skeleton defect, *Hoxa11* inactivation disrupts limb muscle and tendon patterning (Swinehart et al., 2013). Abnormal tendon and muscle formation are observed in heterozygous *Hoxa11* mutants but normal skeleton, demonstrating that the effects on tendon and muscle are independent of the skeleton (Swinehart et al., 2013). However, it cannot be excluded that, in this case, muscle mispatterning could be related to tendon abnormalities rather than to the muscle CT defect. Recently, Gu et al. (2016) have shown that in neonatal muscles, muscle interstitial cells activate NF- κ B, which regulates EPHRINA5 to stimulate myoblast migration towards the end of growing muscle fibers, where they fused to contribute to muscle growth. These results show that muscle CT also contributes to the process of muscle maturation during neonatal development. However, these interstitial cells are characterized by NG2 expression, a neural/glial antigen 2 expressed in pericytes and it cannot be excluded that these cells are of vascular origin (Gu et al., 2016).

Finally, differentiated muscle fibers also control muscle CT formation. In mice deleted for *Lox* (Lysyl-oxidase), an enzyme regulating collagen organization and secreted from the myofibers, TGF β signalling is decreased and promotes muscle CT differentiation at the expense of muscle tissue (Kutchuk et al., 2015).

D. Connective tissue cell involvement in adult muscle homeostasis

In adult, a loss in skeletal muscles is observed in neuromuscular diseases, but also during aging, inactivity and chronic systemic disorders (i.e., diabetes, cancer, rheumatoid arthritis). The regenerative potential of skeletal muscle provides a compensatory response against such pathological muscle loss. The regenerative capacity of skeletal muscle depends on muscle stem cells (the satellite cells), which proliferate in response to exercise to facilitate muscle growth and remodeling, or following myotrauma to repair the injured muscle. Satellite cells are PAX3/7-positive progenitor cells located under the basal lamina that forms around muscle fibers of postnatal skeletal muscle. Satellite cells remain quiescent until the muscle is injured,

when the lamina breaks down and activated satellite cells begin to proliferate before forming new muscle fibers (Relaix et al., 2005). *Myf5* is expressed in most quiescent satellite cells (Cornelison & Wold, 1997; Beauchamp et al., 2000) and activation of satellite cells is characterized by *Myod1* expression and higher levels of *Myf5*, leading to the downregulation of *Pax7*, the activation of Myogenin, and the formation of new muscle fibers (Relaix et al., 2006), reviewed in (Motohashi and Asakura, 2014). In the absence of *Pax7*-positive cells, the process of muscle regeneration failed and fibrotic and fatty infiltration are observed, demonstrating the major contribution of satellite cells in the formation of new muscle fibers. In response to muscle damage, non myogenic cells can also participate to skeletal muscle regeneration, some of them by giving rise to myogenic stem cells, the others by stimulating the activation of resident muscle satellite cells. The first identification of a non-satellite cell population with myogenic capacity comes from the demonstration that bone-marrow-derived cells can participate directly to muscle regeneration (Ferrari et al., 1998). These cells, which normally reconstitute the hematopoietic lineage, can give rise to new satellite cells and myofibers during the muscle regeneration process (Asakura, 2012) and their transplantation into mdx mice (a model for Duchenne muscular dystrophy) improves muscle function (Sampaolesi et al., 2006). A vascular progenitor population, isolated from postnatal muscle, can also participate in muscle repair following arterial delivery in mice (Sampaolesi et al., 2003). Interestingly, pre-treatment of both mesenchymal bone-marrow stromal cells (Galvez et al., 2014) or vascular progenitors (Brzoska et al., 2012) with the CXCL12 chemokine improved the regeneration of injured muscle. In the adult muscle, CXCL12 is expressed by the endomysium, i.e. the CT surrounding each muscle fiber (Hunger et al., 2012). Following muscle injury, CXCL12 secreted by muscle CT rapidly increases (Griffin et al., 2010) and attracts both satellite cells and bone-marrow-derived cells to participate to the regeneration process (Ratajczak et al., 2003). CXCL12 not only chemoattracts stem cells towards the injury site, but also increases their fusion capacity with native muscle fibers (Griffin et al., 2010). These results demonstrate that signals provided by muscle irregular CT are not only crucial for muscle morphogenesis during development but also for muscle regeneration in the adult. More recently, a population of interstitial muscle cells with myogenic potential has been identified (Mitchell et al., 2010). These cells are characterized by the expression of the *PW1/Peg3* gene and named PICs (PW1-positive interstitial cells). They have been shown to contribute to the satellite cell pool during muscle regeneration (Mitchell et al., 2010), although they do not express *Pax3* or *Pax7* genes (Pannerec et al., 2013). PICs can be divided into two different populations: cells that express PW1 and are negative for PDGF α and cells

that express both PW1 and PDGFR α . PW1⁺ PDGFR α ⁻ PICs exhibit a myogenic potential, while PW1⁺ PDGFR α ⁺ cells have been shown to give rise to adipocytes (Pannerec et al., 2013). Interestingly, PW1⁺ PDGFR α ⁺ PICs express the pericyte marker NG2, suggesting a possible overlap between this cell population and muscle pericytes (Pannerec et al., 2013). Pericytes represent perivascular cells located in the muscle interstitium and associated with capillaries. They can be separated into two different populations: type-1 pericytes (NG2⁺ NESTIN⁻ PDGFR α ⁻) and type-2 pericytes (NG2⁺ NESTIN⁺ PDGFR α ⁺), (Birbrair et al., 2013). As observed for PICs, the two different pericyte populations have distinct cell fate potential: type-1 cells contribute to adipose tissue and type-2 cells to myogenesis (reviewed in Birbrair et al. 2014). Type-2 pericytes do not express *Pax7*, *Myf5* and *Myod1*, but activate these genes in regenerative conditions (Cappellari and Cossu, 2013).

The participation of mesenchymal progenitors without myogenic capacity has also been reported during muscle regeneration. In injured muscles, if the regeneration process fails, irregular CT, adipose tissue and even heterotopic bones can develop into skeletal muscle. These CT derivatives all arise from mesenchymal progenitors resident in the adult muscle interstitium (Joe et al. 2010; Uezumi et al. 2010). These mesenchymal progenitors have been isolated from the muscle interstitium, based on the expression of PDGFR α , and it has been shown that they can differentiate in vitro into fibroblasts, adipocytes or osteoblasts, but never into muscle cells (Uezumi et al., 2010). Simultaneously, Rossi's group also identified a cell population with fibroblastic and adipogenic potential, but without myogenic potential (Joe et al. 2010). This population was isolated on the basis of SCA1 and CD34 expression, and named Fibro/Adipogenic progenitors (FAPs), (Joe et al. 2010). Interestingly, mesenchymal PDGFR α ⁺ progenitors express SCA1 (Joe et al. 2010; Uezumi et al. 2010) FAPs express PDGFR α , suggesting that mesenchymal progenitors and FAPs could actually represent a unique progenitor population. FAPs/mesenchymal progenitors are activated upon muscle injury and promote myoblast differentiation in co-cultures (Joe et al., 2010), but also exhibit a strong adipogenic and fibrogenic potential in vitro, indicating a potential contribution of FAPs to fibrosis and adipose accumulation in diseased muscles (Uezumi et al., 2010). The proposed model is that a balance exists between satellite cell-dependent myogenesis and FAPs-dependent adipogenesis/fibrogenesis to control muscle homeostasis and regeneration. After muscle injury, FAPs/mesenchymal progenitors start to proliferate before satellite cells and invade the space between regenerating muscle fibers, where they generate factors promoting myogenesis. When regeneration proceeds efficiently, FAPs/mesenchymal progenitors are excluded through apoptotic signals emanating from satellite cells. If regeneration fails,

FAPs/mesenchymal progenitors persist and differentiate into adipocytes and fibroblasts, leading to fatty and fibrotic degeneration (Judson et al., 2013). Depending on the surrounding environment, FAPs/mesenchymal progenitors will preferentially give rise to fibroblasts or adipocytes. Addition of TGF β to FAPs/mesenchymal progenitors in vitro induces the expression of fibrosis markers leading to fibroblastic differentiation at the expense of adipocyte differentiation (Uezumi et al., 2011). Interestingly, PDGFR α ⁺ expressing FAPs/mesenchymal progenitors accumulate preferentially into fibrotic regions, suggesting a specific role for PDGFR α in muscle fibrosis (Uezumi et al., 2014). This hypothesis is supported by the observation that, in embryonic and adult mouse, an elevated level of PDGFR α leads to an increase in CT differentiation (Olson and Soriano, 2009). It has also been shown that CT fibroblasts identified by *Tcf4* expression proliferate close to muscle satellite cells following injury. Conditional ablation of *Tcf4*-positive cells prior to muscle lesion leads to premature satellite cell differentiation, depletion of the early pool of satellite cells, and small regenerated fibers, indicating that *Tcf4*-positive fibroblasts participate in muscle regeneration (Murphy et al. 2011). It remains unclear whether a direct relationship exists between FAPs/mesenchymal progenitors and TCF4-positive cells. However, *Tcf4*-positive cells express PDGFR α (Murphy et al. 2011) and accumulating evidence suggests that FAPs/mesenchymal and irregular CT progenitors share common features (Sudo et al., 2007); (Haniffa et al., 2009). Extracellular matrix components also contribute directly to the regenerative potential of muscle. It has been shown that a fibronectin-rich fibrosis is essential during the initial step of regeneration to activate the proliferation of muscle satellite cells (Bentzinger et al., 2013). Irregular CT progenitors, FAPs and PICs could be potential sources of fibronectin and might contribute to the transient fibronectin-rich promyogenic fibrosis during muscle regeneration. However, activated satellite myogenic cells themselves release fibronectin into their microenvironment and inactivation of fibronectin using a *Myf5*-Cre reporter impairs the regenerative potential of muscle, suggesting that this effect could be also related to a cell-autonomous role of satellite cell derived-fibronectin (Bentzinger et al., 2013). The importance of muscle CT has been also evidenced in muscle disorders. Mutations in *COL6A1*, *COL6A2* and *COL6A3* genes, the main collagens expressed in muscle CT, have been observed in congenital Ulrich muscular dystrophy and in Bethlem myopathy. Mutant mice for *Col6a1* exhibit alterations of muscle sarcoplasmic reticulum and mitochondria (Pan et al., 2014) and similar myopathic and connective tissue phenotypes are observed in *Col6a3* mutant mice (Pan et al., 2013), demonstrating that collagen VI mutations result in defects

involving both muscle and CT. In addition, *Col6a1* mutant mice showed delayed muscle regeneration and reduced satellite cell self-renewal. Transplantation of wild-type fibroblasts in muscles of *Col6a1* mutant mice rescues muscle satellite cells, indicating that COL6A1 in the muscle environment can modulate satellite cell behavior (Urciuolo et al., 2013). Finally, during muscle hypertrophic activity, satellite cells regulate collagen expression in fibroblasts via exosome secretion, showing that muscle cell progenitors can also act with their surrounding environment to facilitate tissue plasticity. Similarly, (Abou-Khalil et al., 2015) have shown that *Pax7*-positive muscle satellite cells are involved in bone repair, providing a direct evidence of a muscle contribution to specialized CT (bone and cartilage) formation. Taken together, these data evidence interactions between different cell populations promoting muscle progenitor activation during regeneration, with a central role of muscle irregular CT in this process. Changes in CT local environment may contribute to muscle pathologies and age-related loss of muscle stem cell competence through signalling pathways and genes similar to those described to mediate the CT-dependent muscle morphogenesis during development.

IV. The Chemokines CXCL12 and CXCL14

A. Chemokines and their receptors

Chemokines: structure and classification

Chemokines, also known as chemotactic cytokines, represent a group of vertebrate specific molecules that belong to the family of cytokines, which have been first characterized for their role in immune cell trafficking (Baggiolini and Dahinden, 1994; Baggiolini et al., 1994). During evolution, chemokines have highly expanded in higher vertebrates, explaining the differences observed in the list of chemokines between closely related species (DeVries et al., 2006; Nomiya et al., 2013). These small proteins (6 to 14 kDa) have the capacity to induce chemotaxis in many different cell types, mostly related to the immune response (lymphocytes, monocytes, etc), but also to many other migratory mechanisms (germ cells, neurons, neural crest cells, etc). The chemokine family is subdivided into four sub-groups depending on the presence and the position of conserved cysteine residues located in the N-terminal region of the protein (Zlotnik and Yoshie, 2000). Indeed, chemokines have conserved amino acids that are important for their correct conformation. Four cysteine amino acids, spread into the chemokine peptidic sequence, interact together, thanks to disulphide bonds, to confer the typical Greek tridimensional shape, which is characteristic of chemokines (Berry et al., 2000). The N-terminal part is an important region in the chemokine structure, as it contains the binding domain allowing the physical interaction between the protein and its associated

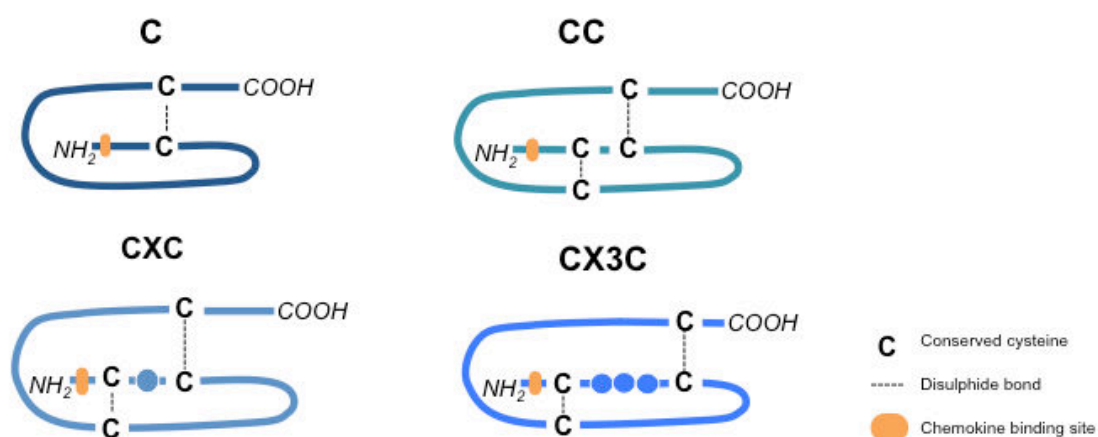


Figure 15: Structural classification of chemokines. Chemokines are classified in four different groups, depending on the distance between the two first conserved cysteines. C chemokines, CC chemokines, CXC chemokines and CX3C chemokines can be distinguished on the basis of this criterion.

receptor. Structural classification of chemokines is based on the distance existing between the two first cysteine residues. Therefore, in the chemokine family, we can distinguish the C chemokines, the CC chemokines, the CXC chemokines and the CX3C chemokines (Figure 15).

Additionally, chemotactic cytokines are functionally classified into inflammatory and homeostatic chemokines. Inflammatory chemokines are produced specifically in an inflammatory context, in order to contribute actively to the inflammation response through recruitment of immune cells to the site of inflammation. Conversely, homeostatic chemokines are constitutively produced. They can also manage immune cell trafficking in inflamed tissues, but they additionally contribute to major mechanisms during embryonic development, for instance in the vascular network formation (Harrison et al., 2015; Ivins et al., 2015; Ojeda et al., 2017), in neuron migration in the central nervous system (Belmadani, 2005; Stumm and Höllt, 2007; Zhu et al., 2002), in germ cell trafficking (Doitsidou et al., 2002; Niu et al., 2016; Stebler et al., 2004) or during limb myogenesis (Vasyutina et al. 2005; Ödemis et al. 2005; 2007; Hunger et al. 2012; Masyuk et al. 2014).

Chemokine receptors: structure and classification

Chemokine receptors belong to the superfamily of seven-transmembrane G-protein-coupled receptors (GPCRs), which are commonly associated with heterotrimeric G-proteins. Such receptors are composed of a small N-terminal part located in the extracellular region,

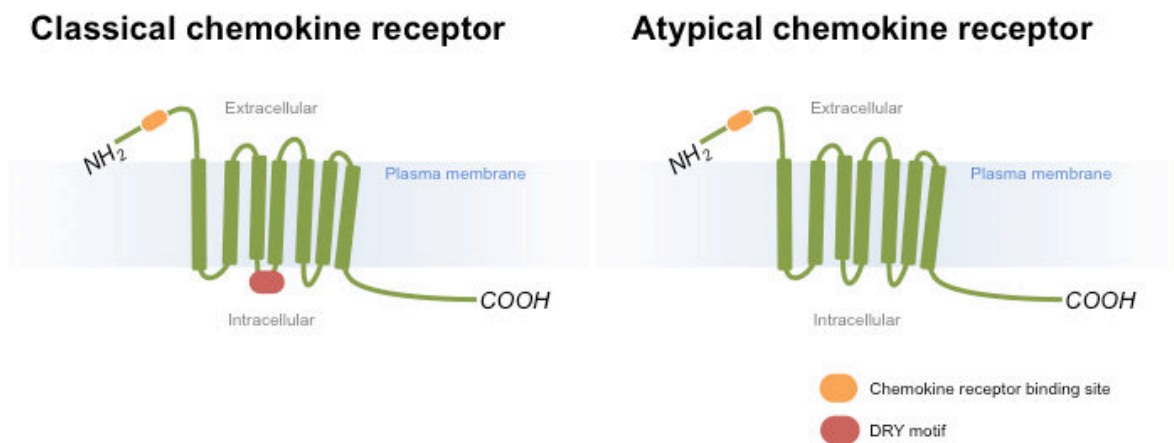


Figure 16: Structure of the chemokine receptors. Chemokine receptors present seven transmembrane domains. The N-terminal region is localized in the extracellular domain, while the C-terminal is localized intracellularly. Chemokine receptors are of two types: classical and atypical receptors. Classical chemokine receptors are G protein-coupled receptors and contain the highly conserved DRY motif in their second intracellular loop, allowing G protein activation. Atypical chemokine receptors do not have the complete DRY motif; hence they cannot activate G protein and the associated downstream signalings.

corresponding to the domain interacting with the ligand. This domain is associated with 3 extra- and intracellular loops. A typical GPCR motif DRY is present in the second intracellular loop. Finally, the C-terminal part of chemokine receptors is located intracellularly and contains a high number of serine and threonine amino acids, which are primordial for the regulation of signal transduction (Figure 16).

Chemokine receptor family can be subdivided into four different groups, depending on the type of chemokines they interact with: the CRs, the CCRs, the CXCRs, the CX3CRs interact respectively with C, CC, CXC and CX3C chemokines. Despite this well-established classification, a fifth class has been added to the superfamily of chemokine receptors, named the atypical chemokine receptors (Bonecchi and Graham, 2016; Zlotnik and Yoshie, 2012). So far, the atypical chemokine receptor (ACKR) subgroup contains 5 receptors: ACKR1 (Duffy Antigen Receptor for Chemokines-DARC), ACKR2 (D6 or CCBP2), ACKR3 (CXCR7 or RDC1), ACKR4 (Chemokine Receptor like 1-CCRL1, or CCX CKR and CCR11) and ACKR5 (Chemokine Receptor like 2-CCRL2 or HCR), (Sozzani et al., 2015; Ulvmar et al., 2011). Although ACKRs are structurally similar to classical chemokine receptors, they cannot transduce GPCR signalling, as they are unable to bind G-proteins (Ulvmar et al., 2011; Zlotnik and Yoshie, 2012).

Interestingly, different chemokines bind the same receptor and one chemokine can bind multiple receptors (Figure 17). This redundancy highlights a putative overlap in between chemokine and chemokine receptor functions. Nevertheless, it has been demonstrated that a few chemokine could bind to only one receptor, and conversely a small number of chemokine receptors only interact with one chemokine (Zlotnik and Yoshie, 2012).

Chemokine signalling

Chemokines exert their biological effect through their binding to specific seven-transmembrane G-protein-coupled receptors, therefore chemokine receptor transduce signals as classical GPCRs. Chemokine receptors are associated with a heterotrimeric G-protein that is composed of three different units G_α , G_β and G_γ linked together in a non-covalent way. The G_α subunit has a GTPase activity. In an inactive state, G_α subunit binds to GDP and possesses high affinity for $G_{\beta/\gamma}$ complex. The interaction with the chemokine leads to a structural rearrangement of the receptor, triggering activation of the G_α subunit that is mediated by GDP to GTP exchange. This activation results in the dissociation of the heterotrimeric G-protein into an active GTP-bound G_α subunit and a $G_{\beta/\gamma}$ dimer. Both induce independently second messengers that activate effector proteins leading to different intracellular signalling events

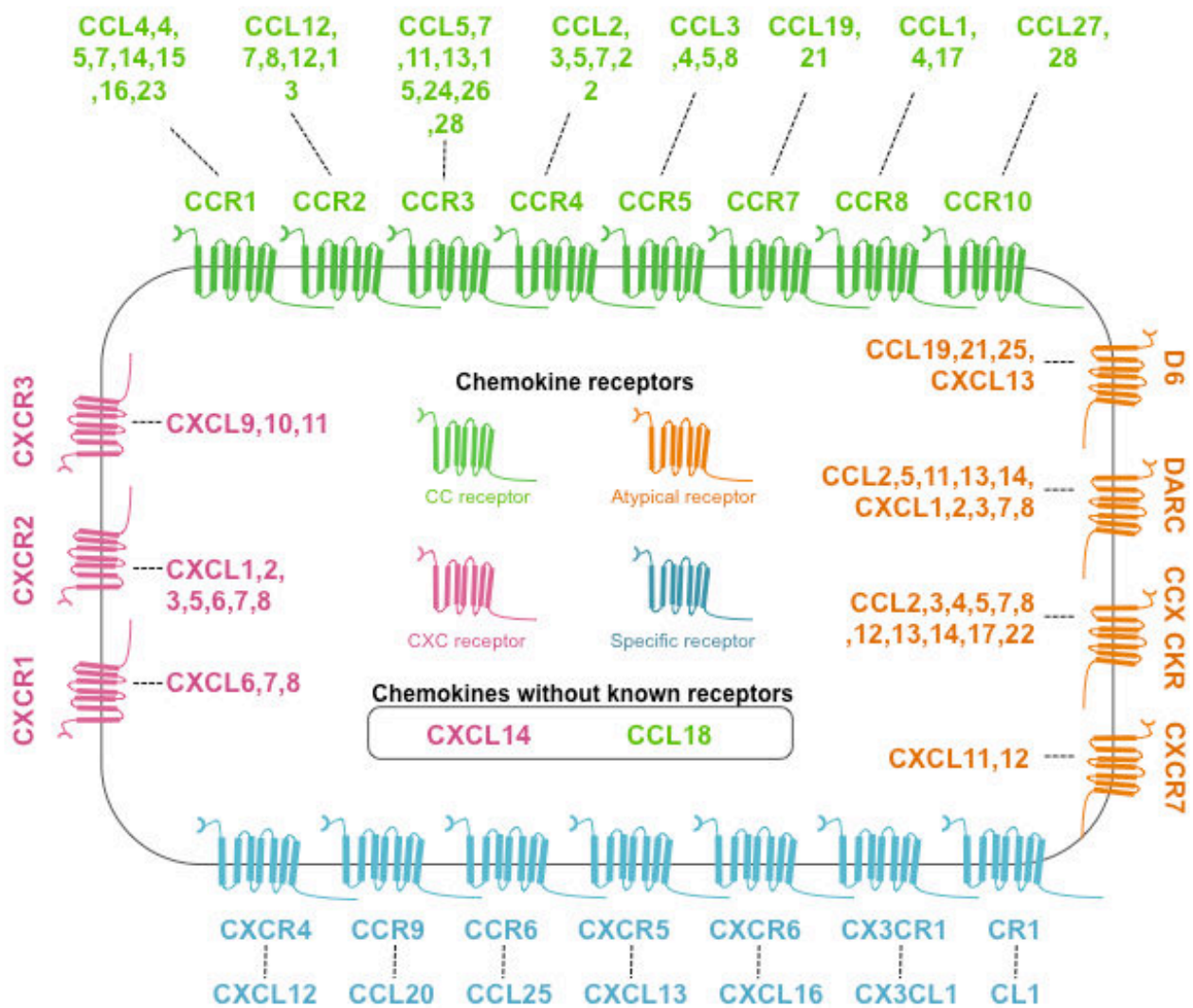


Figure 17: The superfamily of chemokines and their receptors. Schematic representation of chemokines and their associated receptors. CXCR4 chemokine receptor only binds CXCL12 chemokine, however CXCL12 chemokine also binds CXCR7. CXCR5 chemokine receptor and its associated CXCL13 chemokine are specific to each other. Finally, the receptors for CXCL14 and CCL18 have not been identified yet. (Adapted from Lazennec et al., 2010)

(Bennett et al., 2011; Lodowski and Palczewski, 2009). On one hand, the $G_{\beta/\gamma}$ complex triggers intracellular calcium release, as well as the activation of the Erk and Akt pathways. While on the other hand, the G_{α} subunit activates G-protein coupled receptor kinase that phosphorylates the numerous serine and threonine amino acids from the cytoplasmic tail of the receptor. Phosphorylation of the chemokine receptor tail allows the interaction of the receptor with the β -Arrestin molecule to finally induces permanent or transient receptor inactivation/desensitization. Indeed, β -arrestin blocks G-protein-mediated signalling and targets receptor internalization either for degradation or recycling at the membrane (Bennett et

al., 2011; Lodowski and Palczewski, 2009; Pierce and Lefkowitz, 2001), (Figure 18). However, it has also been shown that β -arrestin activation can induce downstream signalling pathways (Pierce and Lefkowitz, 2001).

ACKR signalling differs from pathways induced by classical chemokine receptors. As mentioned above, ACKRs are unable to bind G-proteins. Although ACKRs share a high homology with typical chemokine receptors, they are missing the typical “DRY” motif required for direct interaction of the receptor with G-protein (Zlotnik and Yoshie, 2012), (Figure 16). At first, ACKRs were described only as decoy or scavenger receptors for chemokines, without any signalling ability. However, over the past years, different studies have demonstrated that ACKRs are actually able to activate β -arrestin (Bonecchi and Graham, 2016; Sozzani et al., 2015; Ulvmar et al., 2011) which acts as an adaptor protein to induce activation of downstream signalling cascades (Ma and Pei, 2006; Pierce and Lefkowitz, 2001), (Figure 18).

B. The CXCL12 chemokine

Biological functions of CXCL12

CXCL12, also known as Stromal cell-Derived Factor 1 (SDF1), has been first isolated in a murine bone marrow stromal cell line (Nagasawa et al., 1994; Tashiro et al., 1993). Three CXCL12 isoforms have been reported in mouse: CXCL12 α , β and γ (Gleichmann et al., 2000; Shirozu et al., 1995; Tashiro et al., 1993; Yu et al., 2006), encoding polypeptides of 89, 93 and 119 amino acids, respectively. All isoforms are generated from the same gene, but undergo alternative splicing (Shirozu et al., 1995). They differ only in their C-terminal part, which length varies depending on the isoform. Differences in the C-terminal part of CXCL12 isoforms might play a role on their ability to interact with some proteins of the extracellular matrix. In agreement with this hypothesis, it has been shown that CXCL12 γ possesses four heparan-sulfate binding motifs in its C-terminal part (Gleichmann et al., 2000; Yu et al., 2006). However, no real functional differences have been demonstrated in between CXCL12 isoforms. Importantly, all isoforms share an identical core, in which is included the N-terminal part, holding the binding site that allows the interaction between the chemokine and its receptor. Therefore, CXCL12 isoforms are expected to have the same signalling transduction potential. Among vertebrates, *Cxcl12* possesses a high degree of conservation compared to other chemokines (DeVries et al., 2006; Knöchel et al., 2002; Read et al., 2004;

Shirozu et al., 1995; Tashiro et al., 1993), revealing its significant role. Expression of *Cxcl12* has been detected in human, mouse, chick, frog and zebrafish, both in embryos and adults, classifying CXCL12 as a homeostatic chemokine. During development, *Cxcl12* is present in limb mesenchyme, retina, ectoderm, vessels, heart, kidney and craniofacial regions (García-Andrés and Torres, 2010; McGrath et al., 1999; Ojeda et al., 2013; Read et al., 2004; Rehimí et al., 2008). In adult, CXCL12 has been described in many different organs: skin, kidney, brain and heart ((Abbott et al., 2004; Fedyk et al., 2001; Lotan et al., 2008; Pablos et al., 1999; Tham et al., 2001). This broad spectrum of *Cxcl12* expression, both during embryonic development and at adult stage, suggests a wide range of important functions.

Mutant mice carrying a deletion for *Cxcl12* are non viable and lethal at perinatal stages (Nagasawa et al., 1996), demonstrating an important role for *Cxcl12* during development. Consistent with *Cxcl12* sites of expression during embryonic development, *Cxcl12*^{-/-} mutants present severe defects in heart, nervous system, hematopoietic system and gonad development (Nagasawa et al. 1996; Zou et al. 1998; Tachibana et al. 1998; Ma et al. 1998; Ara et al. 2003). CXCL12 has been initially described for its stimulating effect on B lymphocyte precursors and on bone marrow myelopoiesis (Nagasawa et al. 1994; 1996; Ma et al. 1998). First studies investigating CXCL12 functions mainly focused on its role on hematopoiesis. CXCL12 guides migration of primitive hematopoietic cells from fetal liver to the bone marrow (Zou et al. 1998; Tachibana et al. 1998; McGrath et al. 1999; Jo et al. 2000). In addition to its classical chemoattractant role on hematopoietic cells, CXCL12 has been characterized as a promoting factor for survival and proliferation of circulating primitive hematopoietic cells (Lataillade et al., 2002), by inhibiting apoptosis and promoting G0 to G1 transition, respectively. Those data nicely demonstrate the important role of CXCL12 in homing of hematopoietic stem cells. Over the past years, numerous studies demonstrated the role of CXCL12 in various biological processes including angiogenesis (Salcedo et al. 1999; Salcedo et al. 2003; Salvucci et al. 2002; 2004), cardiogenesis (Escot et al. 2013; Cavallero et al. 2015; Ivins et al. 2015), neurogenesis (Liapi et al., 2007; Lopez-Bendito et al., 2008; Lysko et al., 2011) or primordial germ cell migration (Stebler et al., 2004), mainly through its chemotactic guidance properties. It has been shown for instance that CXCL12 produced by the nerves contribute to vessel branching in developing skin through its chemotactic effect on endothelial cells (Li et al., 2013). Similarly, CXCL12 expressed in the outflow tract of the heart is important to guide peritruncal endothelial cells that contribute to the formation of coronary arteries (Ivins et al., 2015). In the absence of CXCL12, peritruncal endothelial cells are mislocalized and coronary arteries are mispatterned, demonstrating the essential role of

CXCL12 in tissue morphogenesis. Beside its homeostatic functions, CXCL12 also plays a role in tumorigenesis. Indeed, CXCL12 has been established as a key molecule for tumor growth and survival and metastasis formation in many types of cancers (Domanska et al., 2013; Maksym et al., 2009; Teicher and Fricker, 2010). Finally, in adult, CXCL12 displays a classic chemokine activity in immune cell attraction to inflammation sites (Karin, 2010).

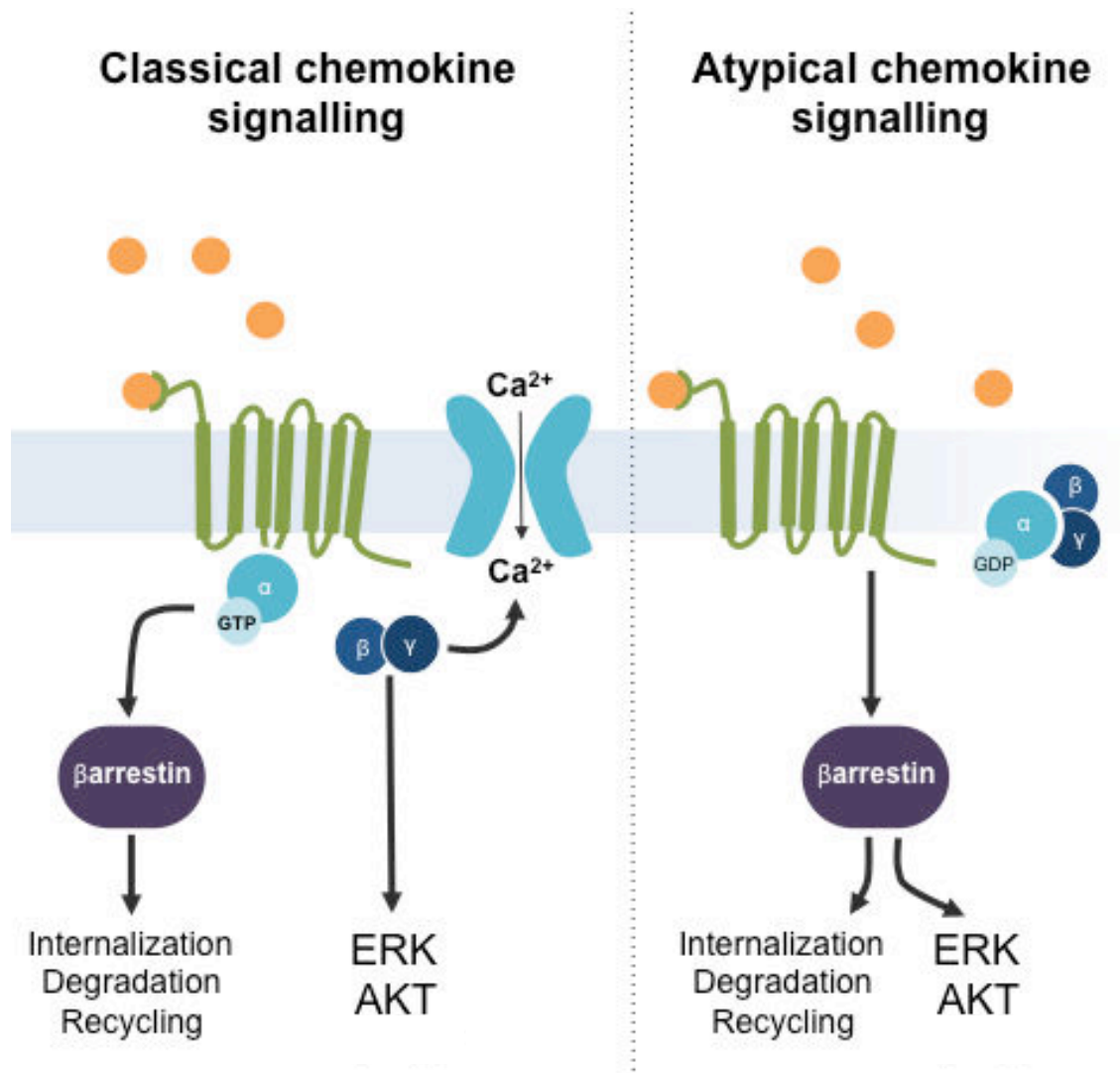


Figure 18: Chemokine signalling. Chemokine binding to its associated receptors triggers activation of downstream signalling pathways. Signal transduction differs between classical chemokine receptors and atypical chemokine receptors. Classical receptors activate G protein-mediated signalling, while atypical chemokine receptor promotes β -arrestin-mediated signalling. Subsequently to their activation, both classical and atypical chemokine receptors induce their internalization and recycling/degradation through β -arrestin recruitment.

CXCL12 signalling

CXCL12 signalling depends on its binding to specific chemokine receptors. So far, two chemokine receptors have been characterized as interacting with CXCL12: CXCR4 and CXCR7.

- **CXCR4/CXCL12 signalling**

Cxcr4 has been isolated in 1993 from a human blood monocyte cDNA library (Loetscher et al., 1994; Nomura et al., 1993) and was first characterized for its high expression level in lymphocytes. At that time, the receptor has been named LESTR for Leukocytes-derived seven-transmembrane domain receptor. To date, only CXCL12 is known to bind CXCR4. It has been demonstrated that CXCR4 receptor can undergo different posttranslational modifications (Farzan et al., 2002). CXCR4 is present in various vertebrate species (Chong et al., 2001; DeVries et al., 2006; Jazin et al., 1997; Yusuf et al., 2005), and has been reported in many different cell types during development and at adult stages as hematopoietic cells, endothelial cells and neuronal cells (Pozzobon et al., 2016).

CXCR4 has been first mainly investigated for its role in HIV infection. The receptor has been identified as a co-factor allowing HIV entry in CD4⁺ T cells (Doranz et al., 1996; Feng et al., 1996). Then, it has been demonstrated that CXCR4 plays essential functions in hematopoiesis, neurogenesis, heart and vascular formation (Zou et al. 1998; Sainz & Sata 2007; Agarwal et al. 2010; Cui et al. 2013). Deficient mice for *Cxcr4* are non-viable and die in utero, exhibiting similar phenotypes than *Cxcl12* mutants, from hematopoietic to cerebellar and heart defects (Nagasawa et al. 1996; Ma et al. 1998; Tachibana et al. 1998; Zou et al. 1998).

CXCR4 interaction with CXCL12 leads to classical G-protein-related intracellular signals. Binding of the chemokine to the receptor leads to the activation of the G-protein heterotrimer. The G_α subunit inhibits adenylate-cyclase and triggers activation of MAPK and PI3K signalling pathways, participating both to cell cycle progression and cell migration (Pozzobon et al., 2016; Ward, 2006). In addition, the G_α subunit is able to recruit G-protein-coupled-receptor kinases (GRKs) that phosphorylate serine and threonine residues of CXCR4 C-terminal part. The phosphorylated tail of the receptor is then associated with the β-arrestin protein, which can induce CXCR4 scavenging (Cheng et al., 2000; Orsini et al., 1999), but also promotes β-arrestin-related MAPK signalling pathways (P38, ERK1/2) (Cheng et al., 2000; Sun et al., 2002), contributing to cell migration. On the other hand, the G_{βγ} dimer

triggers activation of the phospholipase C, which promotes intracellular Ca^{2+} mobilization and activates PKC and MAPK pathways (Pozzobon et al., 2016). Furthermore, $G_{\beta/\gamma}$ is also able to enhance PI3K-induced cell migration (Figure 19).

- **CXCL12/CXCR7 signalling**

Cxcr7 has been isolated from a dog thyroid cDNA library (Libert et al., 1989) and was named RDC1. For a long time, CXCR7 has been considered as an orphan chemokine receptor, until CXCL12 has been identified to bind CXCR7 (Balabanian et al., 2005). Finally, in mammals, a second chemokine has been demonstrated to interact with CXCR7, which is CXCL11 (Burns et al., 2006). Both chemokines are able to bind other receptors. As previously mentioned, CXCL12 can bind CXCR4, and CXCL11 is able to interact with CXCR3. CXCR7 has been classified as an atypical receptor. Indeed, it possesses a modified DRY motif, which does not allow recruitment and activation of G-proteins (Naumann et al., 2010; Thelen and Thelen, 2008). Interestingly, it has been demonstrated that replacement of the non-functional DRY motif of CXCR7 with a classical DRY motif does not induce calcium release upon CXCL12 stimulation (Naumann et al., 2010).

CXCR7 is expressed in many different cell types in both human and mouse (Burns et al., 2006; Gerrits et al., 2008; Libert et al., 1989; Sierro et al., 2007). In the embryo, CXCR7 expression is detected in muscle cells, kidney, heart and brain (Haege et al., 2012; Hunger et al., 2012; Schönemeier et al., 2008; Wang et al., 2011). At adult stages, CXCR7 expression is present in endothelial cells of heart and lung vessels, bone osteocytes, cerebral cortex, heart myocytes, kidney and testis (Burns et al., 2006; Gerrits et al., 2008; Sierro et al., 2007). In the immune system, CXCR7 is only expressed in a subset of B lymphocytes (Sierro et al., 2007). Deficient mice for *Cxcr7* are lethal at perinatal/postnatal stages. Mutant mice display severe heart defects associated with myocardial degeneration and fibrosis. In addition, lung vasculature is impaired (Gerrits et al., 2008; Sierro et al., 2007). Despite CXCR7 expression in bone osteocytes, *Cxcr7* mutant mice do not present obvious bone defects (Gerrits et al., 2008). Conversely to *Cxcr4* and *Cxcl12* mutant mice, impaired hematopoiesis is not observed in *Cxcr7* deficient mice (Sierro et al., 2007). During embryonic development, CXCR7 has been mainly involved in migration processes associated to important morphogenic events (Puchert and Engele, 2014). During zebrafish development, knockout of *Cxcr7* leads to aberrant migration of the primordial germ cells (PGCs), highlighting a crucial role for CXCR7 in the proper migration of PGCs (Dambly-Chaudière et al., 2007). Similarly, CXCR7 is essential to

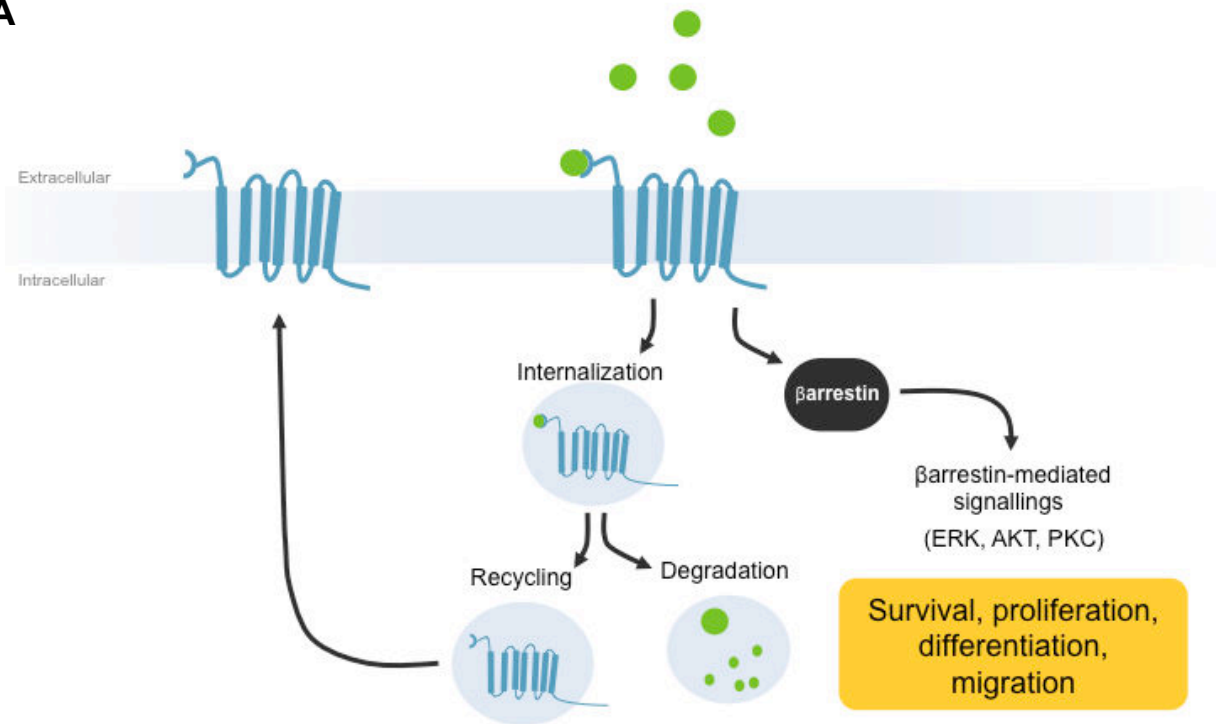
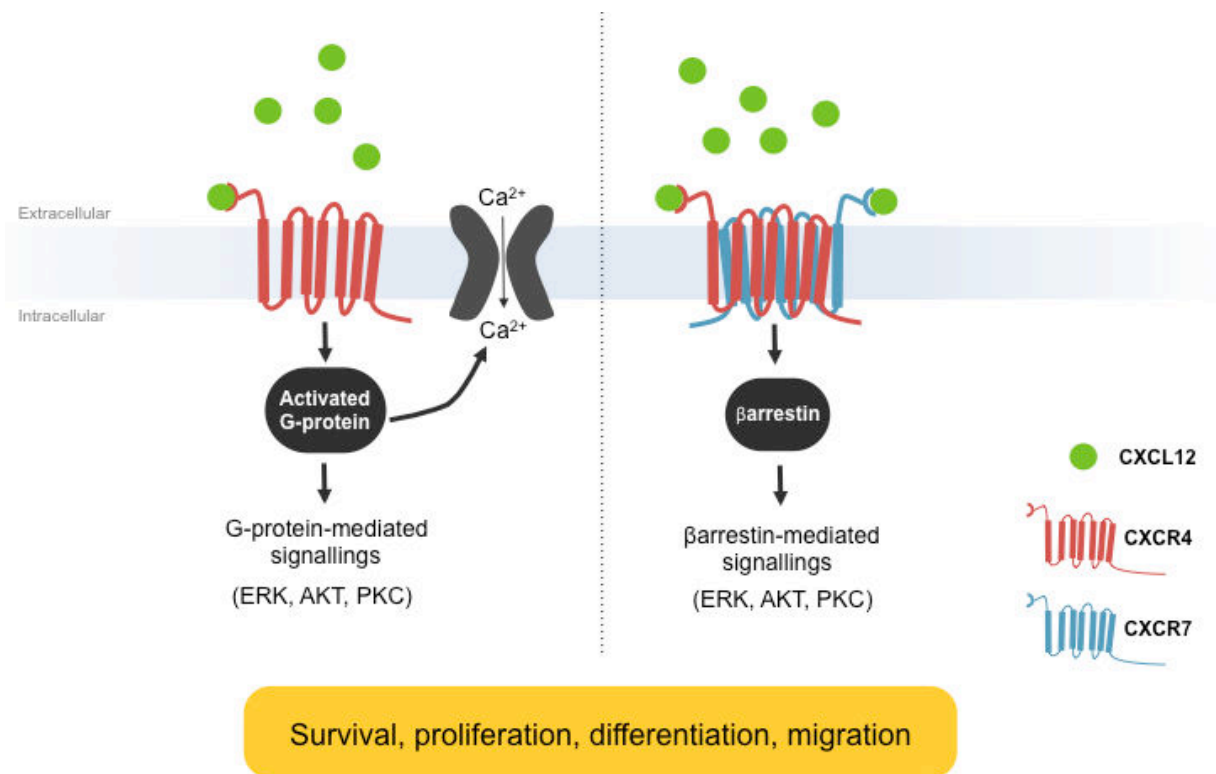
A**B**

Figure 19: CXCL12/CXCR7/CXCR4 axis. CXCL12 is able to bind to CXCR7, CXCR4 and CXCR7/CXCR4 heterodimer. (A) CXCL12/CXCR7 interaction initiates two different type of response. CXCR7 acts as a decoy receptor without downstream signal transduction, or CXCR7 promotes signal activation, mediated through β -arrestin. (B) CXCL12/CXCR4 interaction triggers activation of G protein-related signals. CXCR4 only acts as a signalling receptor. (C) Finally, CXCL12 can binds to CXCR4/CXCR7. In this context, the heterodimer formed by the two chemokine receptors induces β -arrestin-mediated signals.

regulate the correct migration of gonadotropin-releasing hormone neurons during mouse embryonic development (Memi et al., 2013). Finally, in cancer, CXCR7 is described as a positive regulator for tumour growth (Shi et al. 2017; Wang et al. 2008; Gu et al. 2017; Miao et al. 2007).

CXCL12 binding to CXCR7 affects different cellular mechanisms: cell adhesion, cell survival and cell proliferation (Burns et al. 2006; Miao et al. 2007; Wang et al. 2008). These effects are mediated through β -arrestin-dependent activation of signalling pathways. CXCR7 recruits β -arrestin, which activates downstream extracellular signal-regulated kinase (ERK) or protein-kinase B (Akt) (Figure 19), (Miao et al. 2007; Wang et al. 2008; Rajagopal et al. 2010). Besides its signalling receptor activity, CXCR7 has also been described as a non-signalling decoy receptor (Boldajipour et al., 2008; Naumann et al., 2010). In this context, CXCR7 scavenges CXCL12 to finally induce degradation of the ligand (Figure 19). CXCR7 scavenging activity is essential to regulate the gradient of CXCL12 (Boldajipour et al., 2008; Puchert and Engele, 2014). During development, in zebrafish, it has been shown that CXCR7 promotes CXCL12 internalization in order to regulate CXCL12 gradient, and thus affect the migration path of primordial germ cells (Boldajipour et al., 2008; Dambly-Chaudière et al., 2007; Valentin et al., 2007). Similarly, it was also shown that during cortical neuron migration, CXCR7 acts as scavenger receptor, in order to negatively regulate CXCL12 concentration in the extracellular domain and avoid excessive-CXCL12 mediated CXCR4 activation and degradation (Abe et al., 2014). Those data show an important and indirect role of CXCR7 in cell migration, through the regulation of CXCL12 gradient.

- **CXCL12/CXCR4/CXCR7 signalling**

It has been demonstrated that CXCR7 is able to heterodimerize with CXCR4 (Décaillot et al., 2011; Levoye et al., 2009; Puchert and Engele, 2014; Sierro et al., 2007). Such an interaction can either inhibit or enhance CXCR4/CXCL12 axis to modulate signal efficiency upon CXCL12 stimulation (Figure 19), (Décaillot et al., 2011; Levoye et al., 2009). In this context,

CXCR4/CXCR7 heterodimer preferentially triggers the activation of the β -arrestin-related signalling pathways (Puchert and Engele, 2014). It has been proposed that CXCR4/CXCR7 interaction could also trigger internalization of the receptor heterodimer, which would lead to the decrease of CXCR4 receptor at the membrane, in order to avoid CXCL12/CXCR4 binding and thus to inhibit CXCL12/CXCR4 axis (Figure 19), (Puchert and Engele, 2014).

Regulation of CXCL12

Various signalling pathways regulate the CXCL12 chemokine. In mouse stromal cells expressing CXCL12, it has been demonstrated that an overexpression of Wnt3a leads to a decrease in CXCL12 expression and reduces the migration capacity of stromal cells (Tamura et al., 2011). In hematopoietic cells, inhibition of BMP signalling using its antagonist Noggin increases CXCL12 expression. Conversely, an overexpression of BMP ligand leads to a decrease in CXCL12 expression (Khurana et al., 2014). Interestingly, it has been demonstrated that SMAD binding elements are present in CXCL12 promoter. Furthermore, using a chromatin immunoprecipitation assay, Khurana et al. have shown that SMAD4 binds to the CXCL12 promoter, highlighting a direct interaction between CXCL12 and BMP signalling. Moreover, TGF β signalling has been shown as a negative regulator of both *Cxcl12* mRNA and protein in a bone marrow stromal cell line (Wright et al. 2003). However, such regulations seem to be cell specific. For instance, in tumor-associated fibroblasts, BMP signalling positively regulates CXCL12 expression, while TGF β has no effect (Yang et al., 2008).

C. The CXCL14 chemokine

Biological functions of CXCL14

CXCL14 is a small cytokine that belongs to the CXC-motif chemokine subfamily. It has been first isolated from human cell lines (Frederick et al., 2000; Hromas et al., 1999). The chemokine was first named BRAK, due to its isolation from breast and kidney cell lines. CXCL14 presents specific characteristics that differs from the other chemotactic cytokines such as an unusual short N-terminal part of only two amino acids upstream the first conserved cysteine residue and five consecutive additional amino acids (Hromas et al., 1999; Peterson et al., 2007). CXCL14 chemokine is highly conserved among vertebrate species (DeVries et al., 2006; Park et al., 2009). CXCL14 expression pattern has been established in different species during embryonic development and at adult stages, showing a broad expression pattern of this

chemokine. During development, it is detected in paraxial mesoderm, ectoderm, mesonephros, limb, inner ear, optic vesicle, upper eyelid and hindbrain in human, mouse, chick, zebrafish and frog (García-Andrés and Torres, 2010; Gordon et al., 2011; Long et al., 2000; Ojeda et al., 2013; Park et al., 2009), suggesting that CXCL14 might be involved in different developmental processes. At adult stage, CXCL14 is observed in skin, brain, intestine, kidney, white adipose tissue (WAT), immune cells and some tumour cells (Hromas et al., 1999; Nara et al., 2007), highlighting CXCL14 as a likely pleiotropic chemokine. The expression of CXCL14 reveals a homeostatic rather than inflammatory role, during development and at adult stages.

At first, CXCL14 has been mainly investigated for its putative contribution to tumorigenesis (Frederick et al., 2000; Hromas et al., 1999). It has been observed that CXCL14 expression is decreased in many cancer cell lines, compared to normal cell lines (Frederick et al., 2000; Hromas et al., 1999; Tessema et al., 2010). However, further investigations have demonstrated that some tumours exhibit an increase in CXCL14 expression level (Allinen et al., 2004; Frederick et al., 2000; Shellenberger et al., 2004). CXCL14 can act either as a tumour suppressor or as a tumour promotor depending on the type of cancer (Hare et al., 2012). In addition to its role in cancer, CXCL14 is involved in the regulation of glucose uptake and insulin resistance, in the control of body weight, in the regulation of neural control of feeding behavior and during development in neurovascular patterning of the eye (Cao et al., 2013; Nara et al., 2007; Ojeda et al., 2017; Tanegashima et al., 2010). Finally, CXCL14 also displays classical chemokine chemoattractant activity, mostly on immune cells (Cao et al., 2013).

CXCL14 signalling

To date, CXCL14 signalling is poorly understood. This is mainly due to the fact that CXCL14 receptor is not identified yet. However, CXCL14 has been recently characterized as a chemokine ligand for CXCR4 (Tanegashima et al. 2013). So far, CXCL12 was the only chemokine known to bind CXCR4. Tanegashima et al. have shown that CXCL14 is able to inhibit CXCL12/CXCR4 mediated-chemotaxis through its binding to CXCR4 (Tanegashima et al. 2013). Interestingly, CXCL14 binding site on CXCR4 is different from CXCL12 binding site (Figure 20), (Tanegashima et al. 2013). However, these results are controversial as a recent survey has demonstrated that CXCL14 does not affect CXCL12-induced CXCR4 phosphorylation, ERK signalling activation or CXCR4 internalization, in different CXCR4-transfected cell lines (Otte et al., 2014). These data strongly support the fact that CXCL12-

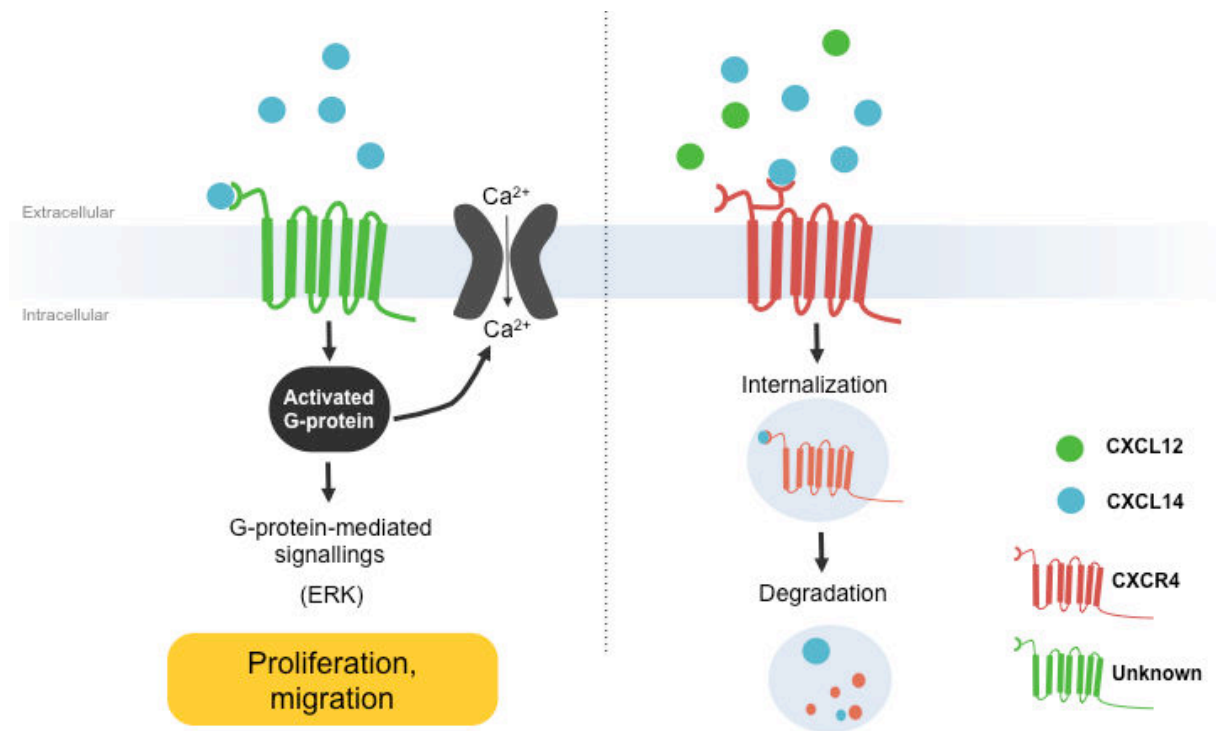


Figure 20: CXCL14 signalling. (A) CXCL14 is able to induce G protein-related cellular response, however its receptor is not identified yet. (B) CXCL14 binds to CXCR4, nevertheless CXCL14/CXCR4 interaction does not mediate signal transduction. Such interaction leads to CXCR4 internalization, thus inhibiting CXCL12/CXCR4 interaction.

mediated CXCR4 signalling is insensitive to CXCL14 and suggest that CXCL14-mediated signalling is not directly related to CXCR4 binding. Although no receptor has been identified to transduce CXCL14 signalling, it has been clearly shown that the chemokine induces signalling activation (Figure 20). For instance, in monocytes, CXCL14 mediates an increase in intracellular calcium (Kurth et al., 2001). Identification of CXCL14 receptor(s) is essential for further investigation on this chemokine, as it would bring new insights on intracellular signalling induced by CXCL14, and hence, on its specific functions.

Regulation of CXCL14

The different mechanisms regulating CXCL14 expression have been poorly investigated. However, some signalling pathways have been already identified for their potential regulation on CXCL14 (Lu et al., 2016). The atypical Rho GTPase RhoBTB2 has been shown to positively regulate CXCL14 expression (McKinnon et al., 2008). Targeting RhoBTB2 by shRNA in primary human epithelial cells leads to a decrease in CXCL14 expression. In head and neck squamous cell carcinoma cell lines, in which loss of RhoBTB2 is also associated with loss of CXCL14, overexpression of RhoBTB2 is sufficient to induce CXCL14 expression (McKinnon et al., 2008). The active phosphorylated form of P38, a member of the

mitogen-activated protein kinase (MAPK) family enhances CXCL14 expression (Ozawa et al., 2010). In addition, transcription factors have been identified to regulate CXCL14 expression. The Iroquois homeobox 1 (IRX1) is activated in metastatic osteosarcoma cell lines. Silencing IRX1 expression using specific shRNA in osteosarcoma cells induces a decrease in CXCL14 expression (Lu et al., 2016). Interestingly, CXCL14 has been defined as a marker of the Middle Zone (MZ) of the growing articular cartilage (Lui et al., 2015) and IRX1 is expressed in joint during limb development in both mouse and chick embryos (McDonald et al., 2010; Zülch et al., 2001). These data suggest that CXCL14 regulation by IRX1 is not restricted to osteosarcoma cells and could be enlarged to developmental processes such as articular cartilage formation. In *Xenopus*, PAX3 expression is necessary and sufficient to induce CXCL14 expression in the hatching gland and Noggin (BMP signalling inhibitor) and Wnt signalling are able to increase CXCL14 expression (Park et al., 2009). Finally, in agreement with these data, CXCL14 expression is inhibited under overexpression of BMP4 in human fibroblasts (Fessing et al., 2010).

D. The role of the chemokines CXCL12 and CXCL14 during myogenesis and repair

Interleukins were the first cytokines to be characterized for their involvement in myogenesis. The absence of Interleukin-4 (IL-4) causes reduced muscle size (Horsley et al., 2003). IL-4 is expressed in a subpopulation of myogenic cells and it has been demonstrated that IL-4 expressed in myotubes recruits myoblasts expressing IL-4 receptor to enhance muscle fusion, and ultimately promotes muscle growth. Interleukin-6 (IL-6) is also involved in myogenesis (Serrano et al., 2008; Tritarelli et al., 2004). Gain- and loss-of-function assays for IL-6 in C2C12 muscle cell line reveal an autonomous promyogenic effect of this cytokine (Tritarelli et al., 2004). More recently, studies conducted on C2C12 muscle cell line or primary muscle cell cultures revealed that numerous cytokines, including TNF-related factors and chemokines are expressed in skeletal muscle cells (Ge et al., 2013; Griffin et al., 2010). Different combinations of cytokine expression are observed during the different phases of myogenesis in vitro but also during muscle regeneration (Table 3), (Griffin et al., 2010), suggesting the importance of these factors in the cell-autonomous regulation of muscle differentiation (Ge et al., 2013; Griffin et al., 2010).

The involvement of CXCL12 during myogenesis

Most informations on the putative role of CXCL12 during myogenesis come from in vitro

studies mainly conducted on the C2C12 cell line. In these cells, CXCL12, CXCR4 and CXCR7 expression is detected on proliferating myoblasts but the expression of ligand and receptors decreased with muscle differentiation (Melchionna et al., 2010). However, another study showed that C2C12 proliferating cells are characterized by the expression of CXCR4 while differentiating muscle cells expressed both CXCR4 and CXCR7 receptors (Hunger et al., 2012). Contradictory results are also observed in the different experiments that have been conducted in vitro on the role of CXCL12 during myogenesis. Indeed, CXCL12 has been shown to induce proliferation of C2C12 cells but to exert a negative effect on myogenic differentiation (Hunger et al., 2012). Phospho-ERK and PKC-related signalling pathways are activated by addition of CXCL12 on C2C12 proliferating myoblasts but not on differentiated muscle cells (Hunger et al., 2012). Moreover, inhibition of CXCR4, using its specific pharmacological antagonist AMD3100, increases myogenic differentiation, showing that CXCL12 inhibits muscle differentiation through CXCR4 activation (Hunger et al., 2012). During muscle differentiation, CXCR7 acts to inhibit CXCL12/CXCR4-mediated signalling, by its scavenger function, which traps CXCL12, then preventing CXCL12/CXCR4 binding (Hunger et al., 2012). However, CXCL12 has been also shown to favour myogenic differentiation by promoting cell cycle withdrawal, an effect mediated through both CXCR4 and CXCR7 (Melchionna et al., 2010; Odemis et al., 2007). In vitro studies also demonstrated the chemoattractive effect of CXCL12 on muscle cells (Griffin et al., 2010; Odemis et al., 2007). Finally, blockade of the CXCR4 receptor, using shRNA or the AMD3100 inhibitor, leads to a decrease in muscle fusion. These results suggest that impairment of CXCL12/CXCR4 axis in muscle cells would impact myoblast migration, which could affect muscle fusion or differentiation (Ge et al., 2013; Griffin et al., 2010).

First evidences for a possible implication of CXCL12 in myogenesis in vivo came from the analysis of CXCL12 and CXCR4 expression patterns. It has been shown that CXCR4 receptor is expressed in migrating muscle progenitors invading the limb bud, both in chick and mouse embryos, while CXCL12 is expressed in the surrounding mesenchyme (Vasyutina et al., 2005). During perinatal and postnatal muscle development in mouse, CXCR4 expression characterizes perinatal muscles while CXCR7 is expressed in postnatal and adult muscles (Hunger et al., 2012). In *Cxcr4* mutant mouse, the number of muscle progenitors that colonize the limb is reduced and limb muscles are impaired, demonstrating the role for the CXCL12/CXCR4 axis in the control of limb myogenesis (Ödemis et al., 2005; Vasyutina et al., 2005). Functional experiments performed in vivo in chick and zebrafish also show that an ectopic source of CXCL12 during somite (Chong et al., 2007), pectoral (Masyuk et al., 2014)

or cloacal (Rehimi et al., 2010) muscles development induces abnormal migration of muscle progenitor cells expressing CXCR4, leading to defects in muscle morphogenesis. To date, there are no data investigating the role of CXCL12/CXCR7 axis during in vivo myogenesis and no defect in muscle development has been reported in the *Cxcr7* mutant mouse (Sierro et al., 2007; Yu et al., 2011).

The role of CXCL14 during myogenesis

The putative role of CXCL14 in muscle development has been poorly investigated. Silencing *Cxcl14* expression in C2C12 muscle cells leads to an increase in myoblast fusion and differentiation, demonstrating the inhibitory effect of CXCL14 on myogenesis (Ge et al., 2013). More recently, it has been shown that C2C12 cells express CXCL14 chemokine and that CXCL14 inhibition induces a decrease in myoblast proliferation and an increase in the expression level of the myotube markers, demonstrating again that CXCL14 is an inhibitor of in vitro myogenesis (Waldemer-Streyer et al., 2017). Interestingly, this study shows that, although ERK phosphorylation is decreased in CXCL14 inhibition conditions, CXCL14 negative effect on myogenesis is not mediated through CXCL12 antagonism. All together, these data demonstrate that CXCL14 inhibits cell cycle exit in undifferentiated muscle cells, thereby preventing myoblast differentiation. So far, no data concerning the involvement of CXCL14 in muscle development in vivo has been reported and no muscle defects have been mentioned in *Cxcl14* deficient mice (Nara et al., 2007).

The contribution of both CXCL12 and CXCL14 during muscle repair

A role for CXCL12 and their receptors during regeneration has been also described. During muscle regeneration transplanted CXCL12-treated primary myoblasts highly contribute to the formation of new myofibers compare to transplanted non CXCL12-treated muscle cells, thus demonstrating that CXCL12 participates and promotes muscle regeneration (Kowalski et al., 2016). In vitro studies have demonstrated that CXCL12 enhances migration potential of satellite cells, but also embryonic stem cells and bone marrow mesenchymal cells (Kowalski et al., 2016) characterizing cell types that have been shown to contribute to the formation of new muscle fibres during muscle regeneration (Brzoska et al., 2015). Furthermore, CXCL12 promotes the ability of those different cell populations to fuse with myoblasts, and finally to contribute to myotubes formation (Kowalski et al., 2016). During muscle regeneration, CXCL12 and their receptors are expressed in skeletal muscles (Ratajczak et al. 2003; Brzoska

et al. 2015; Hunger et al. 2012). Both receptors have been described to be expressed in satellite cells (Ratajczak et al. 2003; Brzoska et al. 2012; Hunger et al. 2012; Kowalski et al. 2016), while CXCL12 is expressed in satellite cells and muscle-associated fibroblasts (Brzoska et al., 2015; Hunger et al., 2012; Ratajczak et al., 2003). All studies assessing the effect of CXCL12 during muscle regeneration showed a positive effect of the chemokine for muscle regeneration. In vivo in mouse, and in vitro in murine satellite cell lines C2C12 and G7 cells, CXCL12 improves skeletal muscle regeneration (Ratajczak et al. 2003; Brzoska et al. 2015; Bobadilla et al. 2014; Kowalski et al. 2016). The beneficial effect of CXCL12 is in part mediated through its capacity to enhance migration of satellite cell-derived myoblasts (Ratajczak et al. 2003; Galvez et al. 2014; Brzoska et al. 2012; Brzoska et al. 2015; Kowalski et al. 2016). This effect is mediated through CXCR4 receptor (Brzoska et al., 2012; 2015; Kowalski et al., 2016). *Cxcr7* silencing does not disturb the CXCL12-induced migration effect (Brzoska et al., 2015). Myoblasts expressing CXCR4 promotes cell engraftment and finally enhances muscle regeneration efficiency (Pérez-Pomares et al., 2009). During muscle regeneration, CXCL12/CXCR4 induces the expression of the tetraspanine CD9, which ultimately increase stem cell migration and fusion ability, hence promoting efficient regeneration (Brzoska et al., 2015). Furthermore, CXCL12/CXCR4 axis requires the activity of the extracellular matrix metalloprotease MMP10 to mediate its beneficial effect on muscle repair (Bobadilla et al., 2014). In deficient mice for *Mmp10*, both CXCL12 and CXCR4 expression is decreased (Bobadilla et al., 2014). In the absence of *Mmp10*, CXCL12 treatment does not induces an increase in regeneration efficiency. The metalloproteases MMP2 and MMP9 are also associated to CXCL12 effects during muscle regeneration (Brzoska et al. 2012). MMP activity is increased in cultures of satellite cells under CXCL12 treatment (Brzoska et al. 2012). Besides its chemoattractant effect, CXCL12 also positively regulates proliferation of satellite cells, as well as their fusion potential (Bobadilla et al., 2014). In co-cultures of CXCL12-expressing COS-1 cells and satellite cells, the number of PAX7/MyoD-positive cells is increased, while PAX7-negative/MyoD-positive cells are less abundant (Bobadilla et al., 2014). Additionally, fusion index was also increased in this context. However, another study showed that CXCL12 does not influence the proliferation of satellite cells-derived myoblasts (Brzoska et al. 2012). So far, the role for CXCR7 during muscle regeneration is poorly understood. Although it has been shown CXCL12/CXCR7 axis regulates the expression of 113 transcripts in the context of muscle repair, it is not clear what is the function of CXCR7 in this process (Kowalski et al., 2016).

A role for CXCL14 during muscle repair has been recently observed (Waldemer-Streyer et al., 2017). Conversely to CXCL12, CXCL14 has a negative effect on muscle regeneration. Indeed, inhibition of CXCL14 expression improves muscle regeneration (Waldemer-Streyer et al., 2017). This effect is associated with a decrease in the proliferation rate of satellite cells (Waldemer-Streyer et al., 2017).

To sum up, the effect of CXCL12 on myogenesis is not clearly established, as it has been demonstrated that the chemokine either promotes or inhibits myogenic differentiation, in vitro. In the contrary it is well characterized that CXCL12 possesses a beneficial effect during muscle repair. The role of CXCR7 has been poorly investigated during developmental and regenerative myogenesis therefore less is known about it. Finally CXCL14 chemokine has been characterized as a negative regulator in in vitro myogenesis and muscle repair.

Aims and objectives of the thesis

Aims and Objectives of the thesis

Due to the lack of molecular markers, the mechanisms underlying the interplay between irregular CT and muscle, during development and adulthood, have been only recently investigated. Different transcription factors such as TCF4, TBX5, OSR1 and OSR2 have been characterized to be specifically expressed in irregular CT (Kardon et al. 2003; Hasson et al. 2011; Stricker et al. 2006). It has been shown that misregulation of these transcription factors impaired muscle development (Kardon et al. 2003; Hasson et al. 2011; Stricker et al. 2006; Mathew et al. 2011), highlighting an intimate link between irregular CTs and the formation of skeletal muscles. However, to date, no secreted signal has been involved in the process of irregular CT-mediated muscle morphogenesis.

Many cytokines have been described for their contribution in myogenesis, among which the two chemokines CXCL12 and CXCL14, although contradictory results concerning their involvement to muscle development emerged from these studies (Ge et al., 2013; Griffin et al., 2010; Hunger et al., 2012; Odemis et al., 2007; Vasyutina et al., 2005; Waldemer-Streyer et al., 2017). Both chemokines have been shown to be expressed in CTs (García-Andrés and Torres, 2010; Gordon et al., 2011; Ojeda et al., 2013; Rehimí et al., 2008). Consequently, CXCL12 and CXCL14 constitute good candidates for driving non-cell autonomous signals coming from the CT to orchestrate myogenesis.

Therefore, the main goal of my PhD project has been to study the involvement of CXCL12 and CXCL14 chemokines in irregular CT-mediated muscle morphogenesis. However, given the previous data emphasizing the expression of CXCL12 and CXCL14 in dense CTs, we also attempted to characterize the putative function of both chemokines in the differentiation of dense CTs. Defects in muscle regenerative ability are often characterized by a progressive substitution of skeletal muscle by an excessive formation of dense CTs constituting a fibrotic tissue. Then, it seems essential to identify the mechanisms driving dense CTs development in order to better understand the specific role of irregular CT during muscle formation. The aims and objectives of my PhD work are detailed below.

Aim 1 – Characterization of CXCL12 and CXCL14 functions in dense CT differentiation

- Establish the precise expression pattern of the CXCL12 and CXCL14 chemokines and their receptors CXCR4 and CXCR7 in CTs of chick embryo forelimbs.

- Define whether CXCL12 and CXCL14 are able to activate the expression of specific dense CT markers in order to promote dense CT differentiation.
- Identify upstream signals that are able to control the expression of CXCL12 and CXCL14 in CTs, in order to better decipher the molecular network involved in the regulation of dense CT differentiation.

Aim 2 – Characterization of CXCL12 and CXCL14 functions in the process of irregular CT-mediated muscle morphogenesis

- Analyse the expression pattern of the CXCL12 and CXCL14 chemokines and their receptors CXCR4 and CXCR7 in relation to limb myogenesis in chick embryos.
- Assess the function of CXCL12 and CXCL14 signalings in the process of irregular CT-mediated muscle morphogenesis in vitro and in vivo, using functional approaches targeting CXCL12, CXCL14 and CXCR7.

Results

Results

I. CXCL12 and CXCL14 chemokines differentially regulate connective tissue markers during limb development

I. CXCL12 and CXCL14 chemokines differentially regulate connective tissue markers during limb development

Connective tissues (CTs) form a large family of tissues, which main roles are to support and connect organs together. Different groups can be distinguished in this family, among which, the dense CT subfamily that is subdivided into regular and irregular CT groups, corresponding to tendon/ligament structures and CT surrounding organs, respectively. Dense CTs are mainly composed of fibroblasts and extracellular matrix (Nassari et al., 2017). During adulthood, fibroblasts constituting CTs are quiescent but their deficient regulation can lead to a pathological phenomenon, called fibrosis (Wynn, 2007). Fibrosis is assimilated to an excessive formation of dense CT, which results from the activation of fibroblasts leading to their proliferation and to the secretion of extracellular matrix components (Kendall and Feghali-Bostwick, 2014; Wynn, 2007). It is commonly admitted that during development, dense CT-fibroblasts are constantly activated to form CT during the different steps of morphogenesis (Yusuf et al., 2013). Consequently, it is important to decipher the different mechanisms underlying dense CT development, in order to better characterize processes associated to the pathological fibrosis in adult tissues. The CXCL12 and CXCL14 chemokines have been shown as implicated in fibrosis (Emblom-Callahan et al., 2010; Ishii et al., 2013; Jia et al., 2017). Nonetheless, although CXCL12 and CXCL14 have been described as expressed in dense CT sub-regions during embryonic development (García-Andrés and Torres, 2010), their role during dense CT development has never been assessed. Therefore, the first aim of my PhD project was to identify the functions of both CXCL12 and CXCL14 chemokines in the establishment of dense CTs during development, using chick limb embryo as model. For this purpose, the specific expression patterns of CXCL12 and CXCL14 in dense CTs were first assessed in the limb of chick embryos. Then we characterized the putative involvement of CXCL12 and CXCL14 on the regulation of the expression of specific dense CT markers by using gain-of-function approaches *in vitro* and *in vivo*. Finally, we investigated the possible upstream mechanisms regulating dense CT-associated expression of CXCL12 and CXCL14.

Introduction

CTs are primarily composed of extracellular matrix and fibroblasts that derive from mesenchymal cells, giving rise to the different types of CT. Among the supportive CT, the specialized CT refers to bone and cartilage elements, while dense CT is divided into regular and irregular CT, which correspond to tendon/ligament and CT embedding organs, respectively (Omelyanenko and Slutsky, 2013). Understanding the specification and differentiation processes of the different types of CT from undifferentiated mesenchymal cells is an important issue as CT deregulation leads to fibrosis, a process attributed to excess deposition of extracellular matrix in response to injury, inflammation or aging (Wynn, 2007). Fibrosis is also a major pathological feature of chronic autoimmune diseases, tumour invasion and many progressive myopathies (Wynn and Ramalingam, 2012).

Until a few years ago, studying the specification of CT types during embryogenesis was still challenging, due to the absence of specific markers to distinguish the different forms of CT. Most studies designed to identify CT-specific genes during development have been conducted on vertebrate limb musculoskeletal system as a model. Indeed, this multicomponent structure formed by muscle, muscle CT (MCT), irregular CT (ICT), tendon, ligament and bone/cartilage is particularly suitable to investigate CT differentiation in the embryo. During limb development, lateral plate mesoderm forms skeleton, tendons, ligaments and other CT (Chevallier et al., 1977; Wachtler et al., 1981), while somites give rise to myogenic cells, which delaminate from the dermomyotome to migrate towards limb buds (Chevallier et al. 1977; Ordahl & Le Douarin 1992). Classical embryological approaches have shown that limb lateral plate mesoderm contains positional information cues for limb formation (Grim and Wachtler, 1991; Michaud et al., 1997). Limb mesenchyme influences muscle patterning (Chevallier and Kieny, 1982), highlighting the importance of CT derivatives for limb musculoskeletal morphogenesis. In the developing limb, studies on CT differentiation first focused on collagen, the major component of the extracellular matrix and showed that type I and type III collagens are both expressed in dense regular and irregular CT (Kieny and Mauger, 1984). In adult tendons, type I collagen tends to replace type III collagen, while mature ICT and MCT are characterized by the expression of both type III and type VI collagens (Gara et al., 2011; Kieny and Mauger, 1984; Zhang et al., 2005).

More recently, the identification of specific molecular markers and genetic tools allowing the labelling and manipulation of CT fibroblasts has largely contributed to a better understanding of the role of CT types during limb development. The *Scleraxis (Scx)* gene, encoding a bHLH

transcription factor, is expressed in tendon and ligament cells (Schweitzer et al. 2001) and *Scx*^{-/-} mice showed severe defects in force-transmitting tendons (Murchison et al., 2007). *Scx* consequently characterizes dense regular CT (tendon and ligament). In contrast to tendon and ligament, the characterization of ICT and MCT remains challenging. *Tcf4*, a member of the *Tcf/Lef* family of transcription factors, is highly expressed in MCT during chick and mouse limb development (Kardon et al., 2003; Mathew et al., 2011). *TCF4* gain- and loss-of-function experiments demonstrated that *TCF4*-expressing cells contribute to limb muscle patterning in chick embryos (Kardon et al., 2003). Similarly, mispatterning and missplitting of limb muscles and tendons are observed after ablation of the T-box transcription factor *Tbx5* in mouse lateral plate-derived cells (Hasson et al., 2010). Interestingly, *Scx* and *Tcf4* genes have been shown to be critical regulators of fibrosis in different adult organs (Bagchi et al., 2016; Contreras et al., 2016). However, although *Tcf4* and *Tbx5* are considered as MCT-associated markers, *Tcf4* is also expressed in myogenic cells (Mathew et al., 2011) and *Tbx5* is also observed in cartilage, tendon and muscle progenitors of mouse limbs (Hasson et al. 2007), showing that both transcription factors are not specific to ICT and MCT. More recently, the zinc finger Odd Skipped-related factors *Osr1* and *Osr2* have been identified as being expressed in ICT during limb development, with a prevalence of *Osr2* expression in MCT (Stricker et al., 2006). *Osr1* and *Osr2* genes are not expressed in cartilage, tendon and ligament (Stricker et al., 2006). Both *Osr1* and *Osr2* drive ICT differentiation at the expense of cartilage and tendon and are required for the differentiation of ICT fibroblasts (Stricker et al., 2012). Overexpression of *OSR1* or *OSR2* in chick limb mesenchymal cells induces the expression of ICT markers such as *COL3A1* and *COL6A1*, while downregulating the expression of cartilage and tendon markers. Conversely, *OSR1* or *OSR2* inactivation downregulates *COL3A1* and *COL6A1* expression, while increasing cartilage formation in chick limb cells (Stricker et al., 2012).

These CT-associated transcription factors are regulated by signalling pathways during limb development. BMP4 represses *Tcf4* (Bonafede et al., 2006) and *Scx* expression (Schweitzer et al. 2001), while *SCX* expression is positively regulated by FGF4 (Edom-Vovard et al., 2002; Lejard et al., 2011). It is important to note that both BMP and FGF signalling pathways are involved in the regulation of tissue fibrosis in adult (Jenkins and Fraser, 2011; Kim et al., 2016). Chemokines are also important regulators of fibrosis (Wynn, 2008) and interestingly, CXCL12 and CXCL14 have been shown to delineate some CT subpopulations in embryonic limbs (García-Andrés and Torres, 2010; Gordon et al., 2011; Vasyutina et al., 2005). Identified CXCL12 functions during embryogenesis include essential roles in the migration

process of hematopoietic stem cells (Nagasawa et al. 1996; Zou et al. 1998), neurons (Bagri et al., 2002; Klein et al., 2001), germ cells (Molyneaux et al. 2003; Boldajipour et al. 2008) and limb skeletal muscle cells (Vasyutina et al., 2005). These functions are mediated by two chemokine receptors, CXCR4 and CXCR7 that signal individually in different cells or act in a cooperative manner in the same cell (Puchert and Engele, 2014). The role of CXCL14 during development remains more elusive but CXCL14 possesses chemoattractive activity for activated macrophages, immature dendritic cells and natural killer cells (Hara and Tanegashima, 2012) and it has been shown that CXCL14-forced expression suppresses tumour growth in mice (Izukuri et al., 2010; Tessema et al., 2010). Specific receptors for CXCL14 have not yet been identified but it is proposed that CXCL14 is a natural inhibitor of the CXCL12–CXCR4 axis (Tanegashima et al. 2013).

In this study, we show that CXCL12 and CXCL14 chemokines display distinct expression pattern in limb CT and regulate the expression of specific CT markers. We provide evidence that CXCL12 positively regulates the expression of *OSR1* and *COL3A1*, a major collagen subtype of the ICT, in chick embryonic limb and fibroblasts, while CXCL14 activates the expression of the tendon marker *SCX* in chick fibroblasts. Moreover, the expression of *CXCL12*, *CXCL14* and *OSR* genes is negatively regulated by BMP4, while the blockade of BMP activity is sufficient for *CXCL12*, *CXCL14* and *OSR1* expression. Lastly, the expression of *CXCL12*, *CXCL14* and CT markers is decreased in the absence of muscle contraction.

Results

***CXCL12* and *CXCL14* chemokines display distinct expression pattern in limb CT during chick development**

We analysed *CXCL12* and *CXCL14* expression pattern in chick limb at different developmental stages. Comparison was made with *OSR1*, *OSR2* and PDGFR α , three ICT-associated markers and with *SCX* for tendon CT. Limb muscles were visualized by myosin expression (MF20 antibody), which reveals differentiated muscle cells. At E5 of development, *CXCL12* displayed a diffuse expression in limb CT, with a strong expression in ICT between cartilage elements, overlapping with *OSR2* expression (Fig. 1A, C). *CXCL12* is also expressed in MCT (Fig. 1D), overlapping with *OSR1* and *OSR2* expression (Fig. 1D-F). *CXCL12* also colocalised with PDGFR α , which appeared widely expressed in the forelimb ICT and at a lower level in MCT (Fig. 1G-I), as previously describe (Orr-Urtreger et al. 1992; Tozer et al. 2007). At E5, *CXCL14* was expressed in forelimb ectoderm and faintly in proximal CT (Fig. 1J, arrow), in a region where *CXCL12* was also expressed (Fig. 1K, arrow). *CXCL14* exhibited a partial overlap with *OSR1* and *SCX* expression but not with that of *OSR2* in proximal limb regions (Fig. 1J, L-N, arrows). From E6, in addition to ectoderm expression, *CXCL14* was expressed in a subpopulation of ICT mostly located in limb ventral regions (Fig. 1O-Q). At E7, *CXCL12* expression was still observed in ICT and MCT, overlapping with *OSR1* expression in ICT and both *OSR1* and *OSR2* expression in MCT (Fig. 2A-F). At this stage, *CXCL12* expression partially overlaps with *COL1A1* but not with *SCX* (Fig. 2G-I). At E10, *CXCL12* expression was reminiscent of that of PDGFR α in ICT and MCT (Fig. 2J-L). In addition to ectodermal expression, *CXCL14* was observed in ICT surrounding cartilage (Fig. 2M), but also in MCT of a subgroup of ventral muscles (Fig. 2M, P) and in ICT at the vicinity of *SCX* expression (Fig. 2R, S). At this stage, *CXCL14* and *CXCL12* expression was not obviously overlapping in ICT, but partially colocalised around cartilage and in MCT (Fig. 2M, N). We then analysed the expression of CXCL12 receptors in chick embryonic limb. At E5, *CXCR7* receptor was observed in limb CT, with a strong expression in CT surrounding cartilage elements, while *CXCR4* was expressed in endothelial cells labelled with MEP21 (Fig. 3A-C), as already described in chick embryos (Escot et al., 2013; Escot et al., 2016). At E10, *CXCR7* appeared strongly expressed in ICT around muscles while *CXCR4* was still expressed in endothelial cells (Fig. 3D-F).

Taken together, our results show that *CXCL12* and *CXCL14* chemokines exhibit distinct and

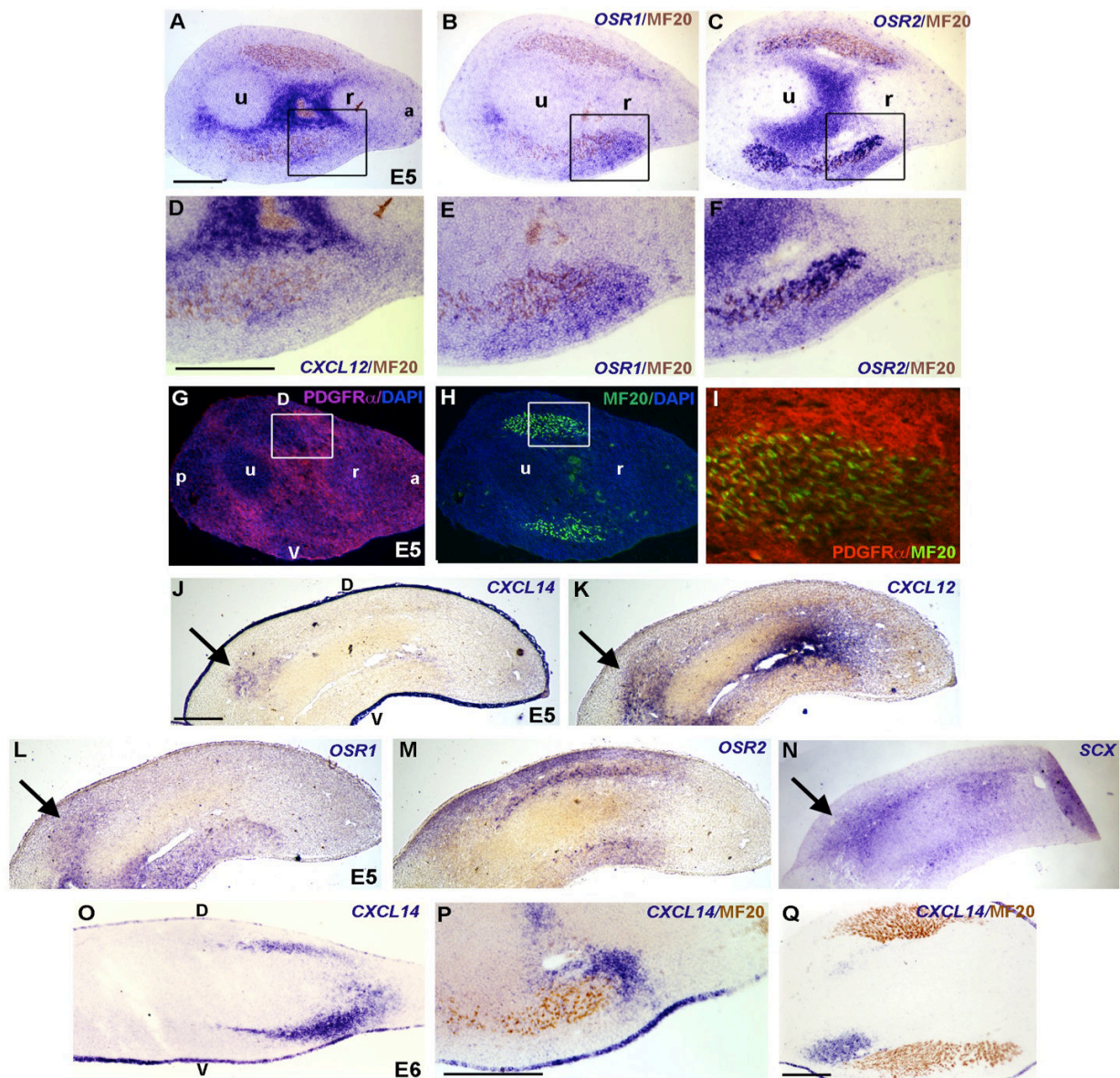


Figure 1: Chemokine expression in relation to CT markers in forelimb of E5 and E6 chick embryo. In situ hybridizations for *CXCL12* (A, D, K), *CXCL14* (J, O, P, Q), *OSR1* (B, E, L), *OSR2* (C, F, M) and immunohistochemical detections for PDGFR α (G, I), MF20 (A-F, H, I, P, Q) on serial transverse (A-I, Q) or longitudinal (J-P) sections. *CXCL12* is expressed in CT and MCT partially overlapping with *OSR1*, *OSR2* and PDGFR α expression. *CXCL14* exhibits a restricted expression in CT (J, O-Q), partially overlapping with *CXCL12*, *OSR1* and *SCX* expression (arrows in J, K, L, N). D, E, F, I represent high magnifications of the squared regions respectively in A, B, C and G, H. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μ m in A-C, G, H, J-O, Q; 100 μ m in D-F, P.

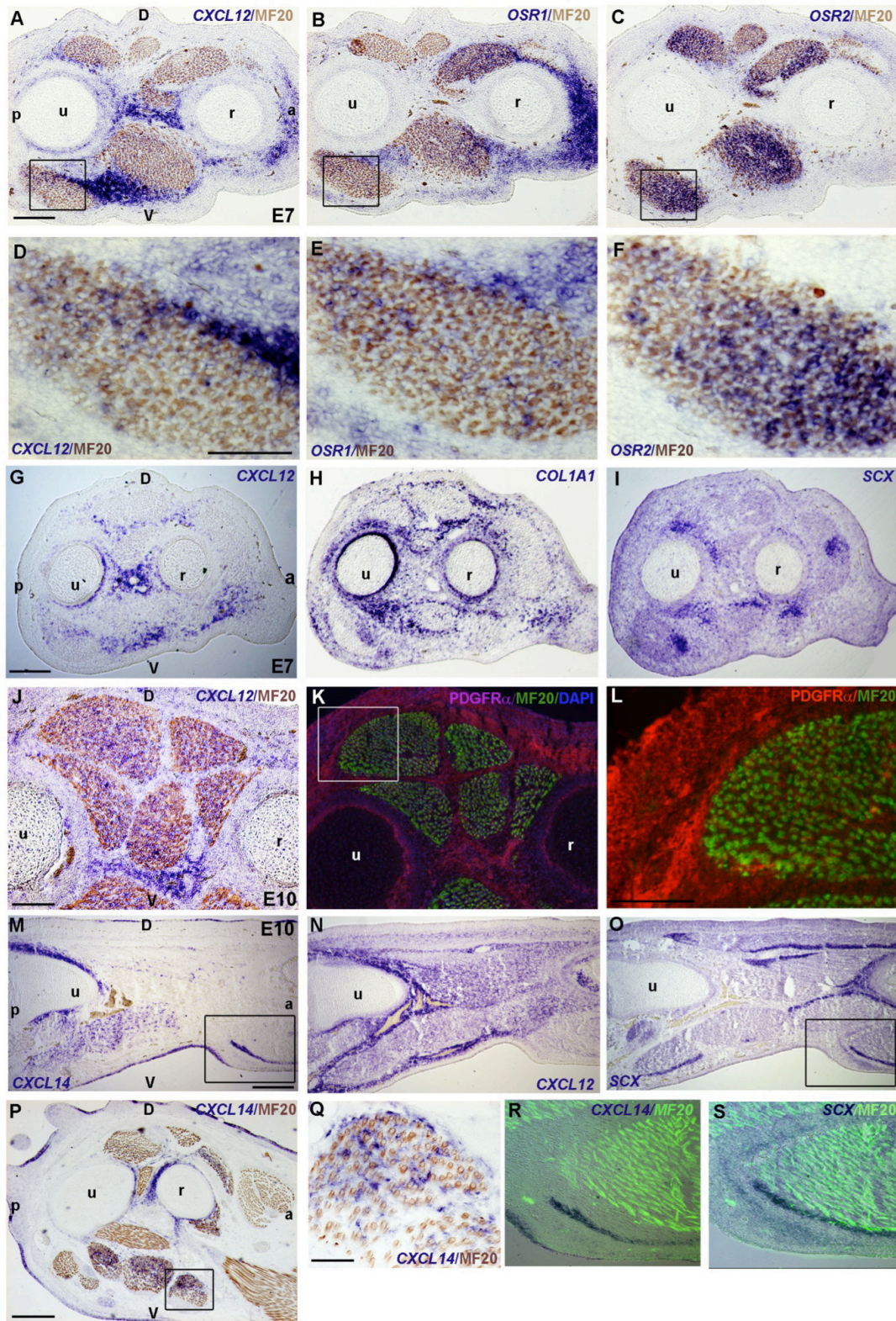


Figure 2: Chemokine expression in relation to CT markers in forelimb of chick embryo from E7 to E10. In situ hybridizations for *CXCL12* (A, D, G, J, N), *CXCL14* (M, P, R, Q), *OSR1* (B, E), *OSR2* (C, F), *COL1A1* (H), *SCX* (I, O, S) and immunohistochemical detections for PDGFR α (K, L), MF20 (A-F, J-L, P-S) on serial transverse (A-L, P, Q) or longitudinal (M-O, R, S) sections. *CXCL12* is expressed in CT and MCT partially overlapping with *OSR1*, *OSR2* and PDGFR α expression but with no evident colocalisation with *COL1A1* or *SCX* expression. *CXCL14* expression partially overlaps with *CXCL12* expression in CT and MCT and is observed in the vicinity of *SCX* expression in CT giving rise to tendon. D, E, F, L, R, S represent high magnifications of the squared regions respectively in A, B, C, K, N, O. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μ m in A-C, G-I, M-P; 100 μ m in D-F, J, K; 50 μ m in Q-S.

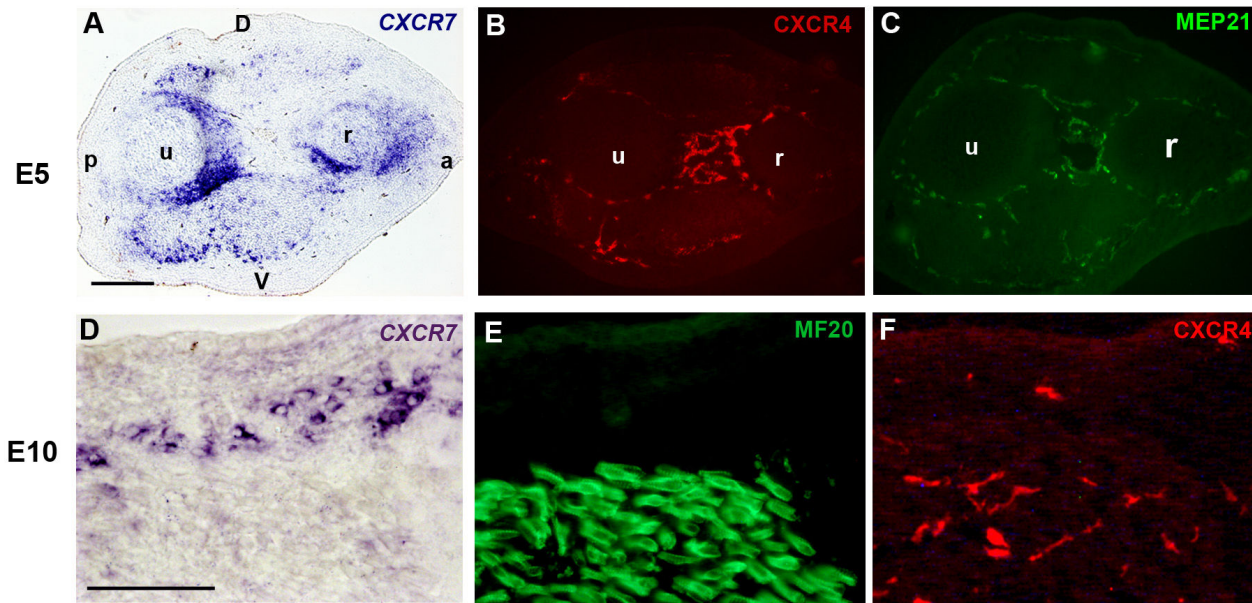


Figure 3: Expression of chemokine receptors during development of the chick forelimb. In situ hybridizations for *CXCR7* (A, D) and immunohistochemical detections for *CXCR4* (B, F), *MEP21* (C) and *MF20* (E) on serial transverse sections from E5 (A-C) and E10 (D-F) chick forelimbs. *CXCR7* is expressed in CT surrounding cartilage and muscles, while *CXCR4* is expressed in a sub-population of endothelial cells, as already described (Escot et al., 2013, 2016). D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μm in A-C, 100 μm in D-F.

dynamic expression patterns during chick limb development in ICT and MCT, as previously described (García-Andrés and Torres, 2010). *CXCL12* expression is widespread in limb ICT including MCT, while *CXCL14* is expressed in the vicinity of a subset of tendons and restricted to MCT of specific muscles, mostly located ventrally in limbs. The comparison of *CXCL12* and *CXCL14* expression with that of limb CT-markers shows a good correlation between the expression of *CXCL12* and *OSR* transcription factors, while *CXCL14* expression appears complementary in some limb regions to the tendon CT-marker *SCX*, suggesting a differential role for the two chemokines in limb CT regulation.

***CXCL12* and *CXCL14* regulate the expression of different CT markers during chick limb development**

To test the involvement of *CXCL12* and *CXCL14* in limb CT differentiation, we performed *CXCL12* and *CXCL14* gain-of-function experiments in vitro and in vivo. We first overexpressed *CXCL12* and *CXCL14* chemokines in primary cultures of chick embryonic fibroblasts using the replication-competent RCAS retrovirus system. Chick embryonic fibroblasts were transfected with recombinant retroviruses that spread in dividing cells, allowing a general expression of the gene of interest after 4 days of culture (Fig. 4A). *CXCL12* overexpression in chick embryonic fibroblasts led to a significant increase in the

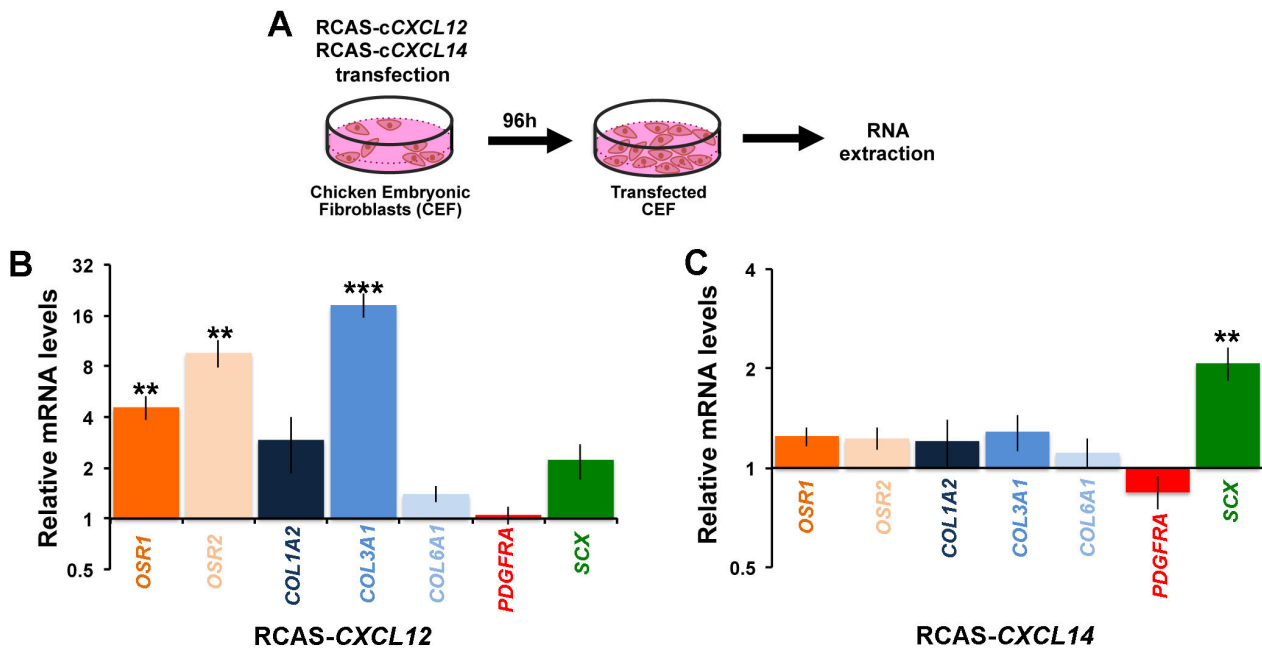


Figure 4: Overexpression of CXCL12 and CXCL14 chemokines increases the expression of CT markers in vitro. A: Experimental scheme of in vitro transfection of chicken embryonic fibroblasts (CEF) by RCAS viruses expressing chick *CXCL12* or *CXCL14* constructs. B, C: RT-qPCR analyses of the expression level of CT markers in CEF overexpressing *CXCL12* (B, n=6) or *CXCL14* (C, n=6) showing that *CXCL12* upregulates significantly *OSR1*, *OSR2* and *COL3A1*, while *CXCL14* increases *SCX*. For each gene, the mRNA levels of control cultures (n=6) were normalised to 1. P values were analysed by two-tail and unpaired Student's t-test using Microsoft Excel. **P<0.01; ***P<0.001; Error bars indicate s.d.

mRNA levels of *OSR1*, *OSR2* and *COL3A1* genes, while the expression of *COL1A2*, *COL6A1*, *PDGFRA* and *SCX* was not significantly modified (Fig. 4B). *CXCL14* overexpression significantly increased the mRNA levels of the tendon marker *SCX*, but did not change the expression levels of the other CT-associated markers (Fig. 4C). We next overexpressed *CXCL12* and *CXCL14* in ovo, by grafting RCAS-*CXCL12* or RCAS-*CXCL14* producing fibroblasts into limb buds of E4 chick embryos (Fig. 5A). The replication-competent retrovirus RCAS spread in chick dividing cells over development and this led to ectopic gene expression in limb regions (Edom-Vovard et al., 2002). Embryos were collected at E10 and the expression of CT markers was analysed by in situ hybridization to transverse limb sections (Fig. 5A). Ectopic *CXCL12* expression (Fig. 5Bb, h) resulted in an increase of *OSR1* and *COL3A1* expression in the infected limb regions (Fig. 5Bd, f, j, l) compared to control limbs (Fig. 5B). Interestingly, while ectopic *COL3A1* expression corresponds to ectopic *CXCL12* expression (Fig. 5Bh, j), *OSR1* expression upon ectopic *CXCL12* was stronger in ICT surrounding muscles than in MCT (Fig. 5Bb, d, f). We did not observe any

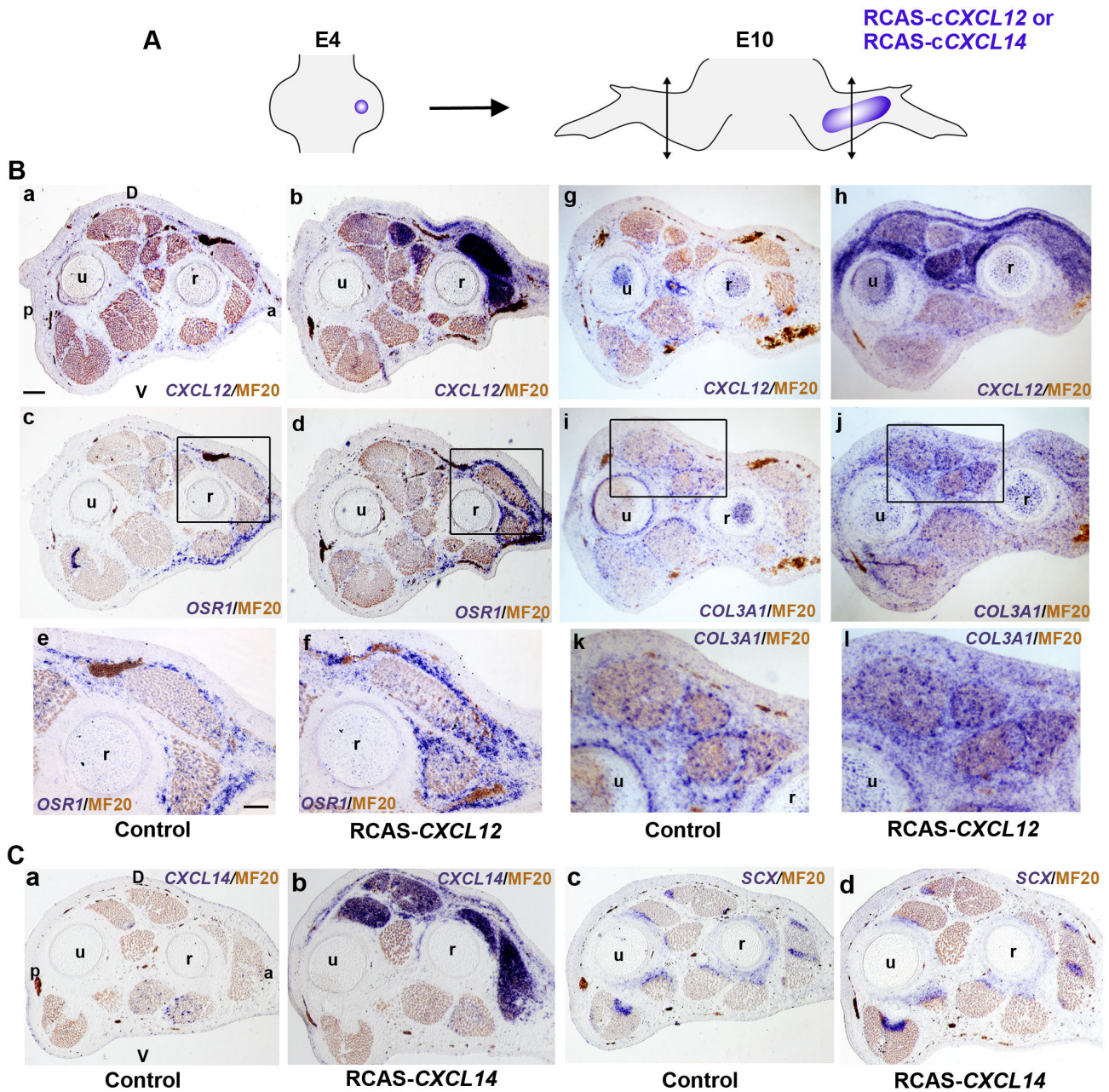


Figure 5: Overexpression of CXCL12 and CXCL14 chemokines in vivo increases the expression of CT markers in the chick embryonic forelimb. A: Experimental scheme of the grafting procedure of CEF pellets expressing chick *CXCL12* or *CXCL14* constructs in the chick embryonic forelimb. B: Ectopic expression of *CXCL12* upregulates *OSR1* and *COL3A1* expression (n=6). In situ hybridizations for *CXCL12* (a, b, g, h), *OSR1* (c-f) and *COL3A1* (i-l) on serial transverse sections of control (a, c, e, g, i, k) and grafted (b, d, f, h, j, l) E10 chick forelimbs. e, f, k, l represent high magnifications of the squared regions respectively in c, d, i, j. C: Ectopic expression of *CXCL14* does not modify *SCX* expression (n=6). In situ hybridizations for *CXCL14* (a, b) and *SCX* (c, d) on serial transverse sections of control (a, c) and grafted (b, d) E10 chick forelimbs. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μ m in B (a-d, g-j), C (a-d); 100 μ m in B (e, f, k, l).

obvious change of *OSR2* expression by in situ hybridization in the RCAS-*CXCL12*-infected regions (data not shown). *CXCL14* overexpression did not induce ectopic *SCX* expression in chick forelimbs when compared to control limbs (Fig. 5C), although we cannot exclude an increase of *SCX* expression in tendons.

Altogether, these results show that *CXCL12* and *CXCL14* chemokines regulate differentially CT-associated transcription factors in vivo and in vitro. *CXCL12* positively regulates the expression of *OSR1* and *COL3A*, two ICT markers, while *CXCL14* regulates *SCX*, a CT-tendon marker.

***CXCL12* and *CXCL14* expression is not regulated by FGF4**

During development, FGF4 positively regulates *SCX* expression (Edom-Vovard et al., 2002; Havis et al., 2016) and the expression of ICT collagens, including *COL1A1*, *COL3A1* and *COL6A1* (Lejard et al., 2011) in chick limbs. Interestingly, FGF has been shown as one of the signalling pathways implicated in fibrosis regulation in different pathological situations (Kim and Braun, 2015). We then analysed the effects of FGF4 overexpression on *CXCL12* and *CXCL14* expression in chick limbs. FGF4 beads were implanted into limbs of E4.5 chick embryos and fixed 48 hours later (Fig. 6A). As previously shown (Edom-Vovard et al., 2002; Havis et al., 2016; Lejard et al., 2011), *SCX* expression was strongly induced at the vicinity of FGF4 beads in grafted limbs compared to normal *SCX* expression in control limbs (Fig. 6Ba, b, g, h). However, no ectopic expression of *CXCL12* and *CXCL14* was observed in ectopic *SCX* expression domains (Fig. 6Ba-f). In addition, we did not observed any *OSR1* and *OSR2* activation around FGF4 beads (Fig. 6Bg-l). Moreover, an inhibition of both *OSR1* and *OSR2* expression was observed in the vicinity of FGF4 beads (Fig. 6Bh, j, l) compared to *OSR* endogenous expression in control limbs (Fig. 6Bg, i, k). Overexpression of FGF4 did not induces *CXCL12* and *CXCL14* expression but leads to the inactivation of both *OSR1* and *OSR2* genes in chick limbs, while increasing *SCX* expression, as previously described (Edom-Vovard et al., 2002; Havis et al., 2016; Lejard et al., 2011). It is likely that *OSR* gene downregulation observed after FGF4 bead graft is independent of *CXCL12* and *CXCL14* chemokines, but, as *OSR1* and *OSR2* overexpression inhibits *SCX* expression in chick limb cells (Stricker et al., 2012), these results suggest that *SCX* and *OSR* genes mutually repress each other in mesenchymal cells during chick limb development.

These results show that ectopic expression of FGF4 does not modify *CXCL12* and *CXCL14*.

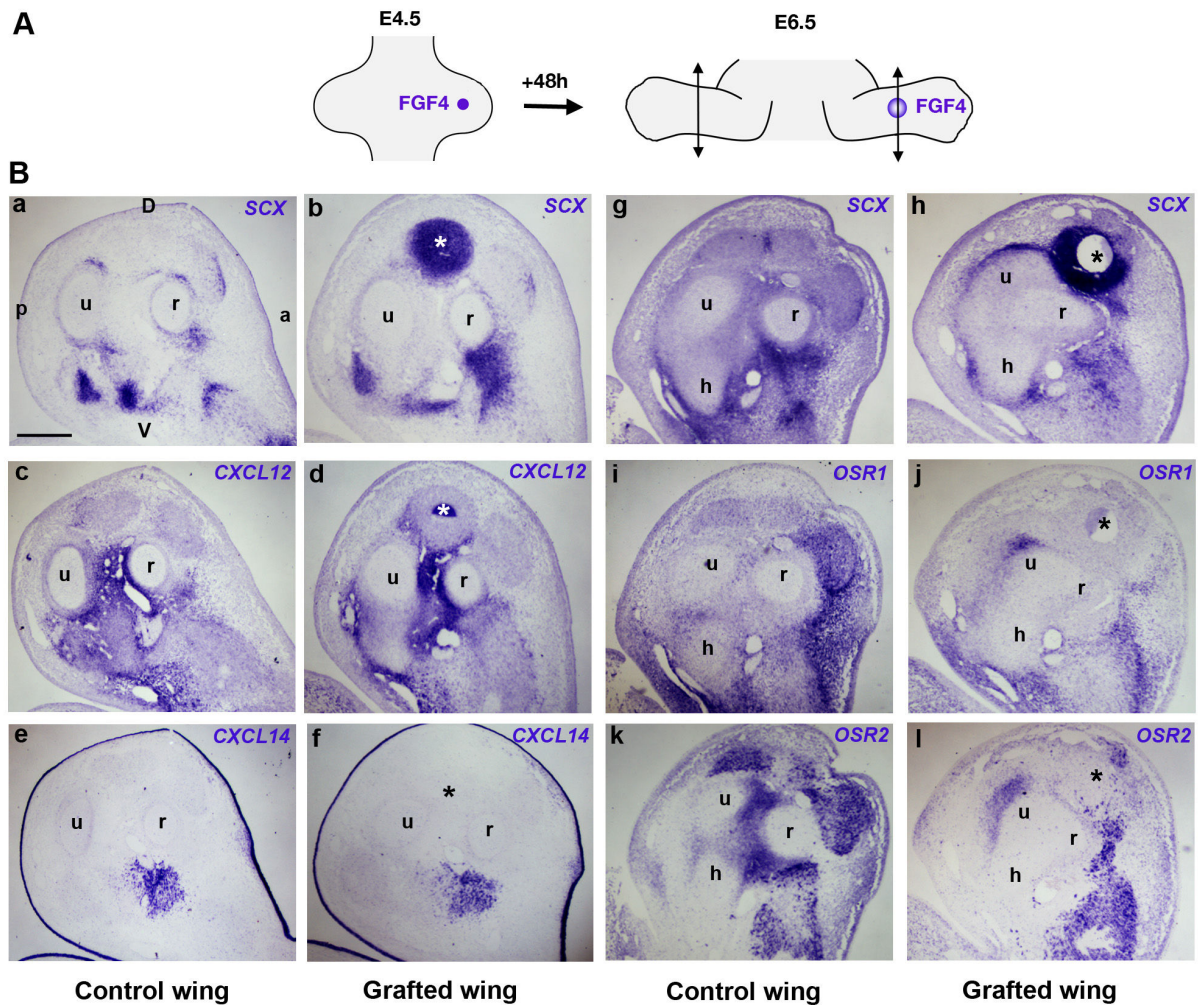


Figure 6: FGF signalling regulates the expression of CT markers independently of chemokines in the chick embryonic forelimb. A: Experimental scheme of the grafting procedure of heparine beads coated with mouse FGF4 recombinant protein in the chick embryonic forelimb. B: 48h after the graft (n=6). FGF signalling activates *SCX* expression and represses *OSR1* and *OSR2* expression without affecting *CXCL12* and *CXCL14* expression in the chick embryonic forelimb. In situ hybridizations for *SCX* (a, b, g, h), *CXCL12* (c, d), *CXCL14* (e, f), *OSR1* (i, j) and *OSR2* (k, l) on serial transverse sections of control (a, c, e, g, j, k) and grafted (b, d, f, h, j, l) E6.5 chick forelimbs. Asterisks indicate the grafted bead. D: dorsal, V: ventral, a: anterior, p: posterior, h: humerus, r: radius, u: ulna. Bars: 250 μ m in C (a-l).

The anti-fibrotic BMP signalling pathway negatively regulates the expression of *CXCL12* and *CXCL14*

BMP signalling has been demonstrated as an antagonist of fibrosis in various organs (Weiskirchen et al. 2009) but very few studies have investigated its potential role in the regulation of CT types during development. Conversely to FGF4, BMP4 inhibits *SCX* expression in chick embryonic limbs (Schweitzer et al. 2001; 2010).

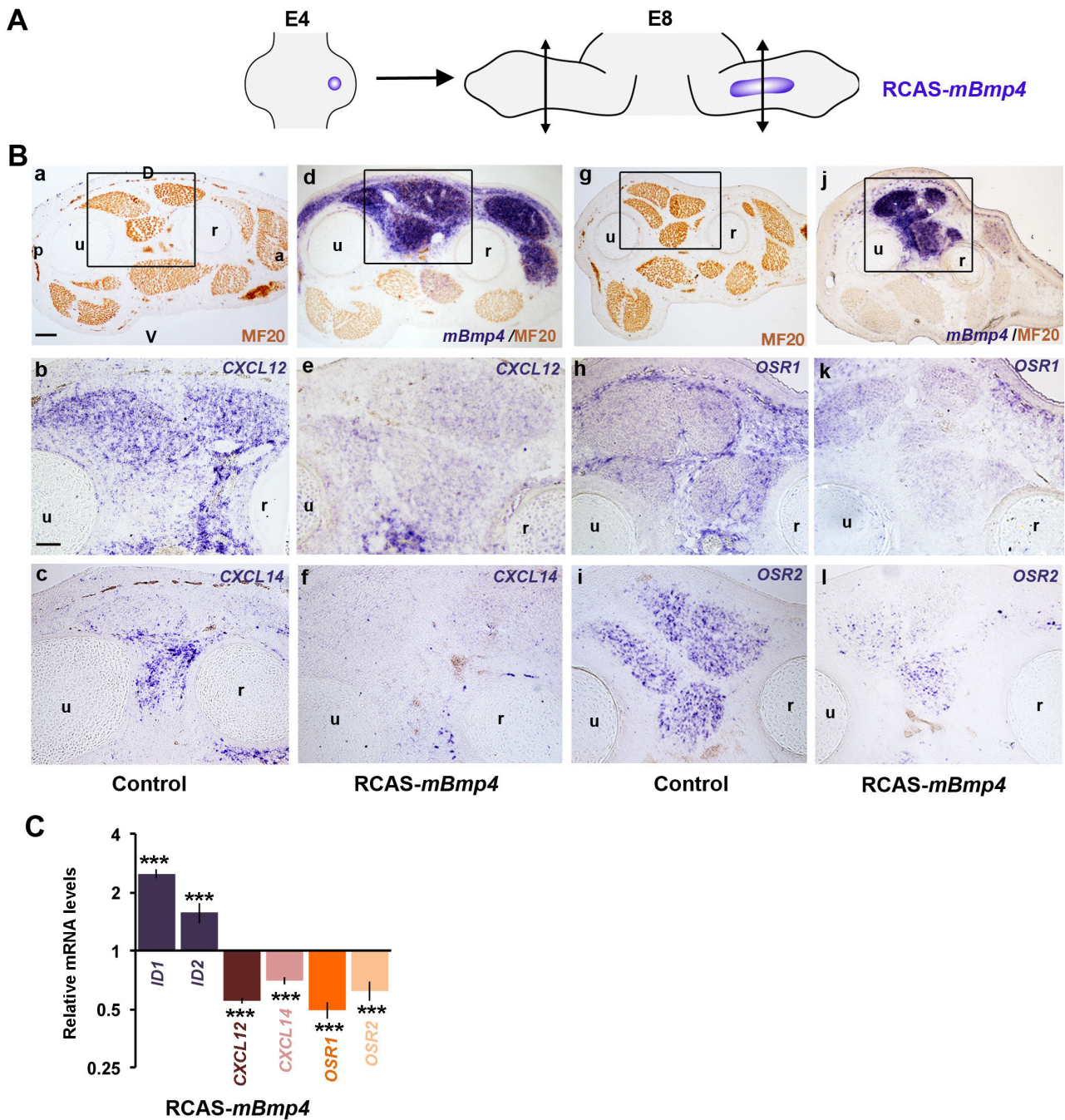


Figure 7: BMP signaling pathway down-regulates the expression of chick CT markers in vivo and in vitro. BMP signalling represses *CXCL12*, *CXCL14*, *OSR1*, and *OSR2* expression in the chick forelimb CT and MCT (n=5). **A:** Experimental scheme of the grafting procedure of CEF pellets expressing mouse *Bmp4* construct in the chick embryonic forelimb. **B:** In situ hybridizations for *CXCL12* (b, e), *CXCL14* (c, f), *OSR1* (h, k), *OSR2* (i, l), *mBmp4* (d, j) and immunohistochemical detection for MF20 (a, d, g, j) on serial transverse sections of control (a-c, g-i) and grafted (d-f, j-l) E8 chick forelimbs. The squared regions in a, d, g, j delineate the high-magnified parts of the adjacent sections revealed for chemokines or CT markers. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μ m in B (a, d, g, j), 100 μ m in B (b, c, e, f, h, k, i, l). **C:** RT-qPCR analyses of expression level of readout targets of BMP4 signalling and of CT markers in CEF expressing RCAS-*mBmp4* construct, showing that BMP4 expression significantly decreases *CXCL12*, *CXCL14*, *OSR1* and *OSR2* expression (n=6). For each gene, the mRNA levels of control cultures (n=6) were normalised to 1. P values were analysed by two-tail and unpaired Student's t-test using Microsoft Excel. ***P<0.001; Error bars indicate s.d.

We then looked at the possible BMP effect on CXCL12 and CXCL14 expression in vivo by overexpressing BMP4 in chick limbs. Pellets of transfected RCAS-*mBmp4* fibroblasts were grafted into limbs of E4 embryos, which were collected at E8 to analyse the expression of chemokines and CT markers (Fig. 7A). *Bmp4* overexpression resulted in a downregulation of *CXCL12* and *CXCL14* expression (Fig. 7Bd-f) and a concomitant decrease in both *OSR1* and *OSR2* expression in limb mesenchyme (Fig. 7Bj-l), compared to control limbs (Fig. 7Ba-c, g-i). These results were supported in vitro by the analysis of mRNA levels of chemokines and CT markers in embryonic fibroblasts overexpressing *Bmp4*, which showed downregulation of *CXCL12*, *CXCL14*, *OSR1* and *OSR2* expression (Fig. 7C). These results evidence that BMP4 gain-of-function experiments lead to a decrease of chemokine and CT marker expression in chick limb cells. In order to test if BMP loss-of-function experiments would provoke the opposite result, BMP signalling pathway was inhibited in vivo by overexpressing the BMP antagonist Noggin in chick limbs (Wang et al., 2010). Pellets of transfected RCAS-*cNOGGIN* producing fibroblasts were grafted into limbs of E4 embryos, which were collected at E9 to analyse the consequences for the expression of chemokines and CT markers (Fig. 8A). *NOGGIN* overexpression led to an upregulation of *CXCL12* and *CXCL14* expression in both ICT and MCT (Fig. 8Ba, b, f, g) and a concomitant increase in *OSR1* expression (Fig. 8Bh), compared to control limbs (Fig. 8Bc-e). In this experimental design, *OSR2* and *SCX* expression was not obviously modified (data not shown).

Taken together, these data show that the anti-fibrotic factor BMP4 negatively regulates the expression of *CXCL12* and *CXCL14* chemokines and *OSR1* and *OSR2* transcription factors, while the inhibition of BMP signalling pathway is sufficient to induce the expression of *CXCL12*, *CXCL14* and *OSR1* in chick limbs.

Mechanical forces act upstream CXCL12 and CXCL14 to regulate CT markers

The musculoskeletal system is sensitive to mechanical loads exerted by muscle contractions. Mechanical forces generated by muscle activity have been shown to be crucial for the formation of components of the musculoskeletal system during development (Esteves de Lima et al. 2016; Havis et al. 2016). In the adult, studies indicate that mechanical forces contributes to fibrosis by regulating CT fibroblasts in bone, cartilage and interstitial tissues of most organs (Carver and Goldsmith, 2013). During development, *SCX* expression is sensitive to mechanical forces in tendons of mouse and chick limbs (Huang et al. 2015; Havis et al. 2016) but no investigation has been conducted concerning the role of mechanical

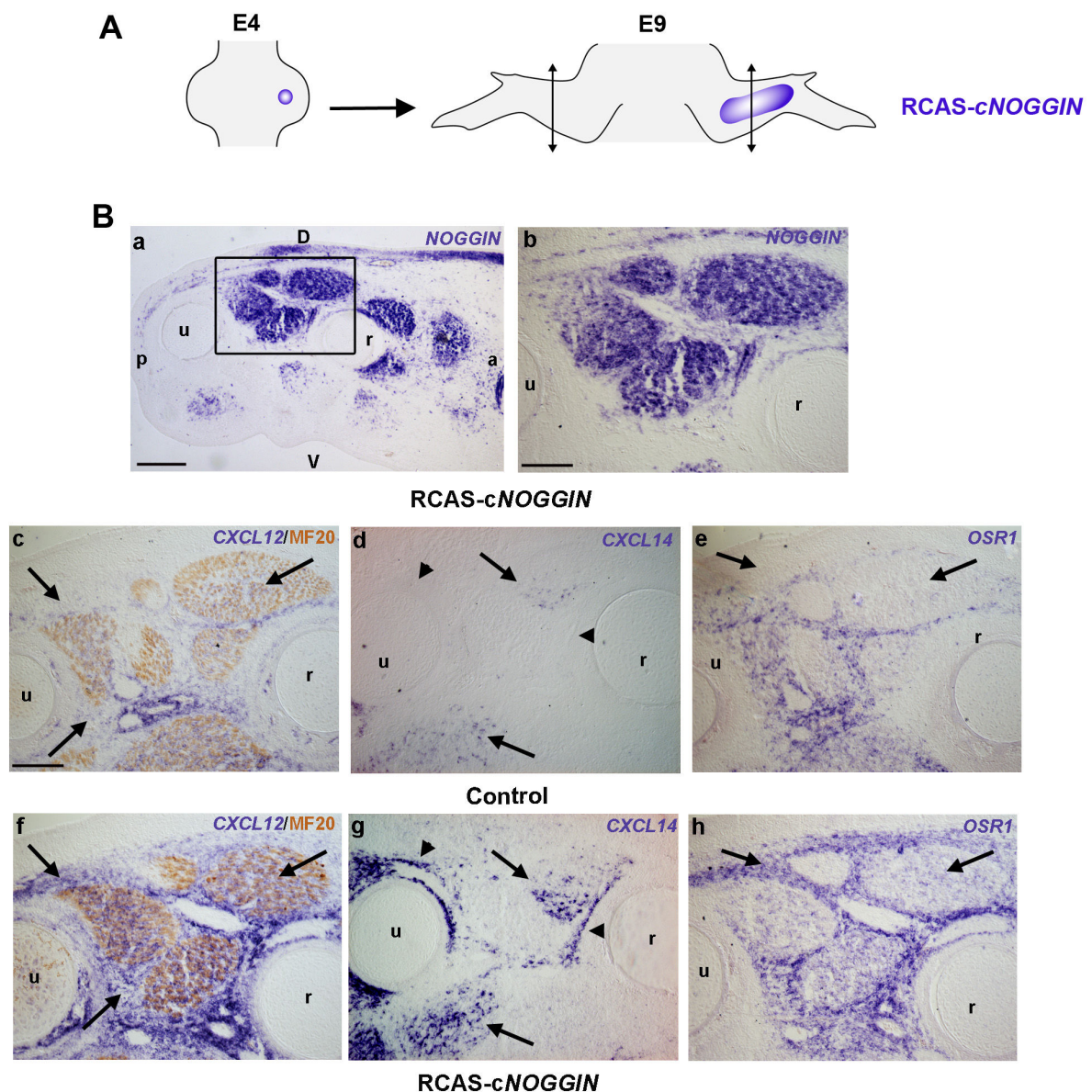


Figure 8: Inhibition of BMP signalling activates the expression of chick CT markers in vivo.

BMP inhibition increases *CXCL12*, *CXCL14* and *OSR1* expression in the chick forelimb CT and MCT (n=4). A: Experimental scheme of the grafting procedure of CEF pellets expressing chick *NOGGIN* construct in the chick embryonic forelimb. B: In situ hybridizations for *NOGGIN* (a, b), *CXCL12* (c, f), *CXCL14* (d, g), *OSR1* (e, h) and immunohistochemical detection for MF20 (c, f) on serial transverse sections of control (c-e) and grafted (f-h) E9 chick forelimbs. The squared regions in a delineate the high-magnified region of the adjacent sections revealed for *NOGGIN*, chemokines or CT markers. Arrows indicate the expression in CT and MCT. Arrowheads indicate the expression in perichondrium. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. Bars: 200 μ m in A (b-h), 100 μ m in A (a).

signals on ICT and MCT during development. To assess the effect of mechanical forces in this context, chick embryos were immobilised using the decamethonium-bromide (DMB) pharmacological agent, an acetylcholine agonist leading to rigid muscle paralysis and immobilisation of the embryo (Nowlan et al., 2010). DMB or control solutions were applied in E4.5 embryos and limbs were collected 2 or 3 days later to analyse the consequences for the expression of chemokines and CT marker by in situ hybridization and RT-qPCR. DMB has been shown to bind motor endplates and block muscle contraction (Pitsillides, 2006). As neuromuscular contractions in chick embryonic limbs are effective from E5.5 (Landmesser and Pilar, 1974), limb muscles were not grossly affected at E6.5 after two days of immobilisation (Fig. 9Ah, l), while paralysis leads to muscle degeneration later during development, as previously described (Fredette and Landmesser, 1991). *CXCL12* and *CXCL14* expression was reduced in MCT and ICT surrounding muscles in paralysed limbs (Fig. 9Ab, d). However, *CXCL12* expression was maintained in CT surrounding bones, while *CXCL14* was still expressed in the ectoderm in immobilised conditions (Fig. 9Ab, d). The expression of *OSR1* and *OSR2* was also decreased in the absence of mechanical activity compared to control limbs (Fig. 9Ae-j). RT-qPCR analyses confirmed the downregulation of the mRNA levels of *CXCL12*, *CXCL14*, *OSR1* and *OSR2* in paralysed limbs (Fig. 9B). RT-qPCR analyses also showed that the mRNA levels of *COL1A2*, *COL3A1* and *COL6A1* genes were significantly reduced in immobilised conditions. The general decrease of CT marker expression cannot be attributed to a loss of CT cells, since TCF4-positive cells were still observed in limbs of immobilised and control embryos (Fig. 9Ah, l). taken together, these results show that the expression of *CXCL12* and *CXCL14* and that of CT markers is sensitive to mechanical forces during limb development.

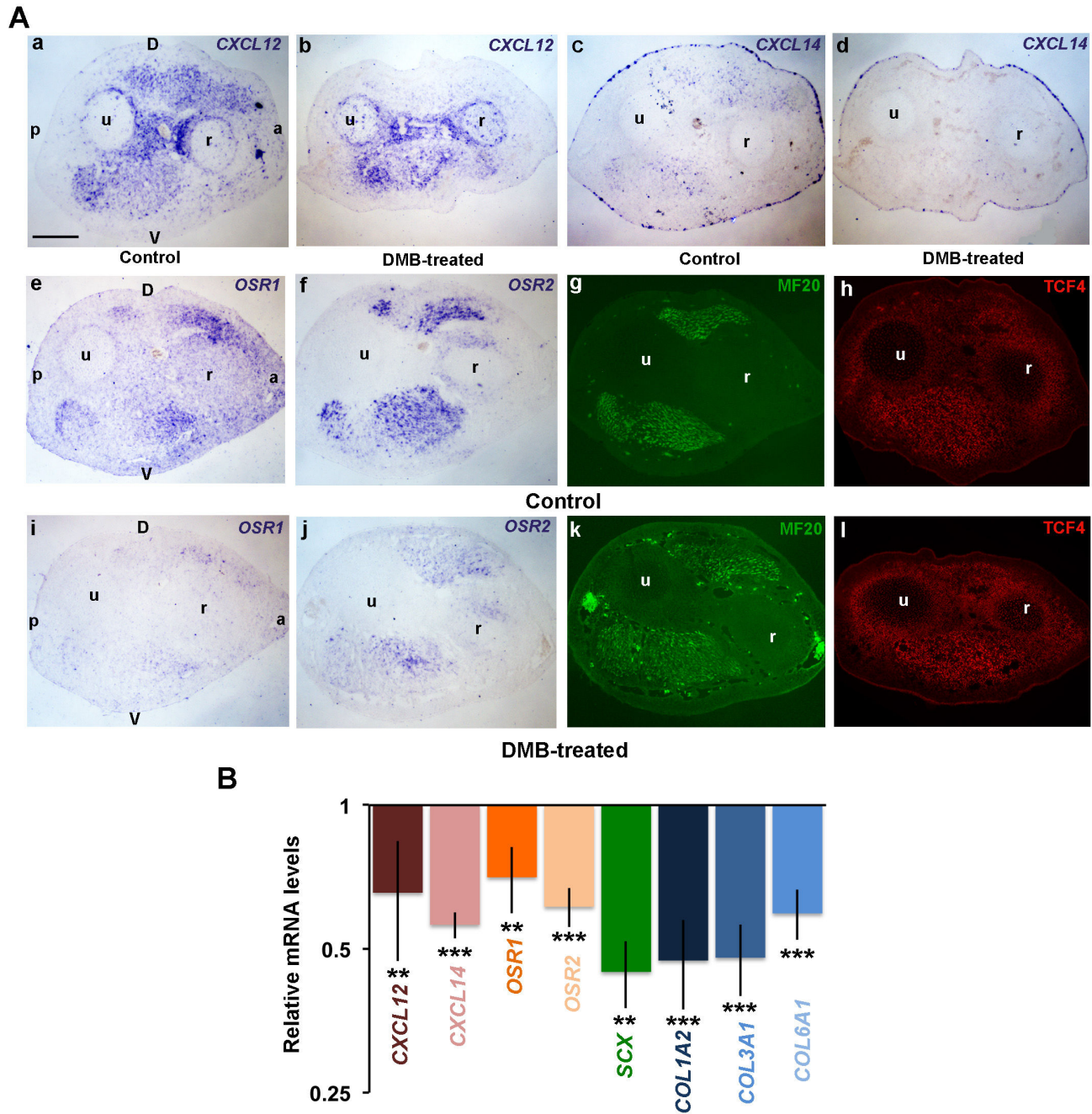


Figure 9: Mechanical forces act upstream CXCL12 and CXCL14 chemokines to regulate the expression of CT markers in the chick embryonic forelimb. A: Immobilisation of the chick embryonic forelimb downregulates the expression of CXCL12 and CXCL14 and of the CT markers (n=3). In situ hybridizations for *CXCL12* (a, b), *CXCL14* (c, d), *OSR1* (e, i), *OSR2* (f, j) and immunodetection of MF20 (g, k) and TCF4 (h, l) on serial transverse sections of control (a, c, e-h) and DMB-treated (b, d, i-l) E6.5 chick forelimbs. D: dorsal, V: ventral, a: anterior, p: posterior, r: radius, u: ulna. B: RT-qPCR analyses of *CXCL12*, *CXCL14* and CT markers in E7.5 immobilized chick forelimbs (n=4) showing that both chemokine and CT marker expressions are reduced in immobilisation conditions. For each gene, the mRNA levels of control limbs (n=4) were normalized to 1. P values were analyzed by two-tail and unpaired Student's t-test using Microsoft Excel. **P<0.01; ***P<0.001; Error bars indicate s.d.

Results

II. The contribution of connective tissue cells to muscle morphogenesis: an unexpected role for CXCL12 and CXCL14 signalling pathways

II. The contribution of connective tissue cells to muscle morphogenesis: an unexpected role for CXCL12 and CXCL14 signalling pathways

Muscle morphogenesis process is mediated by consecutive waves of embryonic and foetal myogenesis, which participate in the establishment of muscle scaffold and growth, respectively. The intrinsic capacities of muscle progenitors highly contribute to muscle development, however, they are not sufficient to build proper muscles with a correct shape, size and location in the body. Besides the intrinsic capacities of muscle progenitors, it emerges years ago that signals emanating from adjacent tissues might be important for the correct development of muscles (Chevallier and Kieny, 1982). The connective tissue (CT), corresponding to a broad family of tissues that maintain and connect organs together, recently became a source of interest in the field of muscle morphogenesis. For a long time, due to the lack of specific markers, the role of CT in muscle formation has been poorly investigated, but recently the identification of specific molecular markers has contributed to renewed interest in the field of CT-contribution to muscle development (Kardon 1998; Kardon et al. 2003; Hasson et al. 2010). Although different transcription factors specific to CT cells have been implicated in the formation of muscle, the molecular signalling involved in CT- muscle interactions during development remains to be elucidated. Chemokines have been shown as important actors involved in CT regulation in adult organ homeostasis and we have previously shown that CXCL12 and CXCL14 chemokines, expressed in the ICT of embryonic chick limb, positively regulate the expression of different CT-associated markers in embryonic fibroblasts (Nassari et al., 2017 submitted).

Consequently, the second part of my PhD project was to study whether CXCL12 and CXCL14 chemokines were involved in muscle development in a non-cell autonomous manner as signals secreted from limb CT.

We first investigated the expression pattern of CXCR4 and CXCR7 receptors during limb muscle morphogenesis in chick embryo. Then, the putative involvement of CXCL12 and CXCL14 in CT-mediated muscle morphogenesis was assessed using gain- and loss-of-function approaches of chemokines and their receptors, both during myogenesis in vitro and limb muscle morphogenesis in vivo. Finally, we tried to decipher the molecular mechanisms underlying CT-derived CXCL12 or CXCL14 mediated muscle development.

Introduction

During development, muscle morphogenesis is an important step in the formation of the musculoskeletal system. This process is mainly driven by the intrinsic proliferation and differentiation capacities of muscle progenitor cells that are orchestrated through the expression of the paired-homeobox transcription factors PAX3/PAX7 and Myogenic Regulatory transcription Factors (MRFs) MYOD, MYF5, MRF4 and MYOG (Buckingham and Rigby, 2014). During embryonic development, PAX3 and PAX7 are expressed respectively in embryonic and foetal muscle progenitors, in which they promote the expression of the MRFs that promote activation of the myogenic program (Relaix et al. 2004; 2005; Buckingham & Rigby 2014). PAX3-positive muscle progenitors constitute the pool of embryonic myoblasts forming the primary fibres that are necessary to constitute the scaffold of muscles (Relaix et al. 2004; 2005). Subsequently, a second wave of myogenesis defined as foetal myogenesis occurs, during which PAX7-expressing muscle progenitors participate in the formation of secondary fibres, contributing to muscle growth and maturation (Relaix et al. 2004; 2005). Although intrinsic capacities of muscle progenitors are critical for the proper formation of muscles, it emerged years ago that signals arising from adjacent tissues, among which blood vessels, nerves and connective tissues (CTs) might contribute in the process of muscle development (Tozer et al. 2007; Ashby et al. 1993; Merrifield and Konigsberg 1987; Robson and Hughes 1999; Chevallier and Kieny 1982; Kardon 1998).

CTs refer to a broad family of tissues, which connect and support organs together (Nassari et al., 2017). The specialized CT corresponds to bone and cartilage while the dense CT is subdivided in regular and irregular CT, referring respectively to tendon/ligament and CT embedding organs. At the muscle level, the irregular CT (ICT) correspond to the ICT surrounding muscles and to the muscle irregular CT (MCT) present inside the muscle (Nassari et al., 2017). Importance of dense CTs on muscle morphogenesis has been highlighted a long time ago using classical embryological approaches, which have shown that CT contributes to initial limb muscle patterning (Chevallier and Kieny, 1982) and that disruption of tendons elements leads to muscle mispatterning (Kardon 1998). More recently, the identification of CT-specific transcription factors has contributed to precisely investigate the role of ICT and MCT in muscle morphogenesis. The TCF4 transcription factor that belongs to the Tcf/Lef family is the first molecular marker that has been identified to be expressed in irregular CT fibroblasts (Kardon et al., 2003). During chick limb development, the TCF4 transcription factor is restricted to MCT (Kardon et al., 2003) and gain- and loss-of-

function experiments in chick and mouse embryos have demonstrated that *TCF4* established a pre-pattern for muscle differentiation (Kardon et al., 2003; Mathew et al., 2011). TBX5, a T-box transcription factor has also been identified as a specific MCT marker (Hasson et al. 2007; Hasson et al. 2010) and deletion of *Tbx5* in forelimbs of mouse embryos leads to defect in muscle and tendon patterning (Hasson, 2011). However *Tcf4* and *Tbx5* are not restricted to ICT as *Tbx5* is also expressed in specialized and regular CT (Hasson et al. 2007) and *Tcf4* is detected at low level in myogenic cells (Mathew et al., 2011).

Embryonic limb ICT also expresses the two Odd-skipped orthologs, *Osr1* and *Osr2* (Stricker et al., 2006), which promotes the expression of ICT markers such as *COL3A1* and *COL6A1* (Stricker et al., 2012). Overexpression of *OSR1* or *OSR2* in muscle cells in vitro or in chick embryonic limbs in vivo leads to a decrease in myogenic differentiation (Stricker et al., 2012). Furthermore, in adult, non-myogenic cells presenting a dense CT identity have been shown as implicated in muscle regeneration (Joe et al. 2010; Uezumi et al. 2010; Judson et al. 2013). Two different groups have characterized adult muscle resident non-myogenic progenitor cells named Fibro/Adipogenic Progenitors (FAPs) (Joe et al. 2010) and mesenchymal progenitors (MPs) (Uezumi et al., 2010) presenting both fibrogenic and adipogenic differentiation potentials. Upon muscle injury, FAPs/MPs are activated and favor muscle regeneration (Joe et al. 2010; Uezumi et al. 2010; Judson et al. 2013), but their prolonged activation can induce a dramatic excess of collagen deposition, leading to fibrosis and ultimately to muscle dysfunction (Joe et al. 2010). These data show that non-myogenic dense CT cells define a myogenic niche that regulates developmental and regenerative myogenesis. However, the molecular pathways underlying the crosstalk between dense CT and myogenic cells are not yet identified.

In adult organs, fibrosis is induced by secreted factors resulting from the inflammation process, among which chemokines appear as important players (Wynn, 2008). Both CXCL12 and CXCL14 have been shown as implicated in inflammatory and cancer-related fibrosis (Rodriguez-Nieves et al. 2016; Östman and Augsten 2009; Lin et al. 2014). Following muscle injury, CXCL12, secreted by the MCT, has been shown to actively participate and promote the regeneration process (Brzoska et al., 2015; Griffin et al., 2010; Kowalski et al., 2016), while conversely, CXCL14 inhibits muscle regeneration (Waldemer-Streyer et al., 2017). During embryonic development, the pleiotropic effect of CXCL12, mostly conducted via its chemotactic guidance properties, has been demonstrated in hematopoiesis (Nagasawa et al. 1996; Zou et al. 1998), angiogenesis (Salcedo et al., 1999; Salvucci et al., 2002) and neural crest cell (Escot et al. 2013; 2016), neuron (Liapi et al., 2007; Lysko et al., 2011) or

primordial germ cell migration (Stebler et al., 2004). Biological functions of CXCL14 during development remain more elusive but it is involved in the patterning of the neurovascular network in the eye of chick embryos (Ojeda et al., 2017) and it has been shown to possess chemoattractant capacities, mostly on immune cells (Cao et al., 2013). CXCL12 can signal through the classical CXCR4 and atypical CXCR7 G-protein coupled receptors (Puchert and Engele, 2014). While CXCR4 mediates classical G-protein-related intracellular signals (Pozzobon et al., 2016), CXCR7 transduces β -arrestin-dependent signalings (Miao et al. 2007; Wang et al. 2008). To date, no signalling receptor has been identified for CXCL14, however it is known that CXCL14 is able to bind to CXCR4 in order to modulate CXCL12/CXCR4 axis (Tanegashima et al. 2013).

Both CXCL12 and CXCL14 have been shown to be involved in myogenesis. CXCL12, emanating from the limb mesenchyme, is implicated in the oriented migration of muscle progenitors expressing CXCR4 (Vasyutina et al., 2005). Contradictory results emanate from in vitro studies, which have shown that CXCL12 can inhibit (Hunger et al., 2012) or enhance myogenic differentiation (Melchionna et al., 2010; Odemis et al., 2007) acting on CXCR4. An in vitro study has demonstrated the benefic effect of CXCL12/CXCR7 axis on myogenic differentiation (Hunger et al., 2012). The role of CXCL14 during myogenesis has been poorly investigated but previous studies performed in vitro characterized CXCL14 as an inhibitor of myogenic differentiation (Ge et al., 2013; Waldemer-Streyer et al., 2017). During chick and mouse embryonic development, CXCL12 and CXCL14 have been shown also to be expressed in limb ICT and MCT (García-Andrés and Torres, 2010; Gordon et al., 2011; Rehimy et al., 2008; Vasyutina et al., 2005). Furthermore, we have previously demonstrated that the distinct expression of CXCL12 and CXCL14 chemokines in limb CT regulates the expression of specific CT markers. CXCL12 enhances the expression of *OSR* genes and *COL3A1*, a major collagen subtype of the ICT, while CXCL14 regulates the expression of the tendon marker *SCX* (Nassari et al., under review). Taken together, these observations suggest that CXCL12 and CXCL14 might be secreted signals emanating from the dense CT, which contribute to limb muscle morphogenesis.

In this study, we have investigated the role of CXCL12 and CXCL14 as non-cell autonomous signals arising from the dense CT cells and acting on myogenic cells. We show that, during chick limb myogenesis, CXCR7 receptor progressively replaces CXCR4 expression in muscle progenitors to be finally restricted to the tips of muscle fibers. Overexpression of CXCL12 leads to defect in muscle morphogenesis via the activation of β 1 integrins at the myotendinous junction, while overexpression of CXCR7 results in enhanced myogenesis and muscle cell

fusion. We propose a mechanism by which a balance between CXCL12 and CXCR7 signalling regulates the differentiation of ICT and muscle during chick limb myogenesis. Finally, we provide evidence that CXCL14 is a paracrine signal emanating from dense CT that negatively regulates myogenic differentiation.

B. Results

CXCR7, a receptor for CXCL12 chemokine, is expressed in myogenic cells

The expression pattern of the CXCL12 chemokine and its receptors, CXCR4 and *CXCR7*, was analysed during limb muscle development of chick embryos by in situ hybridization and immunostaining. PAX7 and MF20 antibodies were used to visualize progenitor and differentiated muscle cells, respectively. At E4, CXCR4 was strongly expressed in the vicinity of the mesenchymal *CXCL12* expressing region (Fig. 1A, B), in scattered cells which delineate the nascent limb vasculature and at a lower level in the forming dorsal and ventral muscle masses of the limb (Fig. 1B), as already described (Vasyutina et al., 2005). At this stage, *CXCR7* also exhibited a complementary expression pattern with *CXCL12*, the receptor being expressed in developing cartilage elements and in restricted regions of the limb, corresponding to muscle masses (Fig. 1C). Simultaneous detection of both receptors in this region revealed that some muscle cells coexpressed CXCR4 and *CXCR7* receptors (Fig. 1D-F), highlighting a putative switch between the two CXCL12 receptors in muscle cells. Simultaneous detection of *CXCR7* with PAX7 and MF20 antibodies at E5 revealed that *CXCR7* is expressed in muscle cells, both in PAX7-positive progenitors and MF20 muscle differentiated cells (Fig. 1G-L). Revelation of MEP21 antibody, which specifically stained endothelial cells, underlined the strong expression of CXCR4 in limb vessels (Fig. 1M-O), while CXCR4 expression was progressively downregulated in muscle cells, as assessed by the simultaneous revelation of MF20 antibody (Fig. 1N-P). At E10, *CXCL12* expression was detected in irregular connective tissue (ICT) both surrounding and into the muscles (Fig. 1Q, S), while *CXCR7* expression was similar to what was observed at earlier stages, overlapping with MF20 staining (Fig. 1R, T, U). However, *CXCR7* expression was restricted to muscle fibre tips (Fig. 1T, U). At this stage, CXCR4 expression only delineated endothelial cells (data not shown). These results show that *CXCR7* is expressed in myogenic cells during limb development in chick embryos, while *CXCL12* is detected in ICT. *CXCR7* expression is first widespread in muscles, being expressed in progenitor and differentiated muscle cells, to be gradually restricted during later stages of development at the tips of muscle fibres. CXCR4 receptor, first expressed in dorsal and ventral limb muscle masses, is progressively downregulated in muscle cells to be only maintained in limb vessels. All together, these data suggest that CXCL12/CXCR7 axis may be involved in limb foetal myogenesis and more specifically in the process of ICT-mediated muscle development.

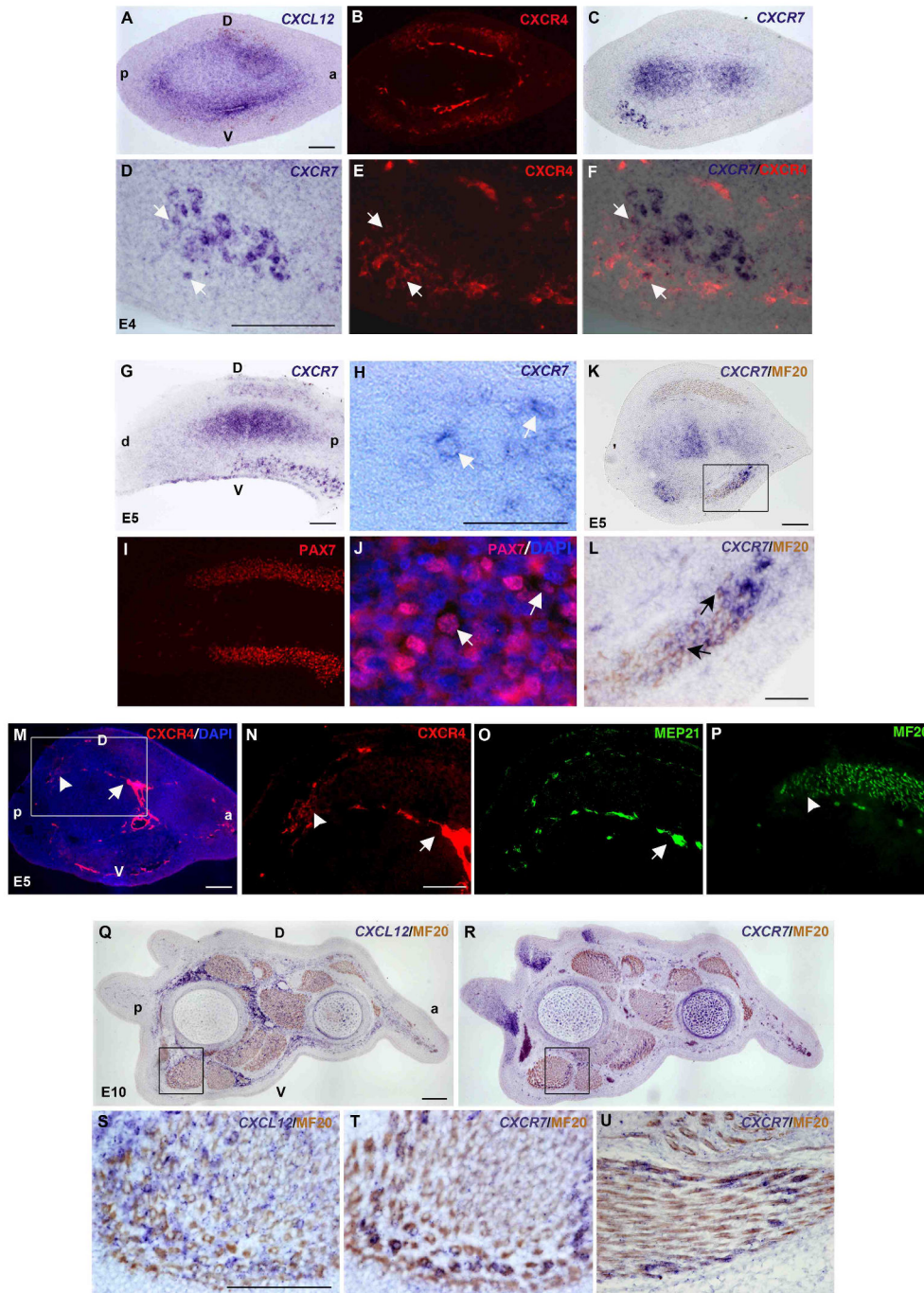


Figure 1: CXCR7 is expressed in myogenic cells during limb muscle morphogenesis in chick embryos. In situ hybridization for *CXCL12* (A, Q, S) and *CXCR7* (C, D, F, G, H, K, L, R, T, U), and immunostaining for *CXCR4* (B, E, F, M, N), *PAX7* (I, J), *MEP21* (O) and *MF20* (K, L, P, Q-U) on transverse (A-F, K-T) and longitudinal (G-J, U) sections of E4 (A-F), E5 (G-P) and E10 (Q-U) chick embryonic limbs. At E4, *CXCL12* is observed in connective tissue and dorsal and ventral muscle masses expressed *CXCR4*. *CXCR7* began to be expressed in muscle cells with some of them coexpressing both *CXCL12* receptors (A-F). From E5, *CXCR4* expression is progressively downregulated in muscle masses and reminiscent with *MEP21* staining, corresponding to endothelial cells, while *CXCR7* is expressed in *PAX7* and *MF20*-positive cells (G-P). At E10, *CXCL12* expression delineated irregular and muscle connective tissue and *CXCR7* became restricted to muscle fibre tips, while *CXCR4* is only detected in endothelial cells. Bars correspond to 100 μ m in A-G, I, K-U; 50 μ m in H, J. D, dorsal; V, ventral; p, posterior; v, ventral; p, proximal; d, distal.

The CXCR7 receptor controls myogenesis in chick foetal myoblasts

We first investigated the putative function of CXCR7 on myogenesis in vitro, using primary cell culture of foetal myoblasts derived from forelimbs of E10 chick embryos. After 5 days of culture, in situ hybridization for *CXCR7* and immunostaining for MF20 revealed that *CXCR7* was expressed in MF20-positive myotubes (white arrows) and MF20-negative muscle cells (black arrows) (Supp. Fig.1). In these in vitro conditions, *CXCR7* expression did not appear restricted at the tips of myotubes, as observed in vivo (Fig.1U). Functional assays were performed by overexpressing *CXCR7* or a dominant-negative form of *CXCR7* (*dnCXCR7*) using the avian RCAS retrovirus system. The *dnCXCR7* construct lacked the carboxy-terminus part of the receptor, impairing *CXCR7* internalization and signalling, without affecting its binding to CXCL12 ligand (Ray et al., 2012). Foetal myoblasts were transfected with *RCAS/CXCR7*, *RCAS/dnCXCR7* or empty RCAS as a control and grown into proliferation medium. At around 70-80% of confluence, muscle cells were cultured into differentiation medium for 5 days (Fig. 2A, 3A). Overexpression of the *dnCXCR7* form in chick foetal myoblasts led to a significant decrease in the number of both muscle progenitors and myotubes, assessed respectively by PAX7 and MF20 immuno staining (Fig. 2Ba-d). Myoblast fusion was also reduced in *dn-CXCR7* transfected muscle cell cultures, as shown by the quantification of myotubes proportion based on the number of nuclei/myotube and the expression of the *MYOMAKER* gene, known to be required for the process of muscle fusion (Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013) which was decreased in muscle cultures overexpressing the *dn-CXCR7* construct (Fig. 2Ca-c). These results were confirmed by RT-qPCR analysis for expression levels of *PAX7*, *MYOD*, *MYOG* and *MYOMAKER* genes, which were all reduced in muscle cells expressing the *dn-CXCR7* construct (Fig. 2Da). Finally, muscle cell proliferation, visualized by immunostaining of Phospho-histoneH3, was not modified in *dn-CXCR7* expressing muscle cells (Supp. Fig. 2A). When *CXCR7* was overexpressed in chick foetal myoblasts, a significant increase in the number of myotubes was observed (Fig. 3Ba-c), while the number of PAX7-positive cells was not significantly modified (Fig. 3Bd). Conversely to *dn-CXCR7* overexpression, myoblast fusion quantification and *MYOMAKER* expression were both increased in *CXCR7* transfected muscle cell cultures (Fig. 3Ca-c), while muscle cell proliferation was not changed (Supp. Fig. 2B). In addition, myotubes overexpressing *CXCR7* appeared slightly larger than in control cultures (Fig. 3Ba, b). RT-qPCR analysis showed that the expression levels of *PAX7*, *MYOD*, *MYOG* and *MYOMAKER* were increased in *CXCR7* expressing muscle cells (Fig. 3Da).

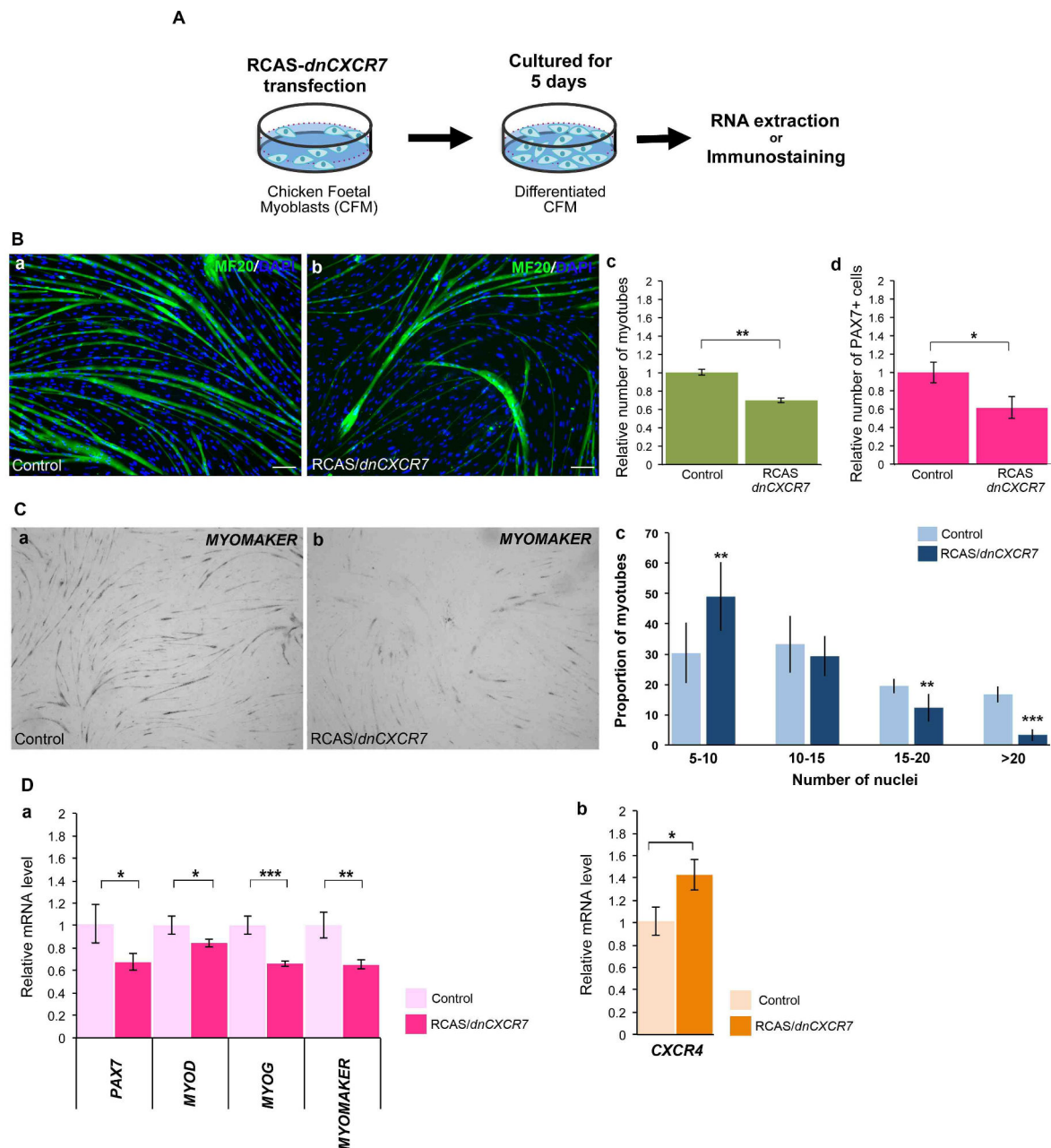


Figure 2: Overexpression of a dominant negative form of CXCR7 inhibits myogenic differentiation in vitro. (A) Chicken foetal myoblasts (CFM) were transfected with RCAS/*dnCXCR7* or empty RCAS as a control. Transfected CFM were cultured for 5 days under differentiation conditions and analysed. (B) Immunostaining for MF20 (a, b) and quantification of myotubes (c) and PAX7-positive cells (d), showing a decrease in both muscle progenitors and myotubes upon *dnCXCR7* overexpression compared to controls (n=6). (C) In situ hybridization for *MYOMAKER* (a, b) and quantification myotube proportion based on nuclei number per myotube (c) illustrating that *dnCXCR7* overexpression led to a decrease in muscle cell fusion (n=6). (D) RT-qPCR analysis of expression levels of myogenic (a) and connective tissue markers (b) and *CXCR4* receptor (c) showing that forced expression of *dnCXCR7* in muscle cells led to a decrease in the expression of *PAX7*, *MYOD*, *MYOG* and *MYOMAKER* genes (a), while *CXCR4* is increased (b) (n=3). Quantification and mRNA levels of controls were normalized to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. *p-value<0.05, **p-value<0.01, ***p-value<0.001. Error bars indicate the standard deviation.

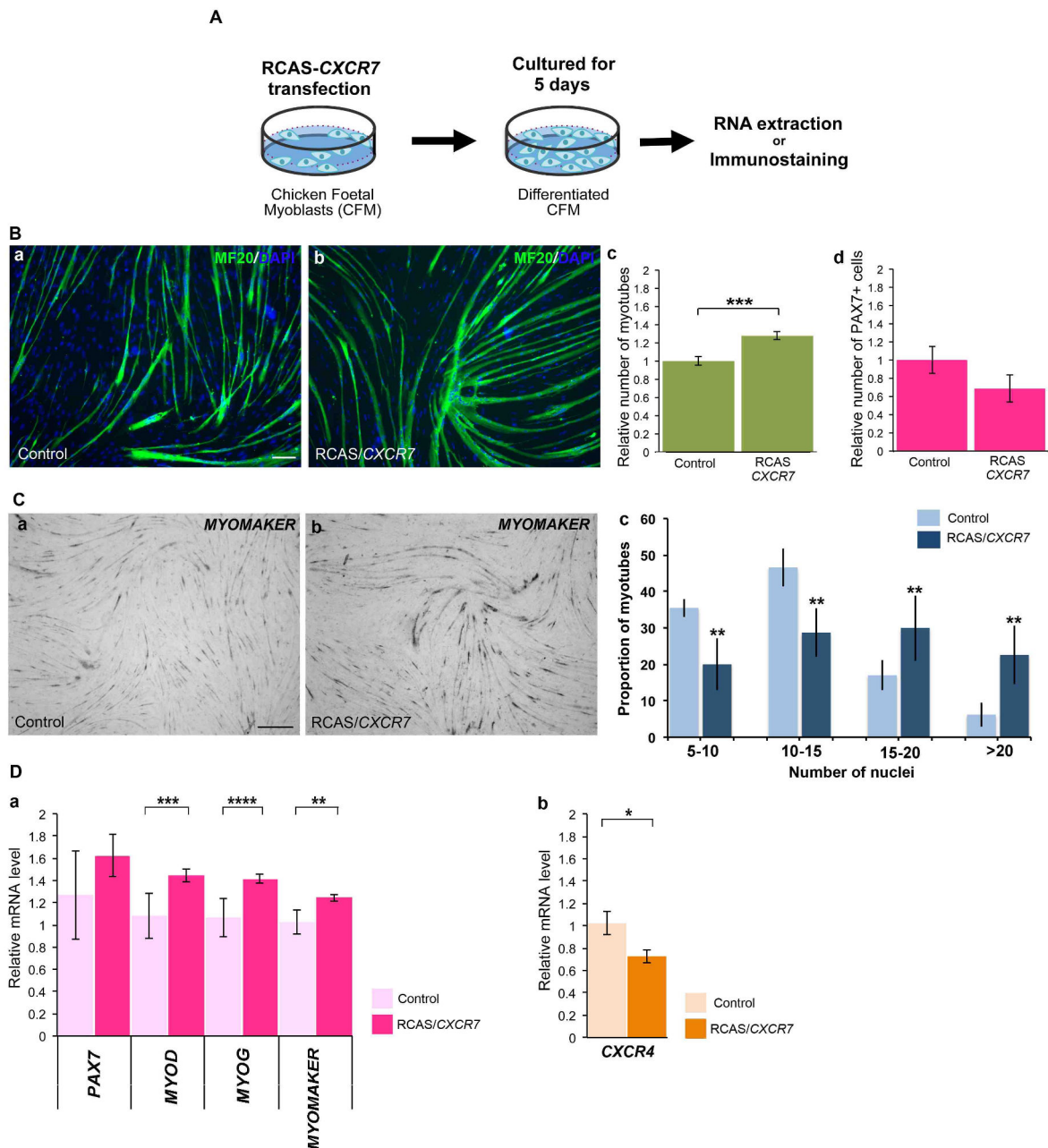


Figure 3: CXCR7 overexpression promotes myogenic differentiation in vitro. (A) Chicken foetal myoblasts (CFM) were transfected with RCAS/*CXCR7* or empty RCAS as a control. Transfected CFM were cultured for 5 days under differentiation conditions and analysed. (B) Immunostaining for MF20 (a, b) and quantification of myotubes (c) showing an increase in myotubes upon *CXCR7* overexpression compared to controls (n=6). Pax7-positive cells are not significantly modified in these conditions (d), (n=6). (C) In situ hybridization for *MYOMAKER* (a, b) and quantification myotube proportion based on nuclei number per myotube (c) illustrating that *CXCR7* overexpression led to an increase in muscle cell fusion (n=6). (D) RT-qPCR analysis of expression levels of myogenic (a) and connective tissue markers (b) and *CXCR4* receptor (c) showing that forced expression of *CXCR7* in muscle cells led to an increase in the expression of *PAX7*, *MYOD*, *MYOG* and *MYOMAKER* genes (a), while *CXCR4* expression is decreased (b) (n=6). Quantification and mRNA levels of controls were normalized to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. *p-value<0.05, **p-value<0.01, ***p-value<0.001. Error bars indicate the standard deviation.

As primary cultures of myoblasts are known to contain a small population of fibroblasts and that we have previously shown that CXCL12 favors the differentiation of limb irregular connective tissue (Nassari et al., submitted), we looked at the expression of connective tissue markers in muscle primary cultures transfected by *CXCR7* or *dn-CXCR7* constructs. Overexpression of *CXCR7* induced a significant decrease in the expression level of *TCF4* and

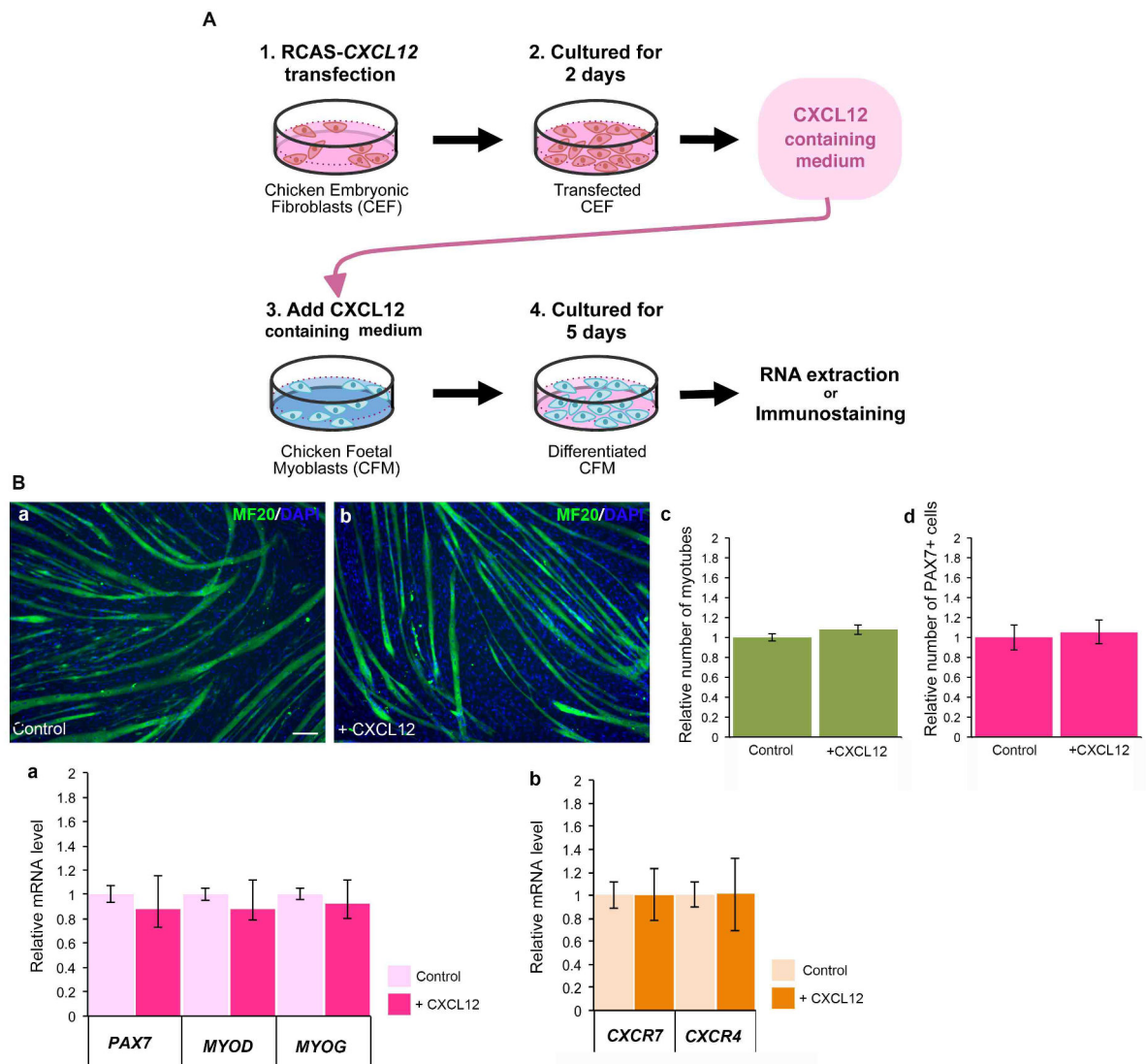


Figure 4: CXCL12 does not impact myogenic differentiation in vitro. (A) Chicken embryonic fibroblasts (CEF) were transfected with RCAS/*CXCL12* or empty RCAS as a control and grown for 2 days to produce CXCL12/control concentrated medium, which was added to chick myoblasts cultured under differentiation conditions for 5 days. Immunostaining for MF20 (Ba, b), quantification of PAX7-positive cells and myotubes (Bc, d) and RT-qPCR analysis for myogenic markers and CXCL12 receptors (C) illustrated that *CXCL12* overexpression did not impact in vitro myogenesis (n=12). Quantification and mRNA levels of controls were normalized to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. *p-value<0.05, **p-value<0.01, ***p-value<0.001. Error bars indicate the standard deviation.

OSRI genes (Fig. 3Db), while expression of *dn-CXCR7* resulted in a slight increase of the expression level of *CTGF* and *OSRI* genes (Fig. 2Db). In addition, muscle cell cultures overexpressing *dn-CXCR7* also exhibited an increase tendency in the expression of *ACTA2* gene, coding for α -SMA, a well-known marker of activated fibroblasts, although samples were quite variable for this gene level (Fig. 2Db). Taken together, these results indicate that *CXCR7* promotes in vitro myogenesis. Finally, since *CXCR4* receptor was expressed in limb muscle progenitors (Vasyutina et al., 2005); Fig. 1), we looked at its expression in muscle cell cultures overexpressing *dn-CXCR7* and *CXCR7* constructs. Although we did not detect *CXCR4* expression in muscle cell culture by immunostaining (data not shown), RT-qPCR analysis showed that *CXCR4* expression level was increased in muscle cells transfected by *dn-CXCR7* (Fig. 2Dc) and decreased in muscle cells overexpressing *CXCR7* (Fig. 3Dc), suggesting that interactions, at least at the RNA level, may exist between expression of both receptors during limb development.

CXCL12 chemokine has no effect on in vitro myogenesis

CXCL12/CXCR4 signalling has been previously shown as implicated in muscle development and regeneration (Griffin et al., 2010; Hunger et al., 2012; Melchionna et al., 2010; Odemis et al., 2007; Vasyutina et al., 2005). To investigate whether *CXCL12* chemokine was involved in the effect of *CXCR7* on in vitro myogenesis, *CXCL12* overexpression was performed in muscle cell cultures. Primary cell culture of chick embryonic fibroblasts were transfected with *RCAS/CXCL12* or empty *RCAS* as a control, cultured for 2 days and the culture medium was collected to prepare *CXCL12*-concentrated supernatant which was mixed to differentiation medium and added to chick foetal myoblasts grown at 70-80% confluency. Primary muscle cells were cultured in the presence of *CXCL12* for 5 days (Fig. 4A). MF20 and *PAX7* immunostaining did not reveal differences in the number of myotubes and muscle progenitors in the presence of *CXCL12*, compared to controls (Fig. 4Ba-d). These results were confirmed by the RT-qPCR analysis of *PAX7*, *MYOD* and *MYOG* expression levels which were not modified in myoblasts incubated with *CXCL12* supernatant (Fig. 4Ca). Additionally, the expression level of connective tissue markers were not grossly modified (Fig. 4Cb). Similarly, both *CXCR4* and *CXCR7* expression remained unchanged (Fig. 4Cc). These data show that *CXCL12* is not able to directly regulate myogenesis in vitro and suggest that the role of *CXCR7* on in vitro myogenesis is independent of *CXCL12*.

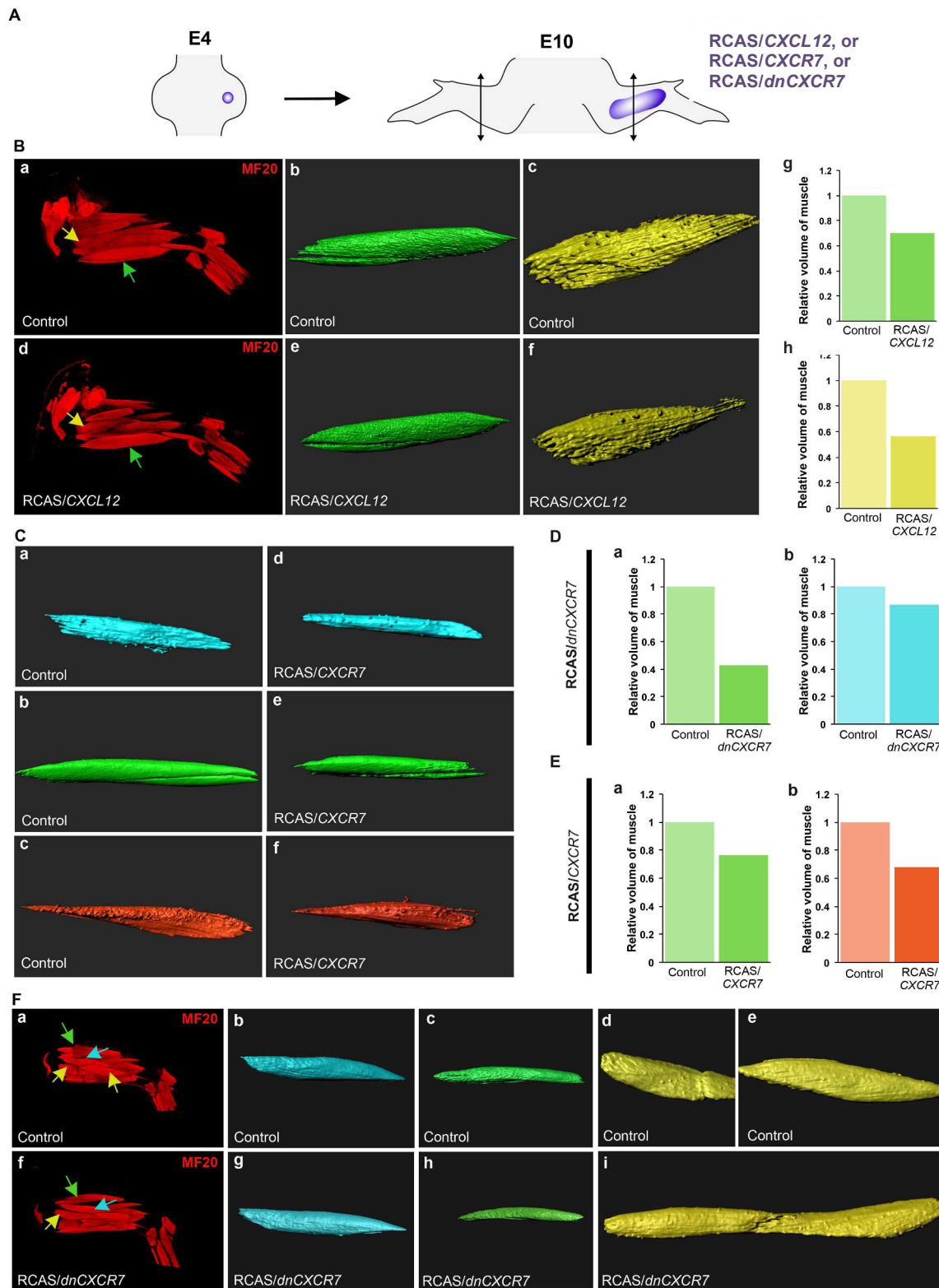


Figure 5: Impairment of CXCL12/CXCR7 signalling in chick embryonic limb induces muscle morphogenesis defects. (A) Pellets of CEF transfected with RCAS/*CXCR7*, RCAS/*dnCXCR7* or RCAS/*CXCL12* were grafted in the forelimb of E4 chick embryos. Infected and control limbs were collected and analysed at E10. 3D reconstructions of whole limb muscles (Ba, f; Fa, d) or individual muscles (Bb-e, g-i; C; Fb, c, e, f) immunostained with MF20 in *dnCXCR7* (B, n=3), *CXCR7* (C, n=1) and *CXCL12* (F, n=4) infected limbs, revealing in all cases differences in muscle patterning and morphogenesis of infected limbs compared to controls. Analysis of limb muscle volume upon overexpression of *dnCXCR7* (D), *CXCR7* (E) and *CXCL12* (Fg, h), showing a decrease in muscle volume in all cases. Color codes in volume data refer to color codes in individual 3D reconstructed muscles.

CXCL12 chemokine and CXCR7 receptor are involved in limb muscle development of chick embryos

In order to elucidate the possible discrepancy between CXCL12 and CXCR7 myogenic phenotypes obtained with gain-and loss-of-function approaches in vitro, the role of CXCL12 and CXCR7 was investigated in vivo by overexpressing *CXCL12*, *CXCR7* or *dn-CXCR7* in the forelimbs of chick embryos. Pellets of RCAS/*CXCL12*, RCAS/*CXCR7* or RCAS/*dnCXCR7* producing chick fibroblasts were grafted into E4 limb buds to allow virus spread into dividing cells and induced massive gene overexpression (Fig. 5A; (Esteves de Lima et al., 2016; Havis et al., 2016)). Embryos were collected 6 days later and muscles from grafted and control limbs were first analysed by performing 3D-reconstruction of MF20 whole-mount immunofluorescence, which allowed to investigate the size, shape and volume of limb muscles. Overexpression of RCAS/*dn-CXCR7*, RCAS/*CXCR7* and RCAS/*CXCL12* all resulted in changes in muscle morphogenesis with shorter, smaller or abnormally fused forelimb muscles associated with changes in muscle shapes, when compared to controls (Fig. 5B, C, F). These results were confirmed by analysis of transverse sections of infected and control limbs. MF20 immunostaining revealed that overexpression of *dnCXCR7* or *CXCR7* led to various phenotypes in infected muscles, which appeared smaller or abnormally cleaved when compared to controls (Fig. 6B, C). Measurements of MF20-positive surface areas showed that 40% of limb muscles were affected by *CXCR7* gain- or loss-of-function. When *dn-CXCR7* was overexpressed, 80% of affected muscles presented a decrease in surface area around 40% while conversely, when *CXCR7* was overexpressed, 70% of affected muscles presented an increase in their surface area around 40% (Fig. 6Da, b; 6Ea, b). Analysis of PAX7-positive progenitors in these experimental conditions showed that 30% of muscles also presented changes in the density of PAX7-positive cells after *CXCR7* misexpression, with 90% of *dn-CXCR7*-expressing muscles presenting an increase in the density of PAX7-positive cells of around 30% while 80% of *CXCR7*-expressing muscles exhibited a decrease in the density of PAX7-positive cells of around 40% (Fig. 6Dc, d; 6Ec, d). Taken together, these results in vivo confirmed those obtained in vitro and revealed that blocking CXCR7 receptor signalling in muscle cells led to a delay in limb muscle differentiation, as shown by the increase in the density of PAX7 progenitor cells and the decrease in MF20-expressing muscle area, while increasing the expression of CXCR7 in vivo enhanced limb muscle differentiation, assessed by the decrease in the density of PAX7 progenitor cells and the increase in MF20-expressing muscle area.

We also investigated the role of CXCR7 associated chemokine CXCL12 on limb myogenesis in vivo. Chick embryonic forelimbs were grafted with pellets of RCAS/*CXCL12*-producing fibroblasts at E4 and analysed 6 days later (Fig. 7A). Conversely to what we observed in vitro where CXCL12 has no effect on myogenesis, MF20 immunostaining on sections of control

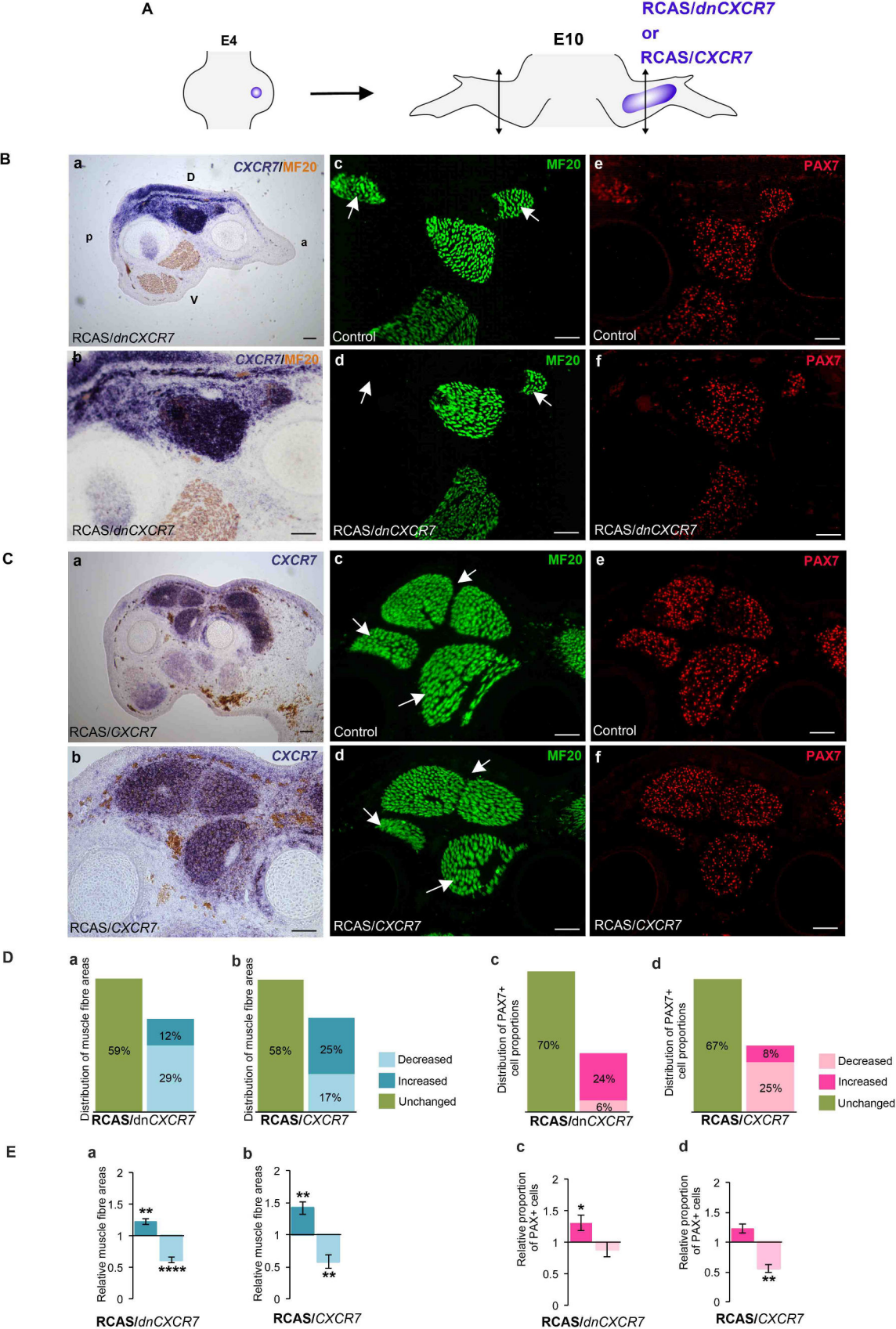


Figure 6: CXCR7 misexpression impacts muscle size, and the number of muscle progenitors during chick limb muscle morphogenesis. (A) Scheme illustrating the graft experiment of RCAS/*dnCXCR7* and RCAS/*CXCR7*-transfected CEF in forelimbs of chick embryos. In situ hybridization for *CXCR7* (Ba, b; Ca, b) and immunostaining for MF20 (Bc, d; Cc, d) and PAX7 (Be, f; Ce, f) on transverse sections of *dnCXCR7* (B) and *CXCR7* (C) infected and control limbs, showing that *CXCR7* misexpression led to defects in limb muscle morphogenesis. (D) Distribution of muscle surface areas (a) and PAX7-positive cell density (b) in infected muscles upon *CXCR7* misexpression. (E) Ratio of muscle surface area (a) and PAX7-positive cell number (b) between infected and control muscles. Muscle surface areas and proportion of PAX7-positive cells were evaluated on 4 successive sections of 3 different muscles at 2 different levels of the limb in 4 embryos in *dnCXCR7* and *CXCR7*-infected limbs and compared to their respective control limbs. D, dorsal; V, ventral; p, posterior; v, ventral.

and grafted limbs revealed that some muscles were missing, while others were misshaped and mispatterned (Fig. 7B). In order to assess specifically the role of CXCL12 as a non-autonomous signal regulating limb myogenesis, a stable vector containing the chicken *CXCL12* sequence and the TOMATO gene as a reporter (Bourgeois et al., 2015) was electroporated in the forelimb lateral plate, to overexpress *CXCL12* specifically in limb CTs. Electroporation was performed at E2.5 and electroporated and control limbs were analyzed at E8.5 (Fig. 7Ca, b). *CXCL12* overexpression was assessed by TOMATO fluorescence (Fig. 7Cc). MF20 immunostaining on transverse sections of electroporated and control limbs revealed that *CXCL12* overexpression in limb CT induced changes in size, shape, and patterning of 40% of limb muscles (Fig. 7D, E), among which 70% presented a decrease in surface area around 30% (Fig. 7Ea; 7Fa). Analysis of PAX7 progenitors showed that 50% of muscles in limbs in which *CXCL12* was overexpressed in CTs exhibited differences in progenitor density with a majority of them presenting a decrease in PAX7-positive cells of around 30% (Fig. 7Fb; 7Fb). These data in vivo clearly show that *CXCL12*, secreted from the CT, negatively impacts limb muscle differentiation. Taken together, our results demonstrate that *CXCL12* and *CXCR7* are involved in the process of muscle differentiation and patterning during chick limb development.

The chemokine receptor CXCR7 regulates muscle fusion during chick limb myogenesis

Our in vitro data showed that *CXCR7* gain- and loss-of –function in muscle cells led to changes in myoblast fusion. In vivo, we observed that modifying *CXCR7* activity in chick limb deregulated the balance between PAX7-positive muscle progenitors and differentiated MF20-positive myotubes. We consequently investigated whether *CXCR7* could control muscle fusion in vivo by looking at *MYOMAKER* expression in limb muscles submitted to

CXCR7 loss-of-function conditions. In situ hybridization on transverse sections of E5 chick limbs showed that *MYOMAKER* expression delineated dorsal and ventral muscle masses expressing PAX7 (Fig. 8Aa-d), while at E10, *MYOMAKER* is expressed both in PAX7-positive progenitors and MF20-positive myotubes (Fig. 8Ae-h). Overexpression of RCAS/*dn-*

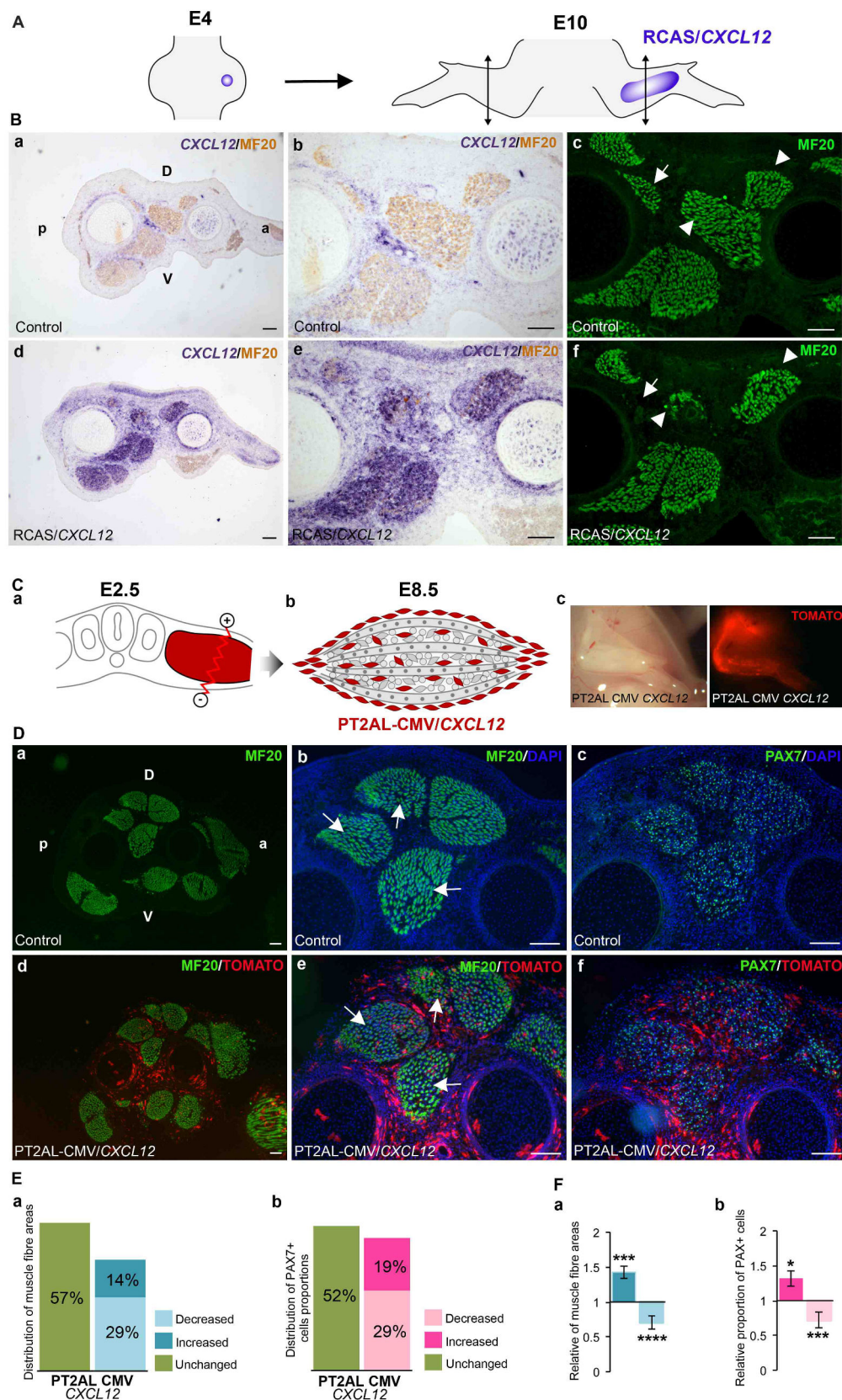


Figure 7: CXCL12 act as a paracrine signal from the connective tissue acting on muscle morphogenesis in limb of chick embryos. (A) RCAS/*CXCL12* producing CEF were grafted in forelimbs of E4 chick embryos and grafted limbs were analyzed at E10. (B) *CXCL12* in situ hybridization (a, b, d, e) and MF20 immunostaining (a-f) showing that *CXCL12* overexpression led to defects in muscle formation. Arrowheads in c, f show reduced muscles and arrows show absent muscles in infected limbs. (C) Schematic representation of lateral plate electroporation of PT2AL-CMV/*CXCL12* performed at E2.5. Electroporated limbs, expressing TOMATO, were collected and analysed at E8.5. (D) Immunostaining for MF20 (a, b, d, e) and PAX7 (c, f) in electroporated (d-f) and control (a-c) limbs, showing a muscle mispatterning after *CXCL12* overexpression in connective tissue (arrows in b, e). (E) Distribution of muscle surface areas (a) and PAX7-positive cell density (b) in muscles of PT2AL-CMV/*CXCL12* electroporated limbs. (F) Ratio of muscle surface area (a) and PAX7-positive cell number (b) between electroporated and control muscles. Muscle surface areas and proportion of PAX7-positive cells were evaluated on 4 successive sections of 3 different muscles at 2 different levels of the limb in 4 embryos in electroporated limbs and compared to their respective control limbs. D, dorsal; V, ventral; p, posterior; v, ventral.

CXCR7 from E4 of development resulted 6 days later in a decrease in the expression of *MYOMAKER* in infected limb (Fig. 8Ba) when compared to control (Fig. 8Bc). These results show that *CXCR7* receptor, as we observed in vitro, regulates muscle cell fusion during chick limb myogenesis.

***CXCL12* expression induces the $\beta 1$ integrin activation at the myotendinous junction**

Previous studies have shown that binding of *CXCL12* to *CXCR4* receptor promotes chemotaxis and motility via integrin clustering and integrin activation in many cell types (Hartmann et al., 2005; Kokovay et al., 2010; Peled et al., 2000; Shimonaka et al., 2004). More recently, it has been shown that *CXCL12*/*CXCR4* activation by cancer-associated fibroblasts promotes $\beta 1$ integrin clustering and invasiveness (Izumi et al., 2015). In addition, *CXCR7* has also been shown to contribute to *CXCL12*-triggered integrin activation in human T lymphocytes (Hartmann et al., 2008). Studies in avian indicate that $\beta 1$ integrins are involved in myoblast migration (Jaffredo et al., 1988), myoblast fusion and sarcomere assembly (Schwander et al., 2003). Skeletal muscle fibers express many integrin subunits in developmentally regulated patterns, including $\beta 1$ and its partners $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$ and αv (Gullberg et al., 1998) and genetic studies in mice have shown that $\alpha 7\beta 1$ is required to maintain myotendinous junctions (Mayer et al., 1997). We investigated whether *CXCL12* was able to regulate integrin activation by overexpressing *CXCL12* specifically in limb CTs thanks to electroporation of the forelimb lateral plate. Immunostaining for TASC antibody, which

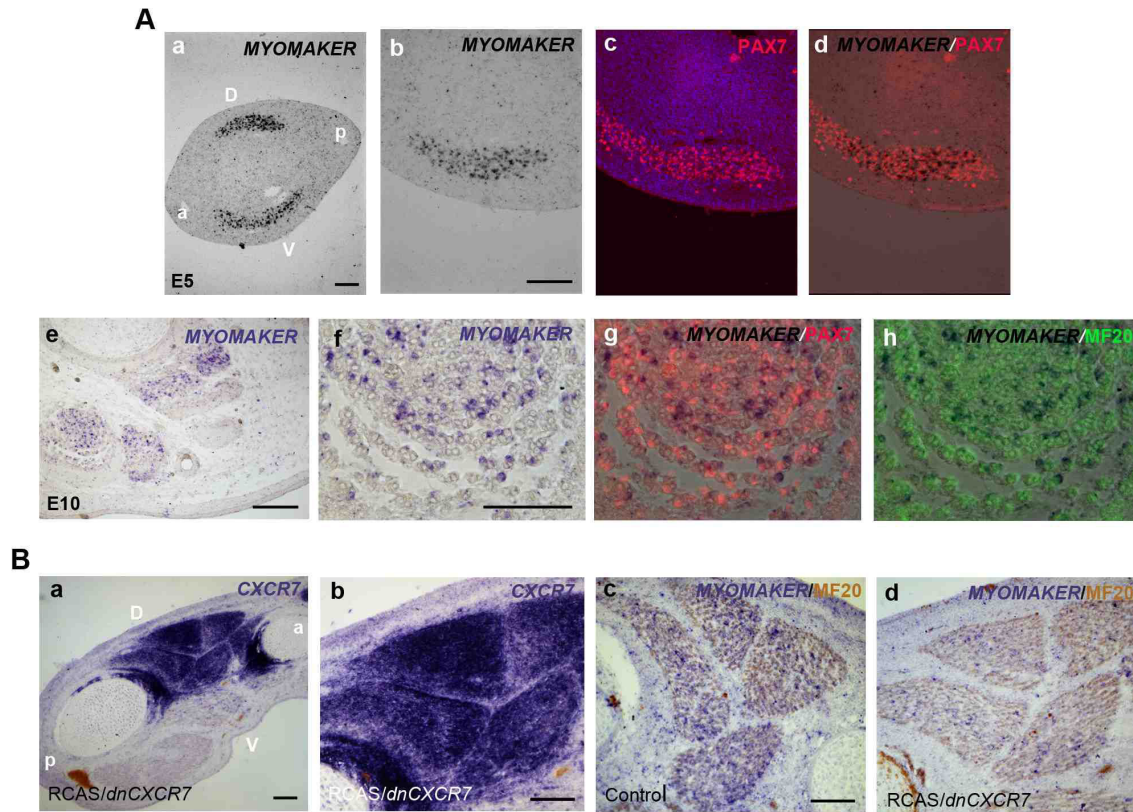


Figure 8: CXCL12/CXCR7 signalling regulates *MYOMAKER* expression in vivo. (A) Expression pattern of *MYOMAKER* during limb development of chick embryos. In situ hybridization for *MYOMAKER* (a, b, d-h) and immunostaining for PAX7 (c, d, g) and MF20 (h) on transverse sections of E5 (a-d) and E10 (e-h) chick forelimbs. (B) Effect of *CXCR7* and on *MYOMAKER* expression. In situ hybridization for *CXCR7* (a, b), *MYOMAKER* (c, d) and MF20 immunostaining (c, d) on transverse sections of *CXCR7* (a, b, d) infected limbs. *MYOMAKER* expression is decreased in RCAS/*dnCXCR7* infected limbs when compared to controls. Bars correspond to 100 μ m. D, dorsal; V, ventral; p, posterior; v, ventral.

specifically recognized the activated form of β 1 integrin, on transverse sections of control limbs revealed that activated β 1 integrins are mostly expressed at the myotendinous junctions (Fig. 9A, E, H, K). CXCL12 overexpression in limb CTs induced an increase in β 1 integrin activation in electroporated limbs (Fig. 9C, F, I, L) when compared to controls (Fig. 9A, E, H, K). These results demonstrate that CXCL12 positively regulates limb myogenesis via β 1 integrin activation at the myotendinous junction and suggest that CXCL12 action on muscle morphogenesis might mediated via activation of β 1 integrin.

Inactive form of CXCR7 enhances CXCL12 in connective tissue

We have previously demonstrated the existence of a cross-regulation between CXCL12 and CXCR4 expression during neural crest migration in chick embryo (Escot et al., 2013; Escot et al., 2016). For this reason, we investigated whether such a cross-talk between CXCL12 and CXCR7 expression could be evidenced during chick limb development. While overexpression

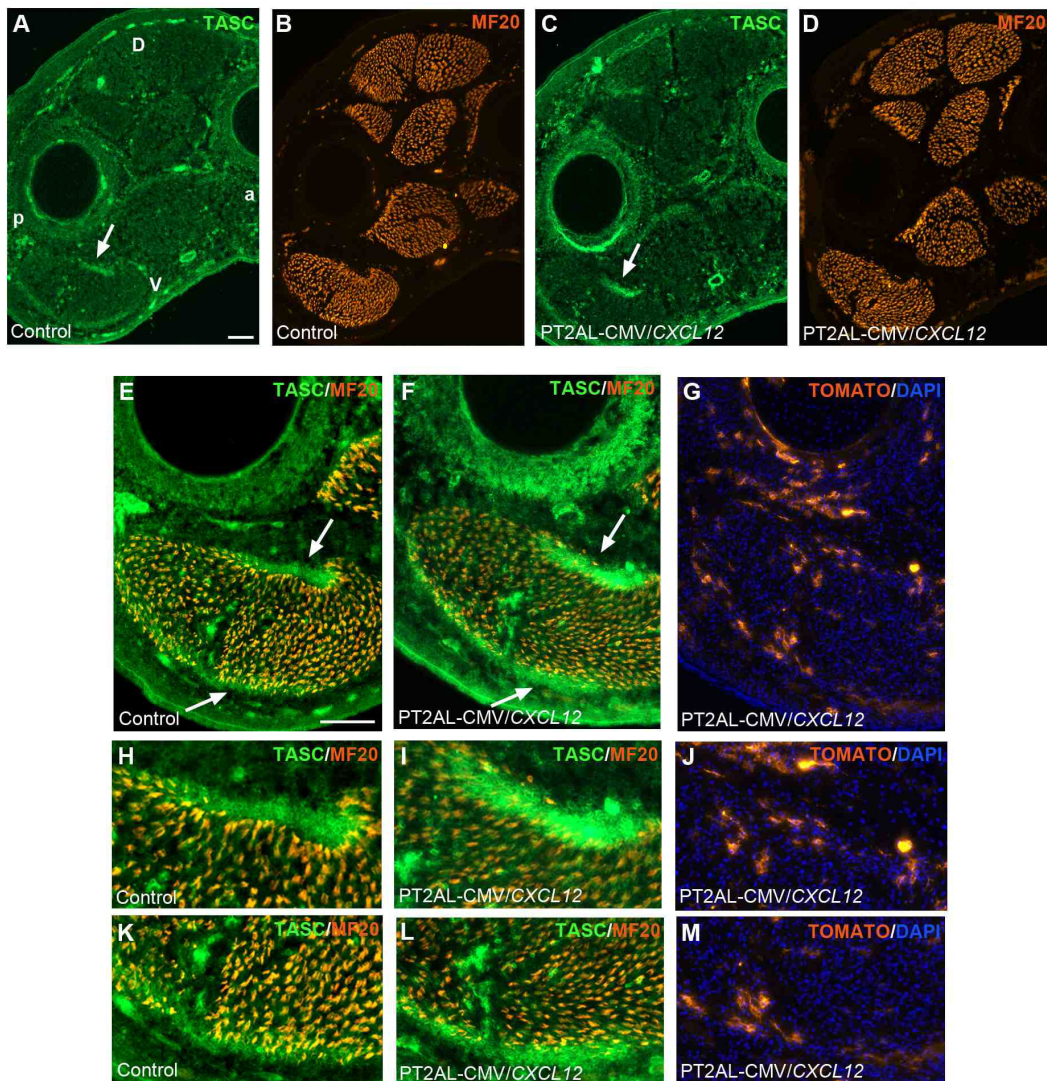


Figure 9: CXCL12 increases β 1 integrin activation at the myotendinous junctions.

Immunostaining for TASC (A, C, E, F, H, I, K, L) and MF20 (B, D, E, F, H, I, K, L) on transverse sections of PT2AL-CMV/*CXCL12* electroporated (C, D, G, I, L, J, M) and control (A, B, E, H, K) limbs. Simultaneous detection of TOMATO and DAPI on transverse sections of electroporated limbs (G, J, M). β 1 integrin activation is increased at the myotendinous junctions near the connective tissue cells overexpressing *CXCL12*. Bars correspond to 100 μ m. D, dorsal; V, ventral; p, posterior; v, ventral.

of *CXCL12* and *CXCR7* did not induced changes in the expression of *CXCR7* and *CXCL12*, respectively, overexpression of the *dn-CXCR7* construct led to a significant increase in the expression of *CXCL12* in infected limb when compared to control (Fig. 10Bc, f). Interestingly, *CXCL12* expression was mostly increased in the ICT surrounding muscles (Fig. 10Cf). This result shows that *CXCR7* contributes to the regulation of *CXCL12* transcripts, especially in ICT surrounding muscles and suggest that *CXCR7* regulates *CXCL12* expression level in ICT, likely to establish the correct balance between limb ICT and muscle formation.

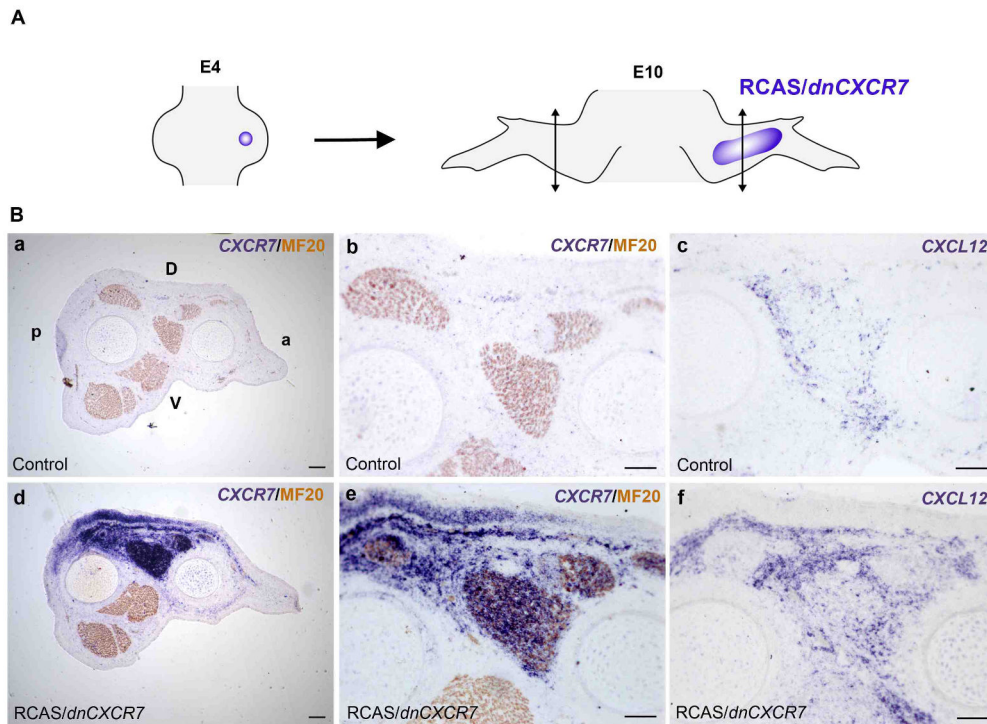


Figure 10: CXCR7 dominant negative form enhances CXCL12 expression in connective tissue surrounding muscles. (A) RCAS/*dnCXCR7* producing CEF were grafted in limbs of E4 chick embryos and grafted and control limbs were analysed at E10. (B) In situ hybridization for *CXCR7* (a, b, d, e) and *CXCL12* (c, f) and MF20 immunostaining (a, b, d, e) on transverse sections of RCAS/*dnCXCR7* infected (d-f) and control limbs (a-c). Overexpression of *dn-CXCR7* induced an increase in *CXCL12* expression in muscle surrounding connective tissue. Bars: 100 μ m in Ba-f. D, dorsal; V, ventral; p, posterior; v, ventral.

CXCL14 inhibits myogenic differentiation in vitro

CXCL14 chemokine has been previously shown to be expressed in ICT mostly in muscles during chick limb development (García-Andrés & Torres 2010; Nassari et al., unpublished data) and to positively regulate the expression of the regular CT marker SCX (Nassari et al., unpublished data). Although CXCL14 receptor has not been yet identified, it has been previously shown that CXCL14 might interact with CXCR4 to modulate CXCL12/CXCR4 axis (Collins et al., 2017; Tanegashima et al., 2013a). We then hypothesized that similarly CXCL14 could bind to the second receptor of CXCL12, CXCR7, to modulate CXCR7 signalling. Thereby we decided to investigate the possible role of CXCL14 on CT mediated-limb myogenesis, both in vitro and in vivo. Cultures of primary foetal myoblasts from embryonic chick forelimbs were incubated with CXCL14-concentrated supernatant produced by chick primary fibroblasts transfected with a RCAS/*CXCL14* vector. At around 70-80% of confluence, muscle cells were cultured into differentiation medium for 5 days (Fig. 11A). MF20 immunostaining showed that CXCL14 dramatically inhibits in vitro myogenesis.

Muscle cell cultures incubated with CXCL14-concentrated supernatant presented around 40% less myotubes than control cultures (Fig. 11Ba-c). In addition, a 60% decrease in the number of PAX7-positive progenitors was observed in CXCL14-treated cultures when compared to controls (Fig. 11Bd). These data were confirmed by the analysis of the RNA levels of *PAX7*, *MYOD* and *MYOG* myogenic markers, which were significantly decreased in the presence of CXCL14 (Fig. 11Ca). Conversely, analysis of muscle fusion index did not show any

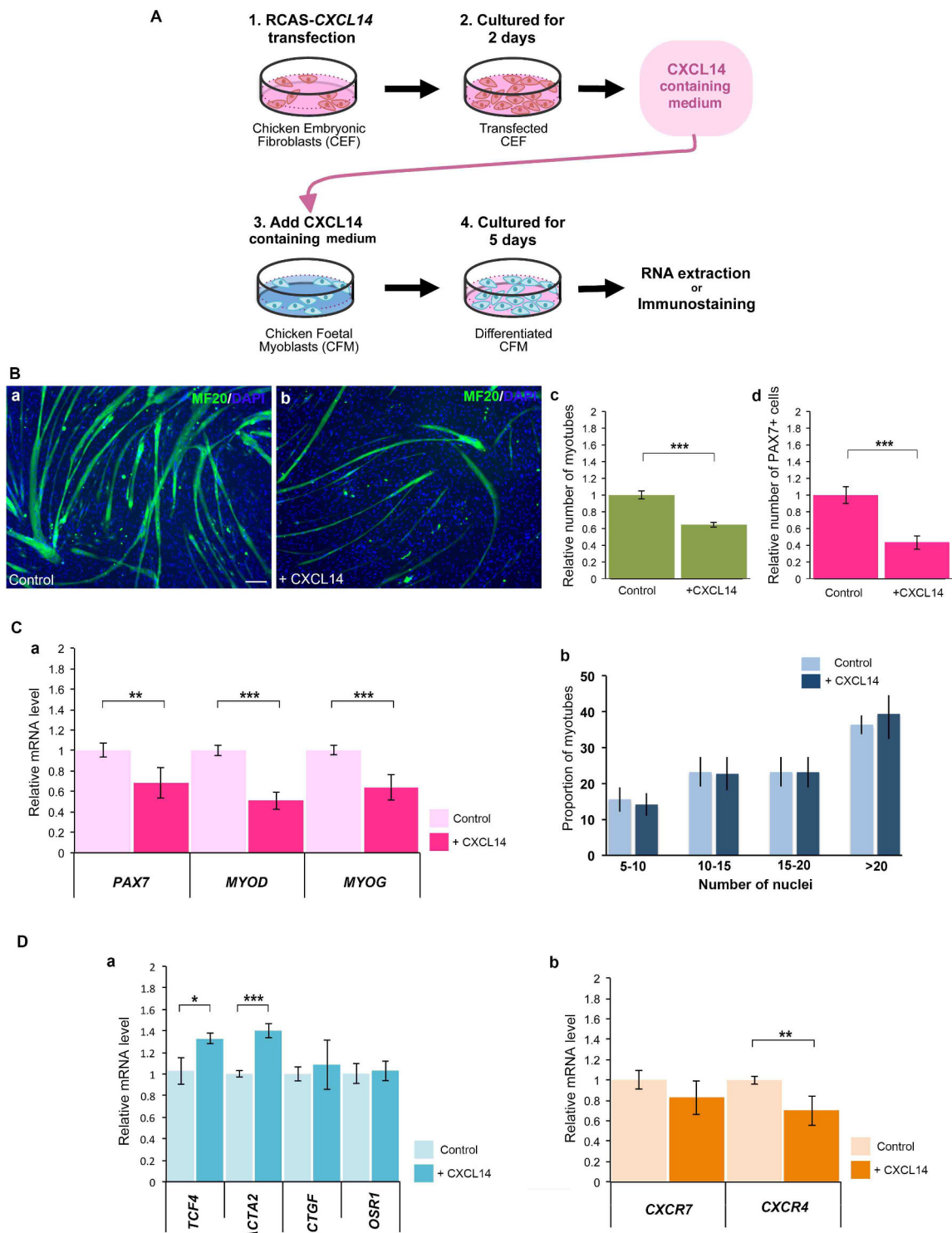


Figure 11: CXCL14 inhibits myogenic differentiation in vitro. (A) Chicken CEF producing RCAS/*CXCL14* or empty RCAS as a control were cultured for 2 days to produce CXCL14/control-concentrated medium, which was added to chick myoblasts cultured under differentiation conditions for 5 days. (B) MF20 immunostaining (a, b) and quantification of myotubes (c) and PAX7-positive cells (d) revealed that CXCL14 inhibits in vitro myogenesis. (C) RT-qPCR analysis for myogenic markers (a) revealed that CXCL14 overexpression induced a decrease in the expression of *PAX7*, *MYOD* and *MYOG*, while quantification of muscle fusion index did not show any change (b). (D) RT-qPCR analysis for connective tissue markers (a) and *CXCR4* and *CXCR7* receptors (b) indicated an increase in the expression level of *TCF4* and *ACTA2* genes and a decrease in the expression of *CXCR4* upon CXCL14 overexpression. Quantification and mRNA levels of controls were normalized to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. *p-value<0.05, **p-value<0.01, ***p-value<0.001. Error bars indicate the standard deviation.

differences between CXCL14-treated myoblasts and controls (data not shown). Although myogenic differentiation was severely decreased immunostaining for Phospho-histoneH3 and Caspase3 revealed no changes in myoblast proliferation (Supp. Fig. 2D) and cell death (data not shown). As we have previously observed that CXCL14 positively regulates the expression of CT markers (Nassari et al., unpublished data), we analysed by RT-qPCR CT markers expression in muscle cell cultures incubated with CXCL14-concentrated supernatant, which revealed no change in *SCX* expression level (data not shown) and a significant increase in the expression level of *TCF4* and *ACTA2*, two markers of activated fibroblasts (Fig. 11Da). Analysis of *CXCR4* and *CXCR7* expression level in muscle cells treated with CXCL14 showed that *CXCR4* level was decreased, while *CXCR7* expression was not changed (Fig. 11Db). These results show that in vitro CXCL14 inhibits muscle cell differentiation and suggest that the chemokine favour phenoconversion of myogenic cells into CT cells, and probably other cell types.

CXCL14 controls muscle morphogenesis in embryonic chick limb

We next analysed the role of CXCL14 on limb muscle morphogenesis in vivo, by electroporating a stable vector containing the chicken sequence of *CXCL14* in forelimb mesodermal lateral plate, in order to specifically overexpress CXCL14 in limb CTs. Chick embryos were electroporated at E2.5 and collected 6 days later. *CXCL14* expression was visualized by TOMATO fluorescence. Whole-mount MF20 immunostaining of electroporated and control limbs revealed that *CXCL14* overexpression led to abnormal limb muscle morphogenesis with extra muscles (arrows) or shorter/smaller muscles (arrowheads) (Fig. 12A).

Analysis of transverse sections of electroporated limbs showed a profound muscle mispatterning, with some muscles missplitted or misshaped (Fig. 12B). Quantitative analysis of muscle surface areas revealed that 75% of muscles were affected by *CXCL14* overexpression, among which 70% presented a decreased surface area of almost 50% (Fig. 12Ca; 12Da). Immunostaining for PAX7 showed that muscle progenitor density was affected in 70% of muscles, among which 70% exhibited a decrease in PAX7-positive cell density of

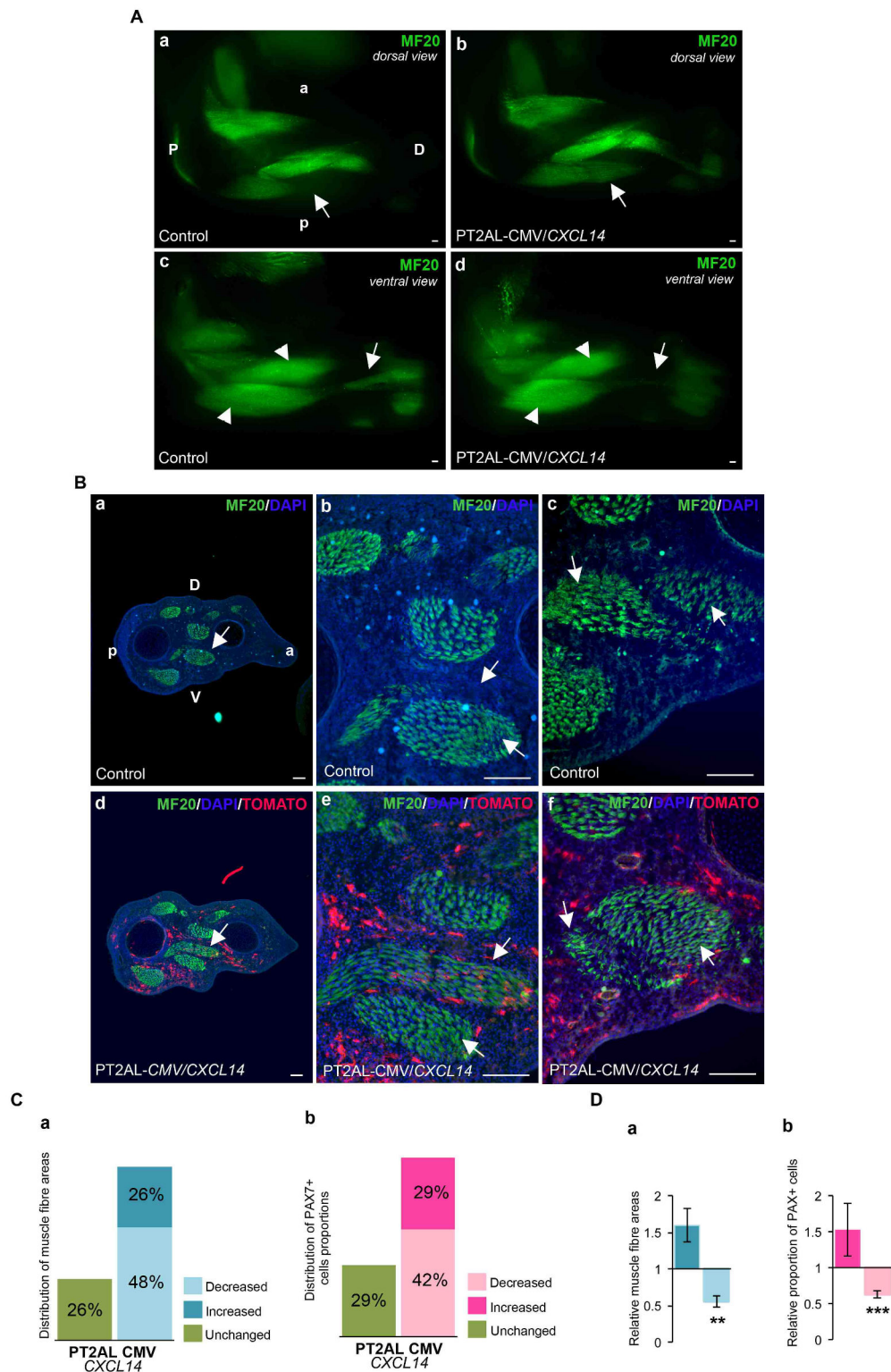
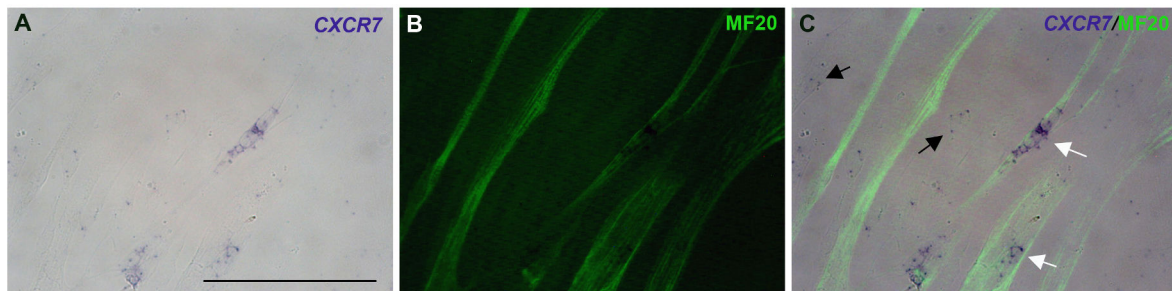


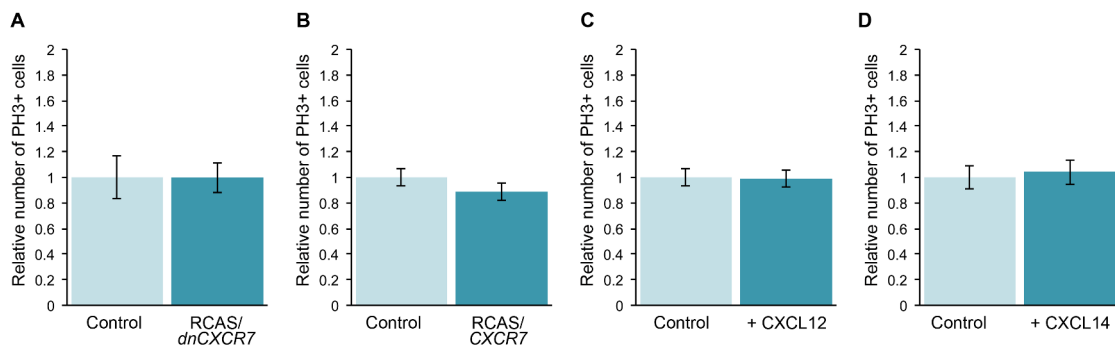
Figure 12: CXCL14 regulates muscle morphogenesis in chick embryonic limbs. (A) Whole-mount MF20 immunostaining in PT2AL-CMV/CXCL14 electroporated (b, d) and control (a, c) limbs, showing the mispatterning of limb muscles after *CXCL14* overexpression in the connective tissue. Arrowheads show reduced muscles and arrows show additional muscles. (B) MF20 immunostaining on transverse sections of PT2AL-CMV/CXCL14 electroporated (d-f) and control (a-c) limbs. Some limb muscles appeared fused and mispatterned (arrows in b, e). (C) Distribution of muscle surface areas (a) and PAX7-positive cell density (b) in muscles of PT2AL-CMV/*CXCL14* electroporated limbs. (D) Ratio of muscle surface area (a) and PAX7-positive cell number (b) between electroporated and control muscles. A decrease in muscle size and progenitors numbers is observed in electroporated limbs. Muscle surface areas and proportion of PAX7-positive cells were evaluated on 4 successive sections of 3 different muscles at 2 different levels of the limb in 4 embryos in electroporated limbs and compared to their respective control limbs. Bars: 100 μ m in Aa-d, Ba-f. D, dorsal; V, ventral; p, posterior; v, ventral.

around 40% (Fig. 12Cb; 12Db). Taken together, these results demonstrate that CXCL14, secreted from the limb ICT negatively regulates muscle morphogenesis, revealing a paracrine function for CXCL14 during limb foetal myogenesis.

Supplementary figures



Supplementary figure 1: CXCR7 is expressed in primary chick foetal myoblast cultures. In situ hybridization for *CXCR7* (A, C), and immunostaining for MF20 (B, C), showing that *CXCR7* is expressed in muscle cells.



Supplementary figure 2: Cell proliferation is not altered in primary cultures of chick muscle cells overexpressing *dnCXCR7* or *CXCR7*, or incubated with CXCL12 or CXCL14 concentrated supernatant. (A, B, C, D) Quantification of Phosphohistone3-positive cells in chick foetal myoblasts transfected with RCAS/*dnCXCR7* (A), RCAS/*CXCR7* (B), or incubated with CXCL12- (C) or CXCL14-concentrated (D) supernatant, showing that misregulation of CXCL12 or CXCL14 signalling did not induce changes in myoblast proliferation. Error bars indicate the standard deviation.

Supplemental results

III. Odd-Skipped-related gene 2 is expressed in myogenic cells during chick development

Odd-Skipped-related gene 2 is expressed in myogenic cells during chick development

A. Introduction

The Odd-Skipped genes were first described in *Drosophila*. They were identified as pair-rule genes in a screen for gene mutations affecting body segmentation (Coulter and Wieschaus, 1988; Nüsslein-Volhard and Wieschaus, 1980). In mammalian, two Odd-Skipped related genes have been characterized: *Osr1* and *Osr2*, which both encode zinc fingers transcription factors (Lan et al., 2001; So and Danielian, 1999).

During mouse embryonic development, the mammalian homolog Odd-Skipped related 2 (*Osr2*) is not detected during development until embryonic day (E9.25), (Lan et al., 2001). In situ hybridization assays in mouse embryos showed that *Osr2* expression is first observed in the mesonephros, the embryonic structure giving rise to the kidney (Lan et al., 2001) and then detected in the mesenchyme surrounding mesonephros ducts (Lan et al., 2001). At E10, *Osr2* expression is also observed in the mesenchyme of some specific mandibular regions, as well as in the mesenchyme of forelimbs and hindlimbs (Lan et al., 2001). Finally, *Osr2* expression is also detected in tissues surrounding the developing eyes (Lan et al., 2001). Such *Osr2* expression pattern during mouse embryonic development allowed to define *Osr2* as a specific marker for mesenchymal tissues. Additionally, RT-qPCR analysis performed in mouse embryos allowed characterization of tissue distribution of *Osr2*, highlighting a high expression level in the uterus and at lower level in the testes, small intestine and prostate. *Osr2* expression was also found in eyes, placenta, lung, thymus, lymph node, and stomach at all embryonic stages from E7.5 to E17.5 (Kawai et al., 2013).

In chick embryos, *OSR2* expression is quite similar to what has been observed in mouse embryos, mostly detected in mesenchymal cells. *OSR2* appears to be expressed in the developing mesonephros, in the branchial arches giving rise to the mandibular craniofacial regions and in the mesenchyme of developing limbs (Stricker et al., 2006; Stricker et al., 2012). During embryonic development, mesenchymal progenitors give rise to different cell lineages among which the connective tissue (CT). During development, different types of connective tissue are formed: the irregular connective tissue (ICT) and the specialized connective tissue. The ICT corresponds to unspecialized loose CT while the specialized CT characterizes dense regular CT corresponding to tendons, ligaments, cartilage and bones. Interestingly, it has been demonstrated that *OSR2* favours the differentiation of mesenchymal

progenitor cells towards an ICT fate at the expense of other cell types such as muscle or specialized CT (Stricker et al., 2012). Mutant mice carrying a deletion of the *Osr2* coding region are lethal at perinatal stages (Lan et al., 2004), demonstrating an important role for *Osr2* during development. Consistent with the regions of *Osr2* expression in mouse embryos, *Osr2*^{-/-} mutants present severe defects in craniofacial development, resulting in open eyelid, cleft palate and thickened tympanic ring (Lan et al., 2004). A reduction in cell proliferation in the regions of palate and eyelid formation was observed in *Osr2*^{-/-} mice, indicating a main function for *Osr2* in growth regulation of these structures (Gao et al., 2009; Lan et al., 2004). Accordingly, microarray assays revealed *Osr2* as a positive regulator of cell cycle progression and cell proliferation during mouse development (Kawai et al., 2010). Targeting deletion of *Osr2* in mice did not affect the development of other tissues that normally express *Osr2*, including the developing kidney, the proximal mandible or the limbs (Gao et al., 2009; Lan et al., 2004; Tena et al., 2007), probably as a consequence of the functional redundancy between *Osr1* and *Osr2* genes in these structures (Gao et al., 2009; Gao et al., 2011).

In this study, we report the expression pattern of *OSR2* during chick limb and head development. We show, as previously described, that *OSR2* is specifically expressed in muscle ICT during limb and head development, but demonstrate that *OSR2* is also expressed in differentiated muscle cells in limb and head muscles during chick development. We confirm the expression of *OSR2* in muscle cells differentiated in vitro from myoblasts isolated from chick limbs. This study highlights a new site of *OSR2* expression in developing chick embryos that has never been observed before (Lan et al., 2001; Lan et al., 2004; Stricker et al., 2006; Stricker et al., 2012) and could bring new insight for *Osr2* function during development.

B. Results and discussion

OSR2 has been described as being expressed in developing limbs of chick and mouse embryos (Lan et al., 2001; Lan et al., 2004; Stricker et al., 2006; Stricker et al., 2012). It has been reported that *OSR2* is expressed in a subpopulation of CT in the developing chick limbs, while being excluded from myogenic cells (Stricker et al., 2006; Stricker et al., 2012). In order to better characterize the sites of *OSR2* expression during chick limb development, we performed in situ hybridisation to limb sections at different developmental stages. Limb myogenic cells originate from the somites and muscle progenitors migrate from from the

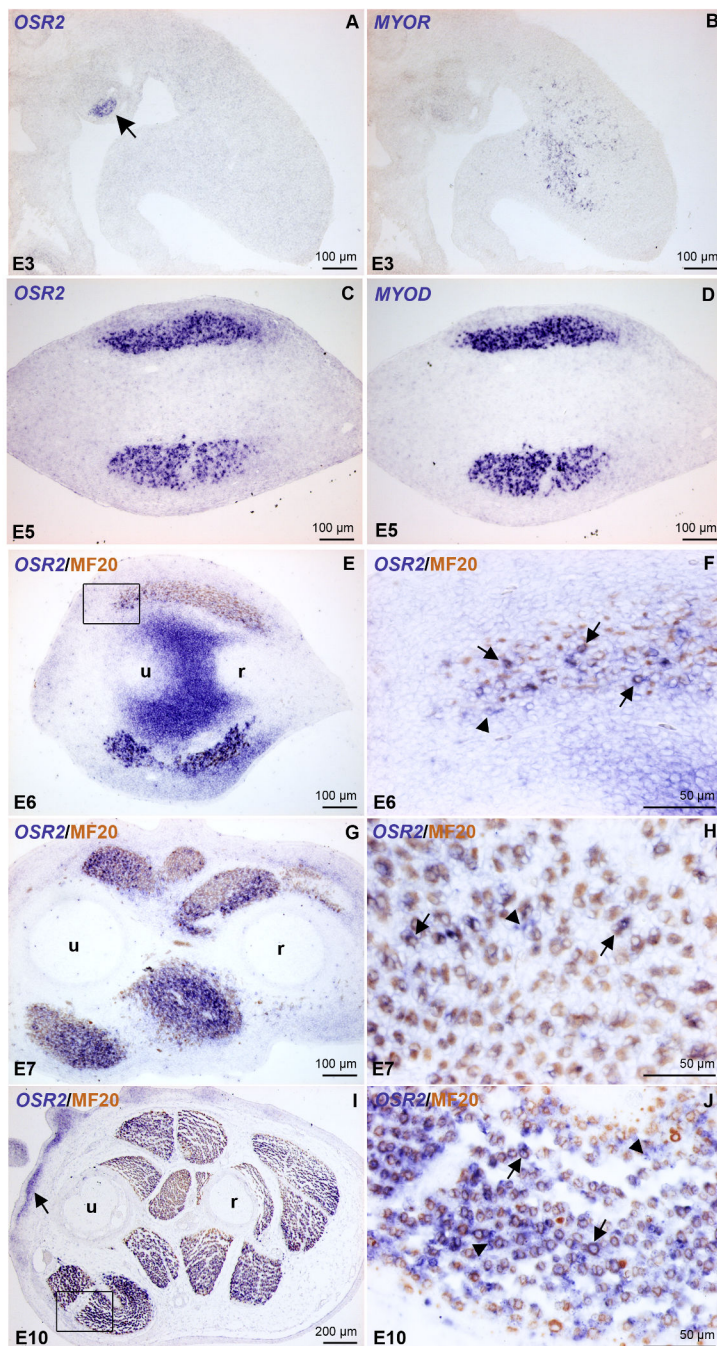


Figure 1: *OSR2* is expressed in differentiated muscle cells in chick limbs. (A,B) In situ hybridization on adjacent longitudinal limb sections of E3 chick embryos with *OSR2* (A) and *MYOR* (B) probes. *OSR2* was not detected in migrating myogenic cells labelled with *MYOR* but was observed in mesonephros (arrow in A). Transverse limb sections of E5 (C,D), E7 (E,F) and E10 (G,H) chick embryos hybridized with *OSR2* probe (purple staining) and immunostained for myosins using the MF20 antibody (brown staining). (D,F,H) represent high magnifications of muscle regions of (C,E,G), respectively. (D,F,H) Arrows indicate *OSR2* expression in MF20-positive cells, while arrowheads point *OSR2*-positive cells in MF20-negative cells. *OSR2* is mainly expressed in ICT and muscle ICT but also in differentiated muscle fibers. r, radius, u, ulna.

hypaxial lips of the somites towards the limb buds from embryonic day 2.5 (E2.5), (Chevallier et al., 1977). Migrating muscle progenitors assessed by *MYOR* expression did not expressed *OSR2* (Fig. 1A, B). At E3, no expression of *OSR2* was observed in chick limb buds (Fig. 1A) but *OSR2* was expressed in mesonephros as previously described (Stricker et al., 2006); Fig. 1A). This shows that *OSR2* is neither expressed in muscle progenitors nor in limb ICT at E4 and consistent with the previous study showing that *OSR2* is not expressed in

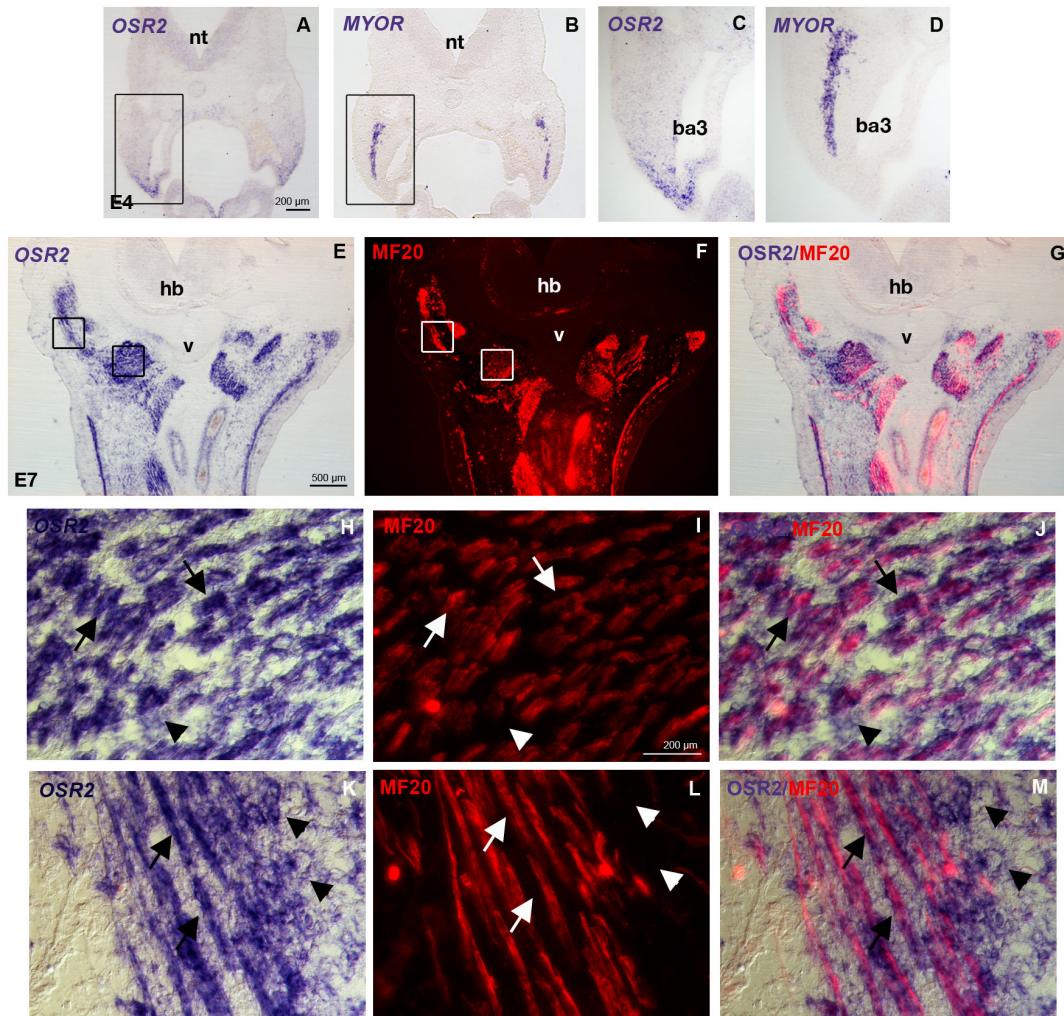


Figure 2: *OSR2* is expressed in differentiated muscle cells in chick head. (A-D) In situ hybridization on adjacent transversal head sections of E4 chick embryos with *OSR2* (A, C) and *MYOR* (B, D) probes. (C-D) represent high magnifications of branchial arch region of (A-B), respectively. *OSR2* was not detected in myogenic cells labelled with *MYOR* but was observed in the ICT of the third branchial arch (ba3). Sagittal head sections of E7 chick embryos (E-M) were hybridized with *OSR2* probe (purple staining) (E, G, H, J, L, M), and then immunostained for myosins using the MF20 antibody (red staining) (F, G, I, J, L, M). (H-M) represent high magnifications of mandibular muscle regions in (E-G). (E-M) Arrows indicate *OSR2* expression in MF20-positive cells, while arrowheads point *OSR2*-positive cells in MF20-negative cells. *OSR2* was detected in both ICT and differentiated muscle cells. nt, neural tube, ba3, 3rd branchial arch, hb, hind brain, v, vertebra.

developing chick limb bud before E4 (Stricker et al., 2006). From E5, *OSR2* was strongly expressed in dorsal and ventral limb muscle masses, revealed by *MYOD* expression (Fig. 2C, D), in the ICT localized between the two cartilage elements, ulna and radius, in the zeugopod limb regions and in the ICT close to the dermis (Fig. 1E). This pattern of expression is consistent with the sites of *Osr2* expression previously described (Lan et al., 2001; Stricker et al., 2006; Stricker et al., 2012). At the level of muscle masses at E6, *OSR2* expression was not only detected in muscle ICT (Fig. 1F, arrowheads), but also in Myosin-positive cells (Fig. 1F, arrows). At later stages of limb development, *OSR2* expression was mostly expressed in muscle areas (Fig. 1G-J), but also in dermis at E10 (Fig. 1I, arrow). Within muscles, *OSR2* was expressed in muscle ICT, characterized by the absence of myosin expression (Fig. 1H, J, arrowheads) but also, as previously observed at E6, in muscle fibres (Fig. 1H, J, arrows). The same pattern of expression was observed during hindlimb development (data not shown). Taken together, these results show that while *OSR2* expression is widely spread in the ICT at early stages of chick limb development, it progressively becomes restricted to muscle ICT at later stages and is additionally expressed in differentiated muscle fibres. Such an expression in chick muscle cells has never been observed (Stricker et al., 2012). During craniofacial development of mouse embryo, *Osr2* expression is restricted to specific mesenchymal tissues,

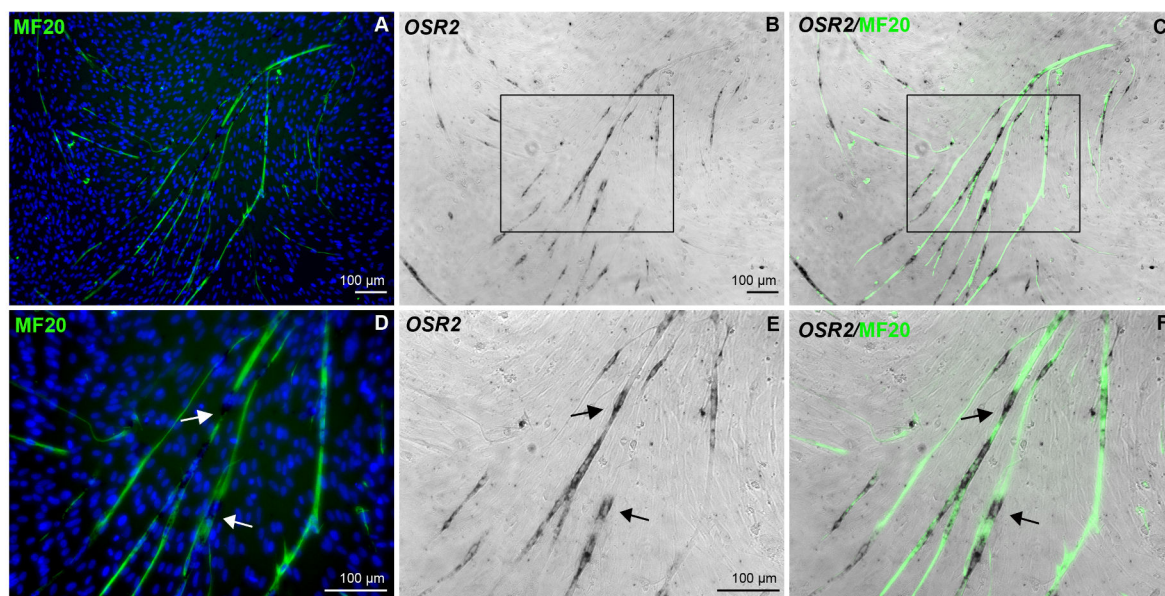


Figure 3: *OSR2* is expressed in differentiated muscle cells *in vitro*. (A-F) Chick foetal myoblasts cultured in differentiation conditions were hybridized with *OSR2* probe (black staining) and immunostained for myosin using the MF20 antibody (green staining). (D,E,F) represent high magnifications of the squared regions in (A,B,C) respectively. Arrows indicate *OSR2* expression in MF20-positive cells.

including the mesenchyme of the developing palatal shelves and the tongue (Lan et al., 2001) and a key role for *OSR2* in the development of specific craniofacial regions has been demonstrated (Lan et al., 2004). In chick embryo, *OSR2* has been described to be restricted to mesenchymal cells of branchial arches from E3 (Stricker et al., 2006). In order to assess whether *OSR2* was also expressed in cranial muscles during chick craniofacial development, we performed in situ hybridisation to head sections of chick embryos at different developmental stages. At E4, *OSR2* was detected in mesenchymal cells of branchial arches (Fig. 2A, C), as already described (Stricker et al., 2006). At this stage, *OSR2* expression did not overlapped with *MYOR* expression revealing muscle progenitor cells, showing that *OSR2* expression is restricted to ICT-derived mesenchymal cells (Fig. 2A-D). At E7, *OSR2* was barely expressed in the ICT surrounding muscles, while it was strongly expressed in the muscle regions (Fig. 2E-G). As previously described ((Lan et al., 2001; Stricker et al., 2006)), *OSR2* expression was observed in the muscle ICT at the level of head muscle masses (Fig. 2H, J, K, M, arrowheads), but also in Myosin-positive cells of muscles (Fig. 2H-M, arrows). Consequently, our results demonstrate that *OSR2* expression in differentiated muscle cells is not restricted to limb muscles but is also observed in developing chick craniofacial muscles, an expression pattern not described previously (Stricker et al., 2006). Finally, to confirm *OSR2* expression in muscle fibres, we analyzed *OSR2* expression in primary cell cultures of chick limb foetal myoblasts. Chicken foetal myoblasts cultures were maintained in differentiation conditions for 2 days, and *OSR2* expression was evaluated by in situ hybridization (Fig. 2B, E). MF20 immunostaining for MF20 (Fig. 2A, D) demonstrated that in in vitro conditions, *OSR2* was expressed in differentiated muscle fibers (Fig. 2B-F). These data confirmed that *OSR2* is indeed expressed in chick differentiated muscle cells.

Previous studies highlighted *OSR2* expression as being restricted to the ICT of developing limb and head of chick embryos (Stricker et al., 2006; Stricker et al., 2012). In this study, we report a new location of *OSR2* expression during chick limb and craniofacial development, demonstrating that *OSR2* is expressed in muscle fibres, in addition to its expression in ICT and muscle ICT during chick limb and craniofacial development.

Discussion

I. CXCL12 and CXCL14 chemokines differentially regulate connective tissue markers during limb development

During embryonic development, CT that is primarily composed of fibroblasts, contribute to tissue remodelling (Yusuf et al., 2013). The formation of CT results from the proliferation of fibroblasts, which produce an important amount of ECM. It is then considered that fibroblasts at the origin of CT during embryonic morphogenesis are constantly activated (Yusuf et al., 2013). Conversely, in adult, CT fibroblasts are mostly quiescent but can be activated under specific stimuli, mostly pathological, which lead to the restoration of proliferation, production of extracellular matrix and growth factors, and migration capacity. This uncontrolled activation of fibroblasts in adult results in fibrosis. Therefore, understanding fibroblast peculiarities and their regulation during embryonic CT formation is an important issue to decipher the mechanisms underlying the fibrosis process. Interestingly, chemokines have been shown to be central mediators of fibrosis initiation and progression, among which CXCL12 and CXCL14 have been shown to promote fibrosis (Emblom-Callahan et al., 2010; Ishii et al., 2013; Jia et al., 2017; Lin et al., 2014b).

One part of my PhD work has been to investigate the role of CXCL12 and CXCL14 chemokines in the formation of CT during chick limb development. Our results show that both chemokines are expressed in dense CT during chick limb development and positively regulate the expression of different CT-associated markers in chick limb fibroblasts. In addition, we reveal that the BMP anti-fibrotic factor inhibits *CXCL12* and *CXCL14* expression in limb CT during development and that mechanical forces, which are essential for the progression of fibrosis (Carver and Goldsmith, 2013), positively regulate the expression of *CXCL12*, *CXCL14* and CT-associated genes.

A. The CXCL12 and CXCL14 expression domains define distinct subpopulations of dense CT.

Although, the expression of *CXCL12* and *CXCL14* has been previously studied in limbs (García-Andrés and Torres, 2010; Gordon et al., 2011; Rehimí et al., 2008; Vasyutina et al., 2005), we show that both chemokines are restricted to specific subpopulations of chick limb dense CT, as evidenced by their overlapping expression with that of specific CT-subpopulation markers. We reveal that *CXCL12* displays expression in non-muscle and muscle CT, colocalizing with *OSR1* and *OSR2* expression, respectively. *CXCL14* expression

is detected in *SCX* expression domains, which delineate tendon CT, but also in regions surrounding cartilage elements, which likely correspond to ligaments. In addition, *CXCL14* expression is also observed in muscle irregular CT of specific muscles, mostly ventrally. The different expression patterns of both chemokines suggest that *CXCL12* and *CXCL14* are involved in the formation of distinct dense CT subpopulations (Nassari et al., 2017 submitted).

B. CXCL12 promotes irregular CT formation during chick limb development

Our results show that *CXCL12* chemokine activates the expression of irregular CT markers *OSR1* and *COL3A1* in chick limb fibroblasts in vitro and in vivo, thus characterizing *CXCL12* as a factor contributing to the differentiation of irregular CT during chick limb development. These results are coherent with the effect of *CXCL12* in the progression of fibrosis in adult (Emblom-Callahan et al., 2010; Ishii et al., 2013; Jia et al., 2017; Lin et al., 2014b). Indeed, *CXCL12* expression is increased in different human fibrotic pathological situations, such as idiopathic pulmonary fibrosis (Tan et al., 2017) and chronic pancreatitis (Neesse and Ellenrieder, 2016). Furthermore, in organizing pneumonia lesions, characteristic of fibrotic pulmonary diseases, *Cxcl12* upregulation is associated with an increase of *Col3a1*, in both human and mouse transgenic models of this pathology (Izykowski et al., 2016).

In the context of fibrosis, *CXCL12* effect is mediated through its CXCR4 receptor. In human lung and prostate fibroblasts, *CXCL12* has been shown to induce, via CXCR4, the expression of α -SMA, the key marker of activated fibroblasts (Lin et al., 2014b; Rodriguez-Nieves et al., 2016). From E6, CXCR4 expression is mainly detected in endothelial cells of developing chick limbs, therefore suggesting that the *CXCL12* effect on irregular CT differentiation is not mediated through CXCR4, but through its second receptor CXCR7.

We demonstrate that *CXCL12* expression induces the expression of irregular CT markers however it is not clear whether this activation is mediated via direct interaction. Stricker et al. (2012) have previously demonstrated that *OSR1* overexpression in mesenchymal progenitors led to an increase in the expression of *COL3A1* (Stricker et al., 2012), indicating that *CXCL12* might activate *COL3A1*, through the induction of *OSR1*. However, *COL3A1* appears to be ectopically expressed both in muscle and non-muscle irregular CT after *CXCL12* overexpression, while the increase in *OSR1* expression is mostly observed in non-muscle irregular CT. One putative intermediate candidate for *COL3A1* regulation by *CXCL12* could be the fibrotic factor PDGFB, since *CXCL12* increases *PDGFB* transcription in human bone marrow cells (Hamdan et al., 2011) and PDGFB is known to activate collagen transcription in

chick limbs (Tozer et al., 2007).

Finally, our data reveal that CXCL12 activates the irregular CT marker *OSR2* in vitro. However, no increase in *OSR2* expression is observed after overexpression of CXCL12 in vivo. Since we demonstrate that *OSR2* is also strongly expressed in myogenic cells in chick limb embryos (supplemental data), in addition to be present in ICT, we cannot exclude that it might hide a putative increase of *OSR2* in muscle irregular CT expression after CXCL12 overexpression in vivo.

C. *CXCL14 is involved in regular CT differentiation during chick limb development*

Previous works have shown that CXCL14 is involved in fibrosis. *CXCL14* expression is upregulated in idiopathic pulmonary fibrosis (Jia et al., 2017) and chronic inflammatory arthritis (Marinova et al., 2014) and has been shown to stimulate prostate fibroblast proliferation and migration (Östman and Augsten, 2009), illustrating a putative role for CXCL14 in fibroblast activation. Our data illustrate a role for CXCL14 in the differentiation of regular dense CT, as it induces the expression of the regular CT marker *SCX* in chick embryonic fibroblasts, without affecting the expression of irregular CT markers. Interestingly, it has been shown recently that *SCX* controls fibroblast activation in heart and could be a potent regulator in fibrotic diseases in the cardiovascular system (Bagchi et al., 2016). In addition, *SCX* is activated by the TGF- β fibrotic factor during chick or mouse limb development (Havis et al., 2014; Havis et al., 2016). Those results indicate that CXCL14 might enhance myofibroblast differentiation rather than dense regular CT differentiation.

Although in vitro experiments indicate a role for CXCL14 in the regulation of *SCX* expression, in vivo CXCL14 overexpression does not induce an obvious increase in *SCX* expression at tendon and ligament levels. During chick limb development, *SCX* expression is first initiated at E3-3.5 (Edom-Vovard and Duprez, 2004). As ectopic CXCL14 expression in limb mesenchyme has been performed at E4, one possibility could be that CXCL14 is only required for *SCX* induction and not involved at later stages of development. In agreement with this hypothesis, previous experiments have shown that *SCX* expression in limb mesenchyme is induced at E3.5 by an unidentified factor coming from the limb ectoderm, but that after E4.5 this factor is not required for *SCX* expression (Schweitzer et al., 2001). As *CXCL14* is expressed in limb ectoderm (our results), one hypothesis is that ectoderm-derived CXCL14 induces *SCX* expression in limb mesenchymal cells.

D. The BMP anti-fibrotic factor regulates the expression of CXCL12 and CXCL14 chemokines during chick limb development

Numerous previous studies have demonstrated the anti-fibrotic effect of BMP signalling pathway (Yang et al., 2011). Activation of the BMP signalling pathway antagonizes the TGF β -induced fibrosis and represses the expression of *Acta2* gene, coding for α -SMA, as well as the expression of the *Ctgf* gene, coding for a fibrotic factor (Weiskirchen, 2009; Yang et al., 2011). During chick limb development, previous data have shown that BMP4 inhibits the expression of the irregular CT gene *TCF4* (Bonafede et al., 2006). We show here that *BMP4* forced-expression induces a decrease in *CXCL12* and *CXCL14* expression, associated with inhibition of both *OSR1* and *OSR2* genes. Conversely, inhibition of BMP signalling by overexpression of its antagonist NOGGIN increases *CXCL12*, *CXCL14* and *OSR1* expression. As we also show that *CXCL12* positively regulates *OSR1*, one hypothesis is that changes in *OSR1* expression is a consequence of *CXCL12* down-regulation. We also show that *CXCL14* induces *SCX* expression. It has been previously demonstrated that *SCX* expression is inhibited by BMP and induced by NOGGIN in chick limbs (Schweitzer et al., 2001). Consequently, we can hypothesized that *SCX* down-regulation in the context of *BMP4* overexpression results from changes in *CXCL14* expression. These results are in agreement with the anti-fibrotic effect of BMP pathway in adult and reveal that BMP signalling inhibits CT differentiation during development. This also indicates a pivotal role for *CXCL12* and *CXCL14* chemokines as downstream targets of *BMP4* signalling pathway to regulate CT markers during chick limb development. In bone marrow stromal cell lines, SMAD-binding elements have been identified in the *Cxcl12* promoter and *BMP4* has been shown to regulate *Cxcl12* expression, as treatment of stromal cells with the BMP antagonist Noggin significantly increased *Cxcl12* levels (Khurana et al., 2014). These data show that BMP signalling elements interact directly with *CXCL12*, favoring a central role of *CXCL12* as a downstream target of the *BMP4* pathway in fibroblastic cells. In chick embryonic limbs, localized BMP signalling restricts the ectodermal signal which induces *SCX* expression (Schweitzer et al., 2001). As previously hypothesized, *CXCL14* is a good candidate for the ectodermal signal inducing *SCX* expression in chick limbs. *SCX* expression might be restricted in limb mesenchyme via the inhibiting effect of BMP signalling on *CXCL14*. In *Xenopus*, it has been demonstrated that *CXCL14* ectodermal expression is partially regulated by BMP signalling. Indeed, *Noggin* overexpression associated with Wnt pathway induction led to an increase of *Cxcl14* expression in the ectoderm (Park et al., 2009). Interestingly, it has been shown that ectodermal Wnts contribute to distal tendon induction (Zhu et al., 2012). Moreover, the Wnt

cascade is also involved in fibroblast activation during fibrosis (Castellone and Laukkanen, 2017). Therefore, it would be then interesting to test the potential role of Wnt signalling pathway in the regulation of *CXCL14* expression during chick limb development.

FGF pathway, as BMP signalling, has been described as being implicated in fibrosis (Kim et al., 2016; Shimbori et al., 2016). However, depending on FGF members and responding cells involved, FGF effect led to activation or inhibition of the fibrosis process (Kim et al., 2016). During embryonic development, FGF positively regulates the expression of different CT markers, including *SCX* and *COL3A1* (Edom-Vovard et al., 2002; Havis et al., 2016; Lejard et al., 2011). Although we observe that FGF inhibits *OSR1* and *OSR2* expression during chick limb development, the expression levels of *CXCL14* and *CXCL12* are not modified. Then, it is likely that *OSR* genes downregulation observed after FGF4 bead grafts is independent of *CXCL12* and *CXCL14* chemokines. However, since *OSR1* and *OSR2* overexpression inhibits *SCX* expression in chick limb cells (Stricker et al., 2012), these results suggest that *SCX* and *OSR* genes mutually repress each other in mesenchymal cells during chick limb development.

E. The expression of CXCL12 and CXCL14 chemokines and CT genes is controlled by mechanical forces in chick limbs

Recently, mechanical forces emerged as principal actor in different morphogenesis events during embryonic development (Hamada, 2015; Happe and Engler, 2016; Sokol, 2016). In chick, muscleless or aneural limbs deprived of muscle activity exhibit reduced *SCX* expression (Edom-Vovard et al., 2002) and embryo immobilisation results in a FGF4-dependent downregulation of *SCX* in limbs (Havis et al., 2016). In addition, mechanical activity has been also shown to modulate *Scx* expression and promote the differentiation of mesenchymal cells towards tendon CT (Chen et al., 2012; Scott et al., 2011). Similarly, mechanical forces have been shown to play a major role in fibrosis progression (Duscher et al., 2014). Under mechanical tension, the TGF- β fibrotic signalling is activated (Wells and Discher, 2008). Moreover, mechanical tension results in α -SMA expression and increase in matrix deposition in multiple tissues (Wells, 2013). Our results provide evidence that inhibition of mechanical forces induces decrease in the expression of *CXCL12*, *CXCL14* and the associated CT markers *OSR1*, *OSR2*, *COL3A1*, *COL1A2*, *COL6A1* and *SCX*. Interestingly, inhibition of *CXCL12* and *CXCL14* after immobilization mainly occurs in muscle CT and irregular CT surrounding muscles, while not affecting chemokine expression in cartilage (*CXCL12*) and ectoderm (*CXCL14*), thus demonstrating that mechanical forces positively impact the expression of both chemokines specifically in dense CT.

Among the mechanisms involved in the adaptative response of adult muscle to exercise is the activation of the fibrotic TGF- β signalling pathway, which leads to an increase in the expression of *CTGF* and collagen genes in muscle irregular CT (Heinemeier et al., 2007). TGF- β has been shown to control dense regular CT differentiation during limb development (Pryce et al., 2009), a mechanism regulated by mechanical activity (Havis et al. 2016). In human fibroblasts, CXCL12 induces *CTGF* expression (Lin et al., 2014b) and TGF- β has been shown to cooperate with CXCL12 in carcinoma-associated fibroblasts to induce α -*SMA* expression (Kojima et al., 2010). Therefore one possibility could be that, downstream of mechanical signals, TGF- β interacts with CXCL12 to regulate irregular CT-associated genes, among which *CTGF* and *OSRs*, during limb development.

This work demonstrates for the first time that CXCL12 and CXCL14 chemokines differentially regulate the expression of specific CT genes (Fig. 1), participating to the orchestration of regular and irregular CT differentiation during chick limb development. A role for CXCL12 and CXCL14 chemokines in CT differentiation during development is completely consistent with their recognised functions in adult fibrosis processes, such as chronic fibrotic pathologies (Jia et al., 2017; Marinova et al., 2014; Neesse and Ellenrieder, 2016; Tan et al., 2017) and cancer-induced fibrosis (Allinen et al., 2004). CXCL12 and CXCL14 regulate the expression of CT-associated transcription factors in chick limbs and are controlled by the BMP anti-fibrotic factor and muscle mechanical forces (Fig. 1). Identification of the unexpected role of CXCL12 and CXCL14 during CT differentiation contributes to a better understanding of the fibrosis mechanisms in adult pathological conditions. To an extent, study of limb CT development could be used as a new model to investigate mechanisms underlying fibrosis formation.

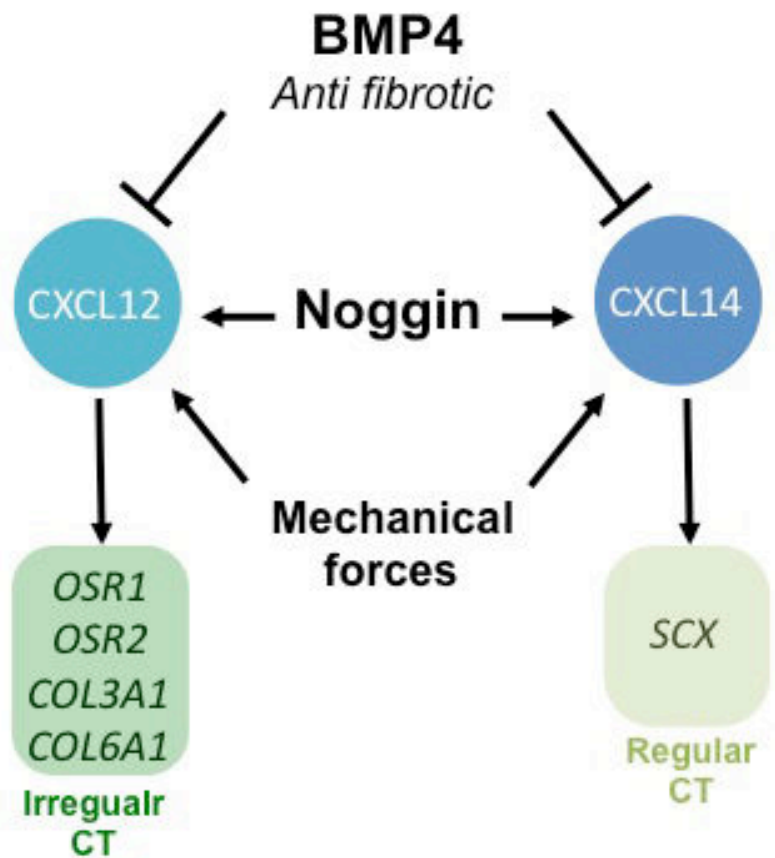


Figure 1: CXCL12 and CXCL14 enhance dense CT markers expression, and are negatively regulated by BMP4 while positively regulated by mechanical forces during chick limb development.

II. The contribution of connective tissue cells to muscle morphogenesis: an unexpected role for CXCL12 and CXCL14 signalling pathways

During myogenesis, the intrinsic capacities of muscle progenitors to proliferate and differentiate are essential (Buckingham and Rigby, 2014), but adjacent tissues also participate in the proper formation of muscles (Ashby et al., 1993; Chevallier and Kieny, 1982; Kardon, 1998a; Merrifield and Konigsberg, 1987; Robson and Hughes, 1999; Tozer et al., 2007). In the past decades, irregular connective tissue (ICT) has been highlighted as an important source of signals for muscle development (Chevallier and Kieny, 1982; Kardon, 1998a), thanks to the identification of ICT-associated transcription factors (Hasson et al., 2010; Kardon et al., 2003; Stricker et al., 2012). However, the molecular mechanisms responsible for the dialogue between ICT and muscle are not completely elucidated.

My PhD work aimed to assess the role of CT-derived CXCL12 and CXCL14 in the process of muscle morphogenesis. We provide evidence that both chemokines affect limb myogenesis, and that one receptor of CXCL12, CXCR7, is expressed in myogenic cells and positively affects myogenesis putatively through beneficial effect on muscle cell fusion.

A. CXCR7 is expressed in muscle progenitor and differentiated cells during chick limb myogenesis

CXCR7 starts to be expressed in limbs of E5 chick embryos, when *CXCR4* expression is progressively down-regulated in muscle progenitors, with some cells co-expressing *CXCR4* and *CXCR7*. Such *CXCR4-CXCR7* co-expression has been already observed in C2C12 muscle cells in vitro (Hunger et al., 2012). Myogenic cells co-expressing both receptors might reveal a possible switch from *CXCR4* to *CXCR7* expression. Interestingly, it has been shown that C2C12 cells preferentially express *CXCR4* under proliferation conditions, while they mostly express *CXCR7* in differentiated state (Hunger et al., 2012). Therefore, one hypothesis is that the switch from *CXCR4* to *CXCR7* indicates a transition from a muscle progenitor to a differentiated state.

Another hypothesis is that *CXCR4* to *CXCR7* transition at E5 stabilizes the myogenic fate. In the vertebrate limb, both endothelial and muscle cells originate from the somites (Chevallier et al., 1977; Christ et al., 1977; Pardanaud et al., 1996) and common bipotent progenitors contribute to both limb muscle and endothelial cell lineages (Kardon et al., 2002). During chick limb development, *CXCR4* is expressed in endothelial cells and myogenic precursors

entering the limb (Yusuf et al., 2006). We show that at E5, CXCR4 is expressed both in limb muscle masses and in endothelial cells, but progressively decreases in muscle cells concomitantly with the appearance of *CXCR7* transcripts. At later stages of limb development, CXCR4 and *CXCR7* are present in endothelial and myogenic cells, respectively. As it has been shown that modulating CXCR4 activity perturbs the formation of limb muscles and vasculature (Yusuf et al., 2006), we suggest that a transition from CXCR4 to CXCR7 expression in myogenic progenitors is important to maintain a myogenic fate and avoid a transition towards an endothelial differentiation at E5. In this context, CXCR7 would act as a lock for the myogenic fate.

Coherent with this hypothesis we reveal that *CXCR7* overexpression down-regulates the expression of *CXCR4* receptor in myoblast cultures, while *dn-CXCR7* overexpression increases the expression of *CXCR4* receptor. Such mechanism at the transcriptional level could account for the transition from CXCR4 to CXCR7 expression in muscle cells.

Alternatively, CXCR4 to CXCR7 transition might be mediated via a downregulation of CXCR4 that could occur through heterodimerization of both receptors. It has been previously shown that CXCR4·CXCR7 complex activate the recruitment of β -arrestin and impair the CXCR4-G-protein-mediated signalling via internalization of CXCR4 (Décaillot et al., 2011).

B. Do CXCL12 and CXCR7 act together in muscle morphogenesis?

During limb muscle formation, CXCL12/CXCR4 axis has been shown to play a role in the migration of muscle progenitors from the ventro-lateral lip of the dermomyotome towards the limb mesenchyme (Vasyutina et al., 2005). However, the role of CXCL12/CXCR7 has not been investigated yet during limb muscle morphogenesis. Previous works have described that CXCR7 is expressed in E18 mouse limb muscle cells and differentiating C2C12 muscle cells (Melchionna et al., 2010; Hunger et al., 2012). In agreement with these data, our results show that *CXCR7* is expressed in myogenic cells during chick limb muscle development. In addition we show that *CXCL12* is expressed in ICT, therefore suggesting that CXCL12 produced by ICT interacts with myogenic cells expressing CXCR7. Using *CXCR7* gain- and loss-of-function approaches in vivo and in vitro, we observe that increasing *CXCR7* expression enhances muscle differentiation, while expressing the *dn-CXCR7* construct results in a decrease in myogenic differentiation. However we observe that CXCL12 has no effect on myogenic differentiation in vitro but induces defects in muscle morphogenesis in vivo.

Although these data appear contradictory, different hypothesis can be proposed for the role of CXCL12/CXCR7 axis in the regulation of muscle morphogenesis. A previous study of

Hunger et al. (2012) has shown that CXCL12 positively regulates myoblasts proliferation via CXCR4 and inhibits differentiation via CXCR7 in C2C12 muscle cells. They demonstrated that CXCR7 acted as a scavenger for CXCL12 to abrogate CXCL12/CXCR4 signalling (Hunger et al., 2012). Such a mechanism could account for (1) the transition from CXCR4 to CXCR7 receptor during chick limb muscle differentiation, (2) the positive effect of *CXCR7* overexpression on limb muscle differentiation and (3) the defects in limb morphogenesis observed after *CXCL12* overexpression in vivo. However, we observe that increasing CXCL12 concentration in the medium of primary muscle cultures does not affect myogenesis. This discrepancy can be explained by a possible saturation of CXCR7 receptors by CXCL12 in the culture conditions. Indeed we use a serum-complemented medium to trigger muscle differentiation, which is an important source of secreted factors among which chemokines, while in the in vitro experiments of Hunger et al. (2012), muscle cells were differentiated in serum free medium.

Based on those published data and our work we can hypothesized that CXCL12 secreted by the ICT negatively regulate muscle differentiation. However this effect would be counteracted by the scavenger function of CXCR7 receptor expressed in muscle cells, allowing a balance between ICT and muscle differentiation. Interestingly, we show that *dnCXCR7* overexpression in developing limbs results in an increase in *CXCL12* expression specifically in the ICT. Such a cross-regulation at the transcriptional level between *CXCL12* and *CXCR4* has been already described during migration of neural crest cells in chick embryo (Escot et al. 2013; Escot et al. 2016) and although it is not clear what mechanisms is involved in the crosstalk between *CXCL12* and CXCR7, it is noteworthy that such a transcriptional regulation of *CXCL12* expression by CXCR7 could also participate in the balance between muscle and ICT differentiation during chick limb development.

C. Does CXCL12 act on muscle morphogenesis via its effect on ICT?

As CXCL12 increases the expression of ICT markers and collagen genes in developing chick limbs (Nassari et al., 2017 submitted), one possibility could be that the CXCL12 effect on muscle morphogenesis is mediated through its positive action on ICT differentiation and not directly via its binding on myogenic cells expressing CXCR7. It has been previously shown that binding of CXCL12 to CXCR4 induces *PDGFB* transcription in human bone marrow cells (Gonzalez et al., 2014). In chick limbs, PDGFB, expressed in endothelial cells of the vessels, promotes the expression of collagen genes and the production of extracellular matrix, allowing separation of the muscle masses during the splitting process (Tozer et al., 2007). As

CXCR4 is expressed in endothelial cells during chick limb development, CXCL12 could act on ICT differentiation via CXCR4 and induces PDGFB secretion, which in turn would favour the differentiation of ICT at the expense of muscle differentiation, independently of CXCL12 binding to CXCR7 receptor (Fig. 2).

During chick embryonic development, $\beta 1$ integrin activation is particularly intense at the myotendinous junction. Furthermore, we provide evidence that *CXCL12* overexpression in ICT induces changes in the composition of the extracellular matrix which in turn induces an increase in $\beta 1$ integrin activation at the myotendinous junction. It is noteworthy that modifying fibronectin by LoxL3 enzyme, located at muscle fibre tips, induces an increase of

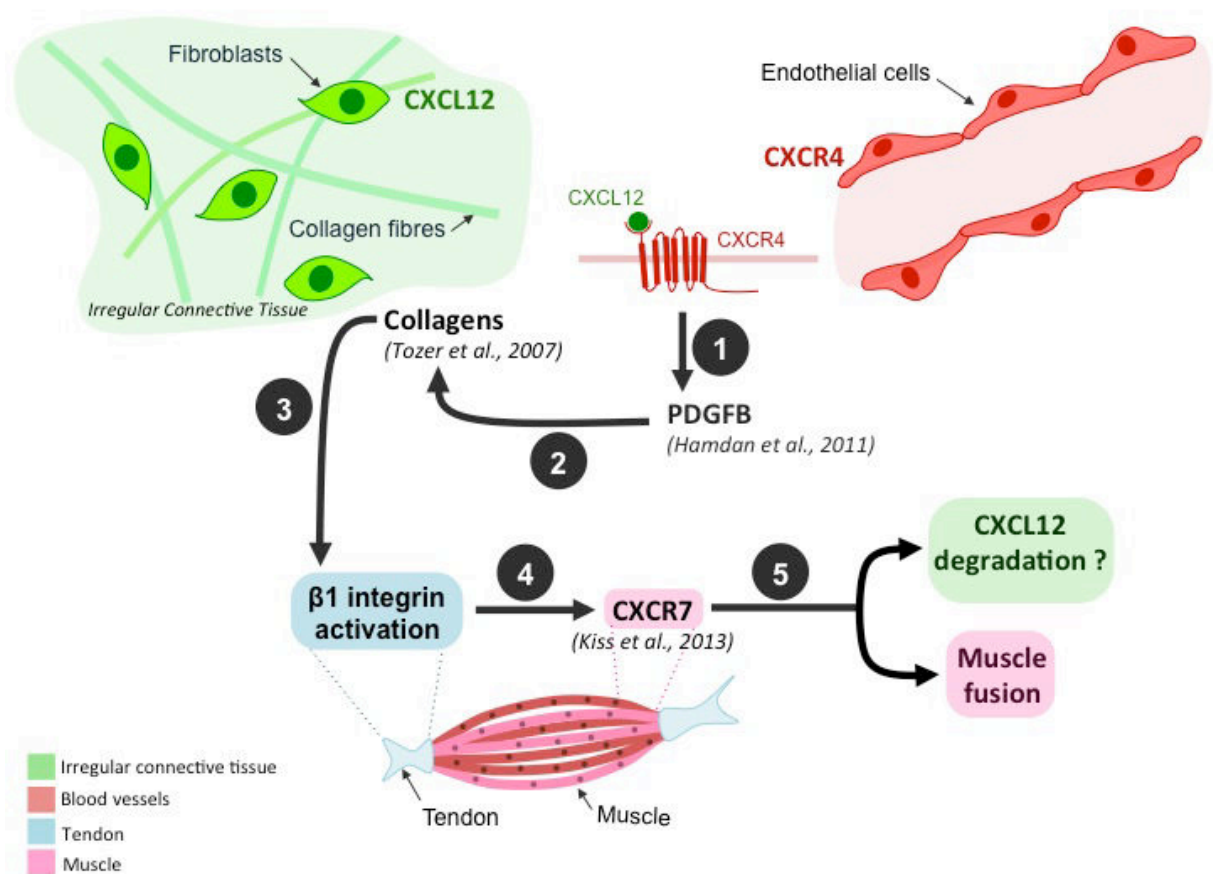


Figure 2: Model illustrating the role of CXCL12 and CXCR7 in the interaction between ICT and muscle development. CXCL12 secreted from the ICT signals through CXCR4 present at the surface of endothelial cells and enhances expression of PDGFB (1) which in turn induces increase in collagens and ICT markers expression in ICT (2). Collagens activate $\beta 1$ integrins at the myotendinous junctions (3), which leads to an increase in the expression of CXCR7 at muscle fibre tips (4). Finally CXCR7 would promote muscle development likely through its effect on myoblast fusion and would negatively regulate CXCL12 expression in ICT possibly through degradation of the chemokine.

integrin activation at the myotendinous junction (Kraft-Sheleg et al., 2016). Among the different integrin subunits, $\alpha7\beta1$ integrin is specifically enriched at the myotendinous junction (Bao et al., 1993), which is closely associated to the localization of *CXCR7* expression at the tips of muscle fibers. Gain- and loss-of-function approaches in mice have demonstrated that $\alpha7\beta1$ integrin is a major receptor connecting muscle cells to tendons, which participate to organize the myotendinous junction (Burkin and Kaufman, 1999). Moreover, increasing $\alpha7\beta1$ integrin promotes myogenesis (Liu et al., 2009) and mutations affecting $\alpha7\beta1$ caused muscular dystrophy (Mayer, 2003). However, these results seem to be contradictory with the *CXCL12* effect, which increases $\beta1$ integrin activation and inhibits muscle morphogenesis. One hypothesis could rely on the observation that activation of $\beta1$ integrins by extracellular matrix increases *CXCR7* expression in invasive prostate cancer cells (Kiss et al., 2013). As we show that *CXCR7* overexpression in muscle cells favour limb myogenesis in vivo and in vitro, one possible model would be that the increase in $\beta1$ integrin activation at the myotendinous junction would be responsible for an increase in *CXCR7* expression at the tips of muscle fibers, which in turn would increase myoblast fusion and myotube differentiation to balance the negative effect of *CXCL12* on muscle morphogenesis (Fig. 2).

D. The chemokine receptor CXCR7 is important for proper muscle formation

Our results show that overexpressing *CXCR7* or *dn-CXCR7* in vivo lead to a misregulation of the balance between muscle cells proliferation and differentiation. Indeed, overexpression of *CXCR7* induces a decrease in the number of PAX7-positive cells and an increase in the surface area of MF20-positive myotubes, while *dn-CXCR7* overexpression induces an increase in PAX7-positive cells and a decrease in the surface area of MF20-positive myotubes, in chick limbs. This suggests that *CXCR7* enhances myoblast fusion, a result which is coherent with the observation that myoblast fusion preferentially occurs at the tips of muscle fibers (Gu et al., 2016; Williams and Goldspink, 1971), where *CXCR7* is preferentially located. Recently, *Myomaker* emerged as a specific marker involved in myoblast fusion (Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013). We show, both in vitro and in vivo, that *CXCR7* gain- and loss-of-function induces an increase and a decrease in *MYOMAKER* expression, respectively. In addition, overexpressing *CXCR7* in vitro increases myoblast fusion, while overexpressing *dn-CXCR7* decreases it.

KLFs (Krüppel-Like Factors) transcription factors have been shown to be involved in muscle cell fusion, via Nephronectin, one of their transcriptional downstream targets (Sunadome et al., 2011). Interestingly, it was shown that *CXCR7* is co-expressed with KLF4 in a

subpopulation of glioblastoma cells (Flüh et al., 2016). Additionally, the database STRING, highlighting known and predicted protein-protein interactions, describes a putative physical/functional interaction between CXCR7 and Nephronectin (Yamazaki et al., 2004), suggesting that CXCR7 could act on muscle fusion through interaction with Nephronectin. Finally, we cannot exclude that CXCR7 acts independently of ligand binding on limb myogenesis. Interestingly, ligand-independent role of CXCR7 has been evidenced in prostate and breast cancer cells (Salazar et al., 2014; Singh and Lokeshwar, 2011) and CXCR7 has been shown to interact with EGFR in breast cancer cells (Salazar et al., 2014). In human muscle cell cultures, EGFR activity is down-regulated during early myoblast differentiation and this event is required for normal differentiation to take place (Leroy et al., 2013). Furthermore, EGFR silencing in proliferation conditions triggers muscle differentiation (Leroy et al., 2013). In addition, β -arrestin proteins have been shown to be essential for cell cycle exit and differentiation through the transactivation of EGFR in human myoblasts (Santos-Zas et al., 2016). One possibility could be that binding of CXCR7 to EGFR or CXCR7 β -arrestin controls down-regulation of EGFR receptor necessary for muscle differentiation. It is noteworthy that EGF has been shown to inhibit myogenesis during chick limb development (Dealy et al., 1998).

E. CXCL14 acts as a non-cell autonomous signal regulating myogenic differentiation and muscle patterning

In agreement with previous studies assessing the role of CXCL14 during myogenesis (Ge et al., 2013; Waldemer-Streyer et al., 2017), our results demonstrate that CXCL14 negatively regulates myogenic differentiation both in vitro and in vivo. Our results clearly illustrate a non-cell autonomous effect of CXCL14 on myogenesis in vitro and in vivo. CXCL14 drastically decreases the number of muscle progenitors and myotubes in primary cultures of chick myoblasts, although cell proliferation and cell fusion are not affected in these conditions. Our data illustrate a likely direct effect of CXCL14 on myogenic cells, suggesting that a CXCL14 receptor is present at the surface of muscle cells. To date, signalling receptor for CXCL14 has not been clearly identified, nevertheless it has been shown that CXCL14 binds to CXCR4 in order to inhibit CXCL12/CXCR4 interactions and associated-signalling (Tanegashima et al., 2013a; Tanegashima et al., 2013b). We observe that CXCL14 decreases *CXCR4* expression in primary cultures of myoblasts, however, as CXCR4 expression was not detected in muscle cells in vivo, it seems unlikely that CXCR4 is involved in CXCL14 effect on myogenesis. Recently, the atypical chemokine receptor ACKR2 (D6 or CCBP2) has been

proposed as CXCL14-signalling receptor (Sjöberg et al., unpublished data). ACKR2 has been shown to signal through β -arrestin as CXCR7 and although its expression has not been reported in adult muscle (Bonavita et al., 2017), it could be a putative candidate for CXCL14 receptor during development.

We demonstrate that while muscle differentiation is severely affected in CXCL14 gain-of-function conditions in vitro (Fig. 11), cell death and proliferation are not affected, suggesting that CXCL14 induces phenoconversion of myogenic cells. Using RT-qPCR analysis we observe an increase in the expression of myofibroblastic cell markers *TCF4* and *ACTA2*, which support the hypothesis that CXCL14 promotes transdifferentiation of primary myoblasts to myofibroblasts. Previous experiments have reported such mechanism in muscle cell cultures: acid retinoic treatment induces myoblast transdifferentiation into premeiotic sperm-like cells in C2C12 muscle cells (Jia et al., 2011), and exposure to vitamin D, long chain-fatty acids and thiazolidinones triggers transdifferentiation of C2C12 cells into adipocytes (Grimaldi et al., 1997; Ryan et al., 2013). Similarly, treatment of porcine satellite cells with ciglitizone promotes adipogenic transdifferentiation (Singh et al., 2007). These data support the idea that transdifferentiation mechanisms occur in muscle cells and suggest that CXCL14 might represent an ICT-secreted factor, which negatively regulates limb myogenesis via its transdifferentiation action on myogenic cells.

In addition, analysis of *Cxcl14* deficient mice revealed a role for CXCL14 in the regulation of glucose metabolism in skeletal muscle. Upon CXCL14 exposure, glucose uptake is decreased in C2C12 cells (Nara et al., 2007). Previous works have demonstrated the involvement of glucose metabolism during muscle development (Viñals et al., 1997), suggesting that CXCL14 regulates myogenesis via regulation of glucose metabolism in muscle cells.

Finally, we demonstrate that CXCL14 is involved in the regulation of *SCX* expression in vitro. Therefore we cannot exclude that CXCL14, in addition to directly regulating myogenesis, also contributes indirectly to limb muscle development through its implication in regular CT development. Regular CT corresponds to tendons and ligaments, and during limb muscle development, interactions between dense CT and myogenic cells are essential for correct muscle morphogenesis (Chevallier and Kieny, 1982; Huang et al., 2013; Kardon, 1998).

Our results provide evidence for the involvement of CXCL12 and CXCL14 chemokines in the process of CT-mediated muscle morphogenesis. In addition, we reveal for the first time an important role for CXCR7 in the control of foetal myogenesis. In adult muscle, several studies

have already underlined the importance of ICT (FAPs/Mesenchymal progenitors) during the process of muscle regeneration. Our findings provide new tools to investigate the interactions between adult CT and muscle cells, thus enabling new perspectives for therapeutic approaches in muscle aging and muscle pathologies associated with deregulation of the CT/muscle cell balance.

Conclusion and future prospects

Conclusion and future prospects

The first part of my work show that secreted factors involved in the regulation of CT homeostasis in adult organs are also involved in the regulation of CT differentiation during development. Using the chick embryonic limb as a model, we demonstrate that CXCL12 and CXCL14 chemokines regulate the expression of specific CT transcription factors and collagen genes in limb ICT and MCT. Furthermore we characterize BMP4, known as an anti-fibrotic factor, and mechanical forces as negative and positive regulators of both chemokines, respectively.

Our proposed model for CXCL12 effect on the differentiation of limb ICT relies on the possibility that it signals through CXCR4 receptor expressed on endothelial cells of limb vessels and activates the secretion of PDGFB which increases the expression of CT markers and collagen genes. Different experiments can be conducted to test this hypothesis. We propose first to study vessel morphogenesis after CXCL12 overexpression in embryonic limbs by black ink injection in the circulation and/or 3D reconstructions of limbs after endothelial immunostaining. CXCR4 involvement in this process could be evaluated by using specific CXCR4 inhibitors in CXCL12 overexpression conditions *in vivo*. Such inhibitors have been shown to have no effect on CXCR7 receptor (Van Hout et al., 2017). The expression of PDGFB and its receptor will be investigated after CXCR4 blockade. Finally, using inhibitors of PDGF signalling (Makino et al., 2017) combined with CXCL12 overexpression could help to decipher whether PDGF signalling is implicated in the positive effect of CXCL12 on ICT differentiation during chick limb development.

The role of CXCL14 on CT differentiation remains more elusive. We show that CXCL14 overexpression *in vitro* induces an increase in *SCX* expression, known to be a specific tendon marker during limb development. However, *Scx* has also been shown to be co-expressed with FSP1, a specific marker of activated myofibroblasts, in adult MCT and a dramatic increase in the number of *Scx*/FSP1 positive cells has been observed following muscle injury, indicating that this population could participate to the fibrosis mechanism during muscle regeneration (Shallal-Ayzin et al., 2010). In order to better understand the role of CXCL14 in limb CT differentiation, it will be interesting to test whether FSP1 is expressed during chick limb development and also regulated by CXCL14 *in vitro* and *in vivo*.

In the second part of my PhD, devoted to investigate the role of CXCL12 and CXCL14 in the CT-mediated muscle morphogenesis in chick limb, we show that CXCR4 and CXCR7 are

sequentially expressed in embryonic muscles, CXCR7 replacing progressively CXCR4 expressed in muscle progenitors during foetal myogenesis. We propose two hypothesis related to explain these observations. First, *CXCR7* expression could be important to initiate myogenic differentiation and abrogate a CXCR4-positive muscle progenitor state and second, the CXCR4/CXCR7 switch could putatively preserve a myogenic fate to prevent a shift to an endothelial fate, CXCR4 expression being maintained only in endothelial cells of limb vessels. During myogenesis, PAX3 characterizes the embryonic progenitors, while PAX7 is expressed in the foetal ones and PAX3 and PAX7 are negative regulators of each other (Galy et al., 2008). Therefore, we propose to test the first possibility by studying CXCR4 and CXCR7 expression in PAX3 and PAX7-positive progenitors and investigating the regulation of CXCL12 receptors by PAX genes, by electroporating the brachial somites with PAX3 or PAX7 constructs. The second hypothesis could be tested by electroporating *CXCR7* and *dn-CXCR7* constructs in chick somites and look at the morphogenesis of muscles and vessels in these experimental conditions. This approach could also allowed us to better understand the role of CXCR7 restriction at the tips of muscle fibers during muscle development. Indeed, using a construct in which *CXCR7* is under the control of the Myosin-light-chain promoter to target specifically *CXCR7* expression in differentiated muscle cells, would allow us to better understand this restricted expression and its function during myogenesis.

Our results show that CXCR7 favors myogenesis in vitro and in vivo, by enhancing myoblast differentiation and muscle fusion. These observations suggest that CXCL12 and CXCR7 could not act independently to each other during the process of CT-mediated muscle morphogenesis, as CXCL12 promotes CT differentiation at the expense of muscle development. We also show that CXCL12 enhances the activation of $\beta 1$ integrin at the myotendinous junction. Our hypothesis is that the remodelling of extracellular matrix consecutive to the increase in collagens after CXCL12 overexpression enhances $\beta 1$ integrin activation which, in turn, increases CXCR7 expression at the tips of muscle fibers, i.e. at the myotendinous junction. As $\alpha 7\beta 1$ integrin is enriched at the myotendinous junction (Bao et al., 1993), it will be interesting to look at the expression of $\alpha 7$ subunit after CXCL12 overexpression. In addition, laminin being the main ligand of $\alpha 7\beta 1$ integrin at the muscle membrane and a reduction in $\alpha 7$ integrin being observed in muscular dystrophies linked to laminin mutations (Mayer, 2003), laminin expression should also be investigated after CXCL12 overexpression. Finally, *CXCR7* expression should be analysed after CXCL12 overexpression in CT to see if, as hypothesized, its expression increases after $\beta 1$ activation.

The positive regulation of CXCR7 on myogenesis is likely mediated through its beneficial effect on muscle cell fusion and muscle differentiation. How CXCR7 regulate myogenesis remains to be elucidated. One possibility would be that CXCR7 acts via its scavenger function to internalize CXCL12, then decreasing CXCL12 effect on CT differentiation to enhance myogenesis. Inhibiting β arrestin via specific inhibitors or siRNA strategy in vitro (Décaillot et al., 2011) could help to answer this question. Another possibility would be that CXCR7 participates to myoblast differentiation and fusion at the tips of muscle fibers where its expression is progressively restricted. Indeed, BMP produced by the tendons has been shown to regulate the differentiation of a subpopulation of muscle progenitors located at the tips of muscle (Wang et al., 2010). As we have also shown that BMP acts as an anti-fibrotic factor in chick embryonic limb, it would be interesting to test whether BMP signalling at the tips of muscle fibres is regulated by CXCR7 activity. Finally, we would like to analyze limb muscles and blood vessels in *Cxcr7* mutant mouse embryos to see whether those structures are affected in the absence of CXCR7.

Overexpression of *CXCL14* drastically inhibits myogenesis in vivo and in vitro. Trying to identify the receptor through which CXCL14 acts is an important step to understand this effect. As the atypical ACKR2 is described as the putative G-protein linked receptor that binds CXCL14, it would be of interest to analyse its expression pattern during chick embryonic limb development. Recent work has demonstrated that RCAS-mediated knockdown of CXCL14 causes severe neural defects in the eye including precocious and excessive innervation of the cornea and iris and that recombinant CXCL14 diminishes CXCL12-induced axon growth in vitro (Ojeda et al. 2017). Hence, it would be interesting to look at nerve patterning in chick embryonic limb overexpressing CXCL14. Preliminary data show defect in the patterning of axonal network, which might be related to CXCL14 effect on muscle morphogenesis.

To test these interactions, we propose to use the classical chemotactic Boyden test to submit myoblasts expressing *CXCR7* or *dn-CXCR7* constructs to CXCL12, CXCL14 or both chemokines, in order to try to decipher the molecular mechanisms implicated in the effect of CXCL12 and CXCL14 in the dialogue between CT and muscle during limb development.

This work provide new cues about the mechanisms regulating the crosstalk between CT and muscle formation and will be helpfull to further decipher the molecular mechanisms underlying skeletal muscle/connective tissue interactions in adults. Finally our data constitute promising therapeutic targets to treat fibrosis and muscular disease associated with abnormal development of ICT.

Material and methods

Constructs

The chicken *CXCL12*, *CXCL14*, *dnCXCR7* and *CXCR7* coding regions were amplified by PCR from an RT-PCR-derived cDNA library made from E5 chick limb, using primers containing *Cla*I restriction site. *CXCL12*, *CXCL14* and *dnCXCR7* amplified sequences were then inserted into pCR-II TOPO vector using TOPO-TA cloning kit (Invitrogen). *CXCR7* sequence was inserted into pGEM vector using pGEM-T easy vector system kit (Promega). Inserted sequences were excised by digestion with *Cla*I and inserted into the *Cla*I site of replication-competent retroviral vector RCASBP(A) previously digested with *Cla*I enzyme (Hughes et al., 1987). Clones containing the *CXCL12*, *CXCL14*, *CXCR7* or *dnCXCR7* coding region in the sense orientation were selected. Mouse RCAS-*mBmp4* and chick RCAS-*cNOGGIN* constructs were described previously (Duprez et al., 1996; Edom-vovard et al., 2001).

The pT2AL-CMV/Tomato-T2A-CXCL12 and pT2AL-CMV/Tomato-T2A-CXCL14 were achieved as followed. The chicken *CXCL12* and *CXCL14* coding sequences were amplified by PCR from a RT-PCR-derived cDNA library made from E5 chick limb, using a forward primer containing the *Bst*BI enzyme restriction site and a reverse primer comprising the *Pml*II enzyme restriction site. Purified PCR products were included into the PCR-II TOPO vector using TOPO-TA cloning kit (InVitrogen), and clones containing *CXCL12* and *CXCL14* sequences with *Bst*BI and *Pml*II restriction sites respectively in 5' and 3' ends of the coding sequences were selected. The TOPO/*Bst*BI-CXCL12-*Pml*II and TOPO/*Bst*BI-CXCL14-*Pml*II were then digested with *Bst*BI and *Pml*II enzymes. Purified digested products were finally inserted into the PT2AL-CMV/Tomato-T2A-GFP plasmid, from which GFP was previously extracted by *Bst*BI and *Pml*II enzymatic digestion. Clones containing *CXCL12* and *CXCL14* coding sequences were selected and generated plasmids were named PT2AL-CMV-*CXCL12* and PT2AL-CMV-*CXCL14*.

Production and grafting of recombinant/RCAS-expressing or control RCAS-expressing cells

Cells expressing RCAS/*cCXCL12*, RCAS/*cCXCL14*, RCAS/*mBmp4*, RCAS/*cNOGGIN*, RCAS/*cCXCR7*, RCAS/*dnCXCR7* and empty RCAS were prepared for grafting as previously described (Delfini and Duprez, 2000; Wang et al., 2010). Pellets of approximately 50 µm in diameter were grafted into the right wing bud of E4 chick embryos. Embryos were harvested at various times after grafting and processed for in situ hybridization or immunohistochemistry to tissue sections. The left wing was used as an internal control.

Owing to certain variability in the virus spread among embryos, the expression of the ectopic gene was systematically checked by in situ hybridization.

Bead implantation in chick limb buds

Heparin beads (Sigma) were soaked in 1 mg/ml of recombinant human FGF4 (R&D Systems) for 30 min on ice. FGF4 beads were grafted into the right wings of chick embryos at E4.5 and embryos were harvested 24 or 48 hours after grafting. Grafted right and contralateral left limbs were processed for in situ hybridization to sections.

Lateral plate mesoderm electroporations

E2.5 chick embryos were electroporated as previously described (Bourgeois et al., 2015). PT2AL-CMV-CXCL12 or PT2AL-CMV-CXCL14 (1,5-2 $\mu\text{g}/\mu\text{l}$) constructs were mixed with the transposase vector CMV-T2TP (molar ratio 1/3) to allow stable integration of genes in the chick genome, in a solution containing 0.33% carboxymethyl cellulose, 1% Fast green, 1mM MgCl_2 in PBS. DNA mix was injected with a glass capillary in the coelomic cavity between somatopleural and splanchnopleural mesoderm, at the level of the forelimb territory. Homemade platinum electrodes were placed above and below the embryo, with the negative electrode inserted into the yolk and the positive electrode localized above the presumptive forelimb region. Electroporation was delivered using a Nepagene NEPA21 electroporator with the following parameters: 2 pulses of 70V, 1ms duration with 100 ms interpulse interval followed by 5 pulses of 40V, 2ms duration with 500 ms interpulse interval. Those settings were delivered using Nepagen electroporator.

Drug administration in ovo

The stock solution for decamethonium bromide (DMB, Sigma D1260) was prepared at 10% dilution in Hank's solution (Sigma H9269). The DMB solution was freshly prepared before each experiment at 0.5% in Hank's solution with 1% of Penicillin-Streptomycin (Gibco, 15140). 100 μl of the DMB or control solution (Hank's solution with 1% of Penicillin-Streptomycin) were administrated in ovo at E4 of development. Embryos were harvested 48 to 72 hours after and processed for in situ hybridization or quantitative real-time PCR.

In situ hybridization and immunostaining to tissue sections

For hybridization and immunostaining on sections, embryos were fixed in a 4% paraformaldehyde solution in PBS supplemented with 4% sucrose and 0.1 mM CaCl_2 , rinsed

in PBS, embedded in a 15% sucrose solution, frozen in chilled isopentane before cryostat sectioning at 10-20 μm . Sections were collected on Superfrost/Plus slides (CML, France) and processed for immunolabelling or in situ hybridization as described previously (Escot et al. 2013). For grafted embryos, grafted and control limbs from the same experimental embryo were positioned in the same orientation for transverse sectioning to allow comparison.

For in situ hybridization, the following digoxigenin-labeled mRNA probes were used: chick *OSR1* and *OSR2* (Stricker et al., 2012), chick *CXCR7* (Escot et al., 2013), chick *CXCL12* (Escot et al. 2013), chick *SCX* (Bonnin et al., 2005), chick *COL1a1*, *COL3a1* (produced from EST clones from ARK Genomics), chick *MYOR* (Grenier et al., 2009) and mouse *Bmp4* (Wang et al., 2010). Chick *CXCL14* probe was produced from an RT-PCR-derived cDNA library made from E5 chick limb, using primers previously described (Gordon et al., 2011). The chick *MYOMAKER* probe was produced from a RT-PCR-derived cDNA library made from chick primary muscle cell cultures.

For immunostaining, the following primary antibodies were used: mouse monoclonal anti-MF20 (Developmental Studies Hybridoma Bank, non-diluted supernatant), rabbit polyclonal anti-PDGFR α (Santa Cruz, sc-338, 1/500 dilution), rabbit polyclonal anti-TCF4 (2569, Cell signalling, 1/100 dilution), mouse anti-CXCR4 (1:1000, Escot et al., 2013), mouse anti-MEP21 (1:200, generous gift from T. Jaffredo), mouse anti-PAX7 (1:200, Developmental Studies Hybridoma Bank), rabbit anti-activated β 1 integrin TASC (1:100, Millipore), rabbit anti-phospho-histone-3 (1:200, Cell Signalling), rabbit anti-cleaved caspase-3 (1:100, Cell Signalling). Immunolabelings were performed using secondary antibodies conjugated to Alexa Fluor 488 and 555 (1:200, InVitrogen). Nuclei were stained using DAPI (1/1000, Sigma). Stained tissue sections were observed with a Nikon microscope, images were collected with the QCapture Pro software (QImaging) and processed using Adobe Photoshop software.

Primary muscle cell cultures

Primary muscle cell cultures were prepared from skeletal muscles of forelimbs from E10 chick embryos. Limb muscles were cut in small pieces in Minimal Essential Medium (MEM) followed by mechanical dissociation. Homogenate was then centrifuged and the supernatant was filtered into a 40 μm filter to collect muscle cells. Centrifugation and filtration steps were repeated several times. Chick myoblasts were seeded on a 0,1% gelatin coated plastic dish. Myoblast primary cultures were first incubated in a proliferation medium (2/3 Minimum Essential Eagle Medium (MEM), 1/3 Hanks' salts medium 199, 10% fetal calf serum, 1%

penicillin streptomycin and 1% glutamine). At 80% of confluence, differentiation was induced using a differentiation medium (2/3 Minimum Essential Eagle Medium (MEM), 1/3 Hanks' salts medium 199, 2% fetal calf serum, 1% penicillin streptomycin and 1% glutamine). Chick primary myoblasts were transfected at around 30-40% confluence with the Calcium Phosphate transfection kit (Invitrogen).

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNAs were extracted from chick limbs. 500ng to 1µg RNAs were reverse-transcribed using the High Capacity Retrotranscription kit (Applied Biosystems). RT-qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences used for RT-qPCR are listed in Table S1. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). The ΔCt s were obtained from Ct normalized with chick *GAPDH* or *SI7* levels in each sample. For in vitro experiments, six cultures were used as independent RNA samples. For in vivo experiments, four forelimbs were used as independent RNA samples. Each sample was analysed in duplicate. Results were expressed as Standard Deviation (SD). Data were analysed by paired student t-test.

3D reconstructions of muscles

Grafted and control forelimbs were immunostained in toto for MF20. Whole-mount immunostained forelimbs were imaged with a Zeiss biphoton microscope and generated files were analysed with the software IMARIS (Bitplane) to perform 3D muscle reconstructions. Reconstructed images were then processed using Adobe Photoshop software.

Quantification of muscle progenitors and myotubes in vivo

Quantification in vivo was realized on transverse sections of control, grafted and electroporated limbs immunostained with PAX7 or MF20 antibodies. Muscle area measurement and determination of the number of PAX7-positive cells were performed on three to four successive sections of three different muscles at two different levels of the limb in four different embryos. The density of PAX7-positive cells was established by doing the ratio between the number of PAX7-positive cells and the surface of the muscle. In vitro, snapshots of muscle cell cultures stained with PAX7, MF20, phospho-histone-3 or caspase-3 antibodies were used for quantifications which were performed on nine biological samples arising from three independent experiments. Quantification analysis was realized with the Cell Counter plug-in of the free software ImageJ and statistical analyses were done using the

two-tails unpaired Student's t test in the Excel Microsoft software. Controls were normalized to one.

Quantification of the proportion of myotubes depending on nuclei number

Quantification was realized on chick foetal myoblast cultures transfected with RCAS/*dnCXCR7*, RCAS/*CXCR7* and empty RCAS immunostained with MF20 antibodies and counterstained using DAPI to label nuclei. The proportion of myotubes, corresponding to the number of myotubes containing 5 to 10, 10 to 15, 15 to 20 and more than 20 nuclei compare to the total number of myotubes, was established on triplicate samples from 3 independent experiments.

Image capturing

Images of labelled sections and culture cells were obtained using a Leica DMI6000 B microscope or a Nikon microscope equipped for epifluorescence. Images were processed using Adobe Photoshop software.

List of qPCR primers

Chick qPCR primers	Forward	Reverse
<i>PAX7</i>	AGAAGAAGGCCAAGCACAGCATAG	ATTCGACATCGGAGCCTTCATCCA
<i>MYOD</i>	CGACAGCAGCTACTACACGGAAT	CTCTTCCCATGCTTTGGGTC
<i>MYOG</i>	AGGCTGAAGAAGGTGAACGAAG	CAGAGTGCTGCGTTTCAGAGC
<i>MYOMAKER</i> (Luo et al., 2015)	TGGGTGTCCTGATGGC	CCCGATGGGTCCCTGAGTAG
<i>TCF4</i>	TCCGATTACAGACCTGAGCG	TGTTGATCAAGGCCAAAGCG
<i>ACTA2</i>	TGCTCCAAGAGCAGTTTTCC	CCCATACCAACCATCACACC
<i>CTGF</i>	AAGACACTTACGGCCCAGAC	AGTAGTCTGCACCAGGCAAT
<i>OSR1</i>	GTGCTGAATCTCCGACTTCTATGA	GTGTAAAATCTGAAGGGCAGGAA
<i>CXCR4</i>	CAGAAGCCCTTGCGTTCTTC	CACCCAGGAAGGCGTAAAGA
<i>CXCR7</i>	TCTGTTGTGGTGTGGGTCAA	CGAGGGAGACAACCCAAACA
<i>RPS17</i>	ACCTGGTTCCTGCACAGGGCTT	TGCCTGAGCGGAGGAGCAAACA

<i>CXCL12</i>	CAAAATCCTTTCCACTCCCAACT	CCATTTGTCTCTTGCCTTACTTGTT
<i>CXCL14</i>	ACCCATTTTGTGTGGAGGAGA	TGCTCACCTCTCACTTTTCGT
<i>OSR1</i>	GTGCTGAATCTCCGACTTCTATGA	GTGTAAAATCTGAAGGGCAGGAA
<i>OSR2</i>	CTTGCCTTGCAGGTACATCCA	TCCTGGCACTTGAAGGGTTT
<i>COL1A2</i>	GCAGTAACTTCATACCTAGCAACAAGC	TGCAGATGCCTCACTCACATG
<i>COL3A1</i>	GCGTCCTGTTGTGCCAAAA	GTTCAATTCTTGCCGTGTTTCAA
<i>COL6A1</i>	GCATGCCTAAACAAGCGATGT	GGAACAACCCAAACCCAGATC
<i>PDGFR α</i>	GCTAGTGCTTGGTCGAATCC	TGTCCCTTCCACCACTTTTC
<i>SCX</i>	CACCAACAGCGTCAACACC	CGTCTCGATCTTGGACAGC
<i>ID1</i>	CCGGAGGGTCTCTAAAGTGG	GCAGGTCCCAGATGTAGTCG
<i>ID2</i>	GAAGAACGGCCTTTTCGGAG	TCATGTTGTACAGCAGGCTCA
<i>ID3</i>	GCTGGAGGAACCCATGAATCT	TCCCGCAATTTGGAGTAGCA

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