Dynamics of cell-extracellular matrix interactions during epaxial muscle morphogenesis

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Abstract

All muscle cells of the trunk and limbs are derived from the delamination of myogenic precursor cells (MPCs) from the dermomyotome of somites. Myogenic differentiation is then activated and orchestrated by myogenic regulatory factors. In the trunk, myogenesis begins with the formation of the myotome, a segmented muscle which progressively differentiates, reorganizes and translocates to form the definitive adult skeletal muscles. Nevertheless, external factors such as the extracellular matrix (ECM) are also important in myogenesis. Previous studies have proved that ECM plays important functions in primary myogenesis. It is thought that the fibronectin matrix may help muscle cell reorganization through its integrin receptors, in particular through α4, α5 and αv subunits. Here we mapped the 3D distribution of the fibronectin matrix in embryos throughout myotome development until its final reorganization. We also described the distribution pattern of the αv integrin subunit. To assess the effect of fibronectin during the translocation of myocytes, we performed explant cultures, using the 70kDa fragment and the RGD peptide to inhibit fibronectin matrix assembly and cell-ECM interactions, respectively. Our data highlights a very dynamic behavior of fibronectin matrix in myogenesis. Fibronectin seems to be used as a guide by the MPCs entering the myotome from the central dermomyotome. αv subunit is present in all elongated myocytes and enriched at their tips, showing that myocytes are able to attach to the thick fibronectin matrix at intersegmental borders, possibly serving as a tendon structure. Disruption of fibronectin assembly induces the collapse of the elongated muscle cells and disruption of the myogenic program. Similarly, inhibiting cell-ECM interactions does not perturb muscle morphology, but affects myogenic differentiation. Together, these observations highlight the importance of fibronectin during muscle development, including skeletal muscle morphogenesis.

Key-words: fibronectin matrix; integrins; extracellular matrix; myogenesis; epaxial muscles; muscle morphogenesis; mouse embryo
Resumo

A morfogénese dos músculos esqueléticos é um processo complexo que se inicia no embrião e se prolonga por toda a vida do indivíduo, envolvendo um conjunto de factores celulares autónomos e extrínsecos, bem como a diferenciação e maturação de vários tipos de precursores celulares. Os músculos esqueléticos dos vertebrados derivam da segmentação da mesoderme paraxial, alinhada lateralmente pelo tubo neural e notocorda, estendendo-se ao longo do eixo embrionário e medialmente localizada em relação à mesoderme intermédia e lateral. Este processo conhecido por miogénese, encontra-se bem estudado em ratinho iniciando-se com a formação do miótomo, ao fim de 8,5 dias de gestação. A formação progressiva e faseada do miótomo ocorre a partir da delaminação coordenada das células progenitoras miogénicas (CPMs), que simultaneamente expressam os factores de transcrição Pax3 e Pax7. Actuando a montante e em sincronia com estes factores, são activadas uma pleutropia de vias de sinalização em resposta a factores extrínsecos às células, provenientes dos tecidos adjacentes, que regulam o início do desenvolvimento miogénico. Assim, o crescimento do miótomo dá-se por vagas sucessivas e sincronizadas de CPMs provenientes do dermomiótomo. No miótomo, as CPMs podem diferenciar-se em mioblastos, através da activação de uma cascata de factores de regulação miogénica (FRMs) ou continuam a proliferar dentro das massas musculares, constituindo um nicho residente de células progenitoras, essencial para o crescimento e desenvolvimento dos músculos. Em ratinho a hierarquia de expressão dos vários FRMs encontra-se bem estudada. Myf5 e MyoD são os primeiros factores responsáveis pela especificação das células miogénicas e Mrf4 e miogenina controlam a manutenção das populações miogénicas, actuando na fase final do programa de especificação miogénico. Após acção dos FRMs, as células miogénicas começam a expressar proteínas estruturais musculares, como a desmina e miosina. Nesta fase, no miótomo, os mioblastos diferenciam-se em miócitos que se extendem paralelamente ao longo de toda a porção do miótomo até às fronteiras que delimitam o segmento. Estes continuam a diferenciar-se, fundindo-se entre si atingindo sucessivamente a maturação, originando as massas musculares multinucleadas. A partir de um determinado estádio de E11.5/E12.5, as massas musculares epaxiais sofrem uma reorganização estrutural, alterando a sua orientação em relação ao eixoembrionário, mudando para os seus destinos finais de diferenciação. Este processo, designado por translocação muscular define a natureza transitória do miótomo, que acaba por desaparecer, formando os precursores das massas musculares adultas. Assim, a sequência de todo o programa miogénico pode ser definida como uma entidade complexa dependente da regulação e activação de factores intracelulares. Todavia, tal entidade também se baseia em processos de comunicação intercelular e interacções entre as células e o meio ambiente, onde se sabe que a matriz extracelular (MEC) representa um papel importante no desenvolvimento. Tal importância deve-se à grande versatilidade funcional da matriz, podendo
actuar como suporte físico-mecânico para as células ou reter sinais bioquímicos. A MEC é constituída por uma rede dinâmica e variada de macromoléculas, maioritariamente definida por glicoproteínas, proteoglicanos e colagénios. A fibronectina é uma das glicoproteínas mais comuns da matriz intersticial. A sua montagem é controlada pelas próprias células que secretam diméros de fibronectina, que se ligam às superfícies celulares dessas células ou outras adjacentes através de receptores membranares específicos, designados por integrinas, unindo-se ente si, formando uma rede fibrilar complexa e compacta de fibronectina. Estudos anteriores realçam a importância da fibronectina na somitogénese dos vertebrados, assim como na diferenciação miogénica de linhas celulares in vitro. As integrinas são heterodímeros transmembranares, constituídos pela combinação múltipla de uma subunidade α e outra β, sendo um dos principais elos de ligação entre as células e a MEC, através do motivo de ligação RGD. A combinação de cada subunidade perfaz um total de 24 integrinas funcionais com diferente especificidade. As integrinas α4β1, α5β1 e as de subunidade αv, destacam-se por serem receptores da fibronectina e por se saber que são expressas durante a miogênese. Pretendemos, determinar de que modo a matriz e as suas interacções com as células influenciam o desenvolvimento miogênico, afectando a diferenciação, migração e o comportamento das células miogénicas. Numa primeira fase, tentámos entender a organização da fibronectina durante o desenvolvimento muscular, desde a formação do miótomo até à fase de translocação, através de experiências de imunofluorescência in toto em embriões e reconstruções 3D. Em estádios precoces, a fibronectina surge em abundância a delimitar as fronteiras intersomíticas, assim como todos os sómitos. Mais tarde notámos que pequenos feixes de fibronectina se propagam para o interior do miótomo, adquirindo a mesma orientação dos mióctitos e crescendo em complexidade à medida que as massas musculares se desenvolvem. Em estádios miogénicos tardios observámos que a fibronectina ocupa totalmente o espaço deixado pelo dermomiótomo, após a dissociação deste e que continua presente à volta das massas musculares. Precedentemente à dissociação do dermomiótomo, notámos a formação de pequenos feixes de fibronectina na parte lateral do miótomo, isto é, entre o dermomiótomo e o miótomo, atentando uma possível interacção entre as CPMs do dermomiótomo e a fibronectina. Para averiguar esta questão, realizámos experiências de imunofluorescência, em cortes de embriões, onde observámos a presença de feixes fibrilares a envolver as CPMs em translocação. Além dessas experiências, também recorremos à técnica de imunofluorescência para detectar a integrina αv. Verificamos que, durante todas as fases em estudo está presente nos mióctitos alongados, sendo especialmente enriquecida nas pontas dos mesmos, possivelmente promovendo o seu ancoramento à matriz de fibronectina. Em conjunto, os dados apontam para a possibilidade de um papel altamente dinâmico da fibronectina na morfogénese do músculo epaxial, tal como permitir a entrada das CPMs no miótomo, conferindo, mais tarde, suporte as células miogénicas para promover a sua diferenciação, alinhamento, maturação reorganização e eventual migração.

Numa fase posterior, decidimos averiguar a influência que a MEC tem no comportamento das células durante a translocação do miótomo. Mais concretamente pretendemos caracterizar a
forma como a MEC, em particular como a ligação à fibronectina condiciona o desenrolar da miogénese. Para tal, realizámos culturas de explantes onde os mesmos foram sujeitos à acção de um fragmento amino terminal de fibronectina de 70kDa e um péptido com o motivo RGD, que respectivamente, bloqueiam a fibrilogénese da fibronectina e as ligações às integrinas que contêm este domínio de ligação à fibronectina, onde se seguiu a imunofluorescência in toto dos referidos explantes e reconstruções 3D. Notoriamente, obtivemos um efeito significativo no fenótipo das massas musculares, uma vez que estas se apresentavam colapsadas e os miócitos encurtados, sugerindo uma interrupção drástica no desenvolvimento das mesmas. Além disso, nestes explantes também se destacou a ausência da matriz de tenascina e de células miogenina-positivas, salientando a falta de suporte estrutural das células miogénicas e a inibição do respectivo programa de diferenciação. Quando incubados com o péptido RGD, os explantes apresentavam uma morfologia muscular normal, com presença de uma matriz de tenascina. Contudo, não identificamos quaisquer células positivas para miogenina, de entre os explantes analisados, sugerindo que a inibição da ligação à matriz via RGD não afecta significativamente o desenvolvimento dos músculos esqueléticos mas é suficiente para induzir uma interrupção no programa de diferenciação miogénico.

Neste trabalho evidenciamos a existência de relações directas entre as células miogénicas com a matriz de fibronectina envolvente, essenciais ao seu próprio desenvolvimento, atentando para as funções de suporte estrutural que a esta desempenha. Mais ainda, revelamos que a matriz de fibronectina é essencial para a morfogénese dos músculos esqueléticos.

**Palavras-chave:** fibronectina; integrinas; matriz extracelular; miogénese; músculos epaxiais; morfogénese muscular; embrião de ratinho
Introduction

Somitogenesis: Segmenting the musculoskeletal system in vertebrates

Somite formation

Muscle morphogenesis is a complex process that can extend throughout adulthood. Since muscles regulate several important biological functions, their study is important to understand the molecular and cellular networks that are behind their development. Among the different muscle types that constitute the vertebrates, skeletal muscles are the most abundant (Biressi et al., 2007). Vertebrate skeletal muscle formation, also known as myogenesis, is a highly regulated process. It directly depends on a plethora of autonomous and extrinsic signals, differentiation and maturation of different types of uncommitted multipotent cells and also involves extensive extracellular matrix (ECM) remodeling (Bryson-Richardson and Curie, 2008; Cossu et al., 2000; Czirák et al., 2004; Deries et al., 2011; Linker et al., 2003; Müstenberg and Lassar, 1995; Thorsteinsdóttir et al., 2011).

The myogenic commitment program is activated in paraxial mesoderm and is regulated by neighboring tissues such as the ectoderm, neural tube and notochord. The paraxial mesoderm lies on each side of the neural tube and notochord, extending along the embryonic axis (Kalcheim and Ben-Yair, 2005; Stockdale et al., 2000). The non-segmented, caudal part of the paraxial mesoderm (presomitic mesoderm; PSM) undergoes a mesenchyme to epithelium transition, at its rostral end leading to the formation of a pair of somites (figure 1). Each somite is surrounded by a fibronectin matrix and is connected with the ECM, which stabilizes its epithelial morphology (Rifes et al., 2007; Martins et al., 2009; Christ et al., 2007). Somites give rise to a variety of structures such as the dermis, all muscles of the body and a few of the head, the vertebral column, ribs, some tendons and ligaments and some blood vessels (Baker et al.,

Figure 1: Caudal differentiated somites (asterisks) and somite boundaries (arrows) in a E9.5 mouse embryo. Abbreviations: psm-presomitic mesoderm; tb-tailbud. Aulehla and Herrmann, 2004
Somite maturation and differentiation: specification of myogenesis

As development proceeds, the ventral part of the somites de-epithelializes and forms the sclerotome and the dorsal part is now called the dermomyotome (Munsterberg and Lassar, 1995; Dockter and Ordahl, 2000).

The sclerotome is the precursor of vertebrae and ribs. The epithelial dermomyotome possesses four contiguous lips and is delimited by a basement membrane enriched in laminin (Tosney et al., 2004; Bajanca et al., 2004; Kalcheim and Ben-Yair, 2005). The dermomyotome will generate another somitic compartment, called the myotome, which is the first skeletal muscle that forms in vertebrates (figure 2).

Dermomyotome cells express Pax3 and Pax7 and can give rise to several different mesodermal cell lineages such as all the myogenic cells of limbs and body, dorsal dermis, smooth muscle, endothelial cells and brown fat precursors (Buckingham, 2006; Christ et al., 2007; Kalcheim and Ben-Yair, 2005; Relaix et al., 2003; Relaix et al., 2005). Pax3/Pax7 expressing cells were identified as myogenic progenitor cells (MPCs) of all muscles of the body (Relaix et al., 2005, Kassar-Duchossoy et al., 2005, Gros et al., 2005, Kalcheim and Ben Yair, 2005). MPCs of axial muscles migrate in distinct waves from the different dermomyotome domains and invade the area of the myotome (figure 3).

The ability of dermomyotome to generate a large amount of precursor lineages depends on a balance between processes which promote the proliferative (uncommitted) versus the myogenic differentiated state (Buckingham, 2006; Buckingham and Vincent, 2009; Venters and Ordahl, 2005). Therefore, these cells can have two specific fates: either they become committed to skeletal muscle program and activate the myogenic regulatory factors (MRFs), differentiate into myocytes (Kalcheim and Ben Yair, 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Gros et al., 2005) or they continue to proliferate within the muscle mass during prenatal and adult development as a pool of muscle progenitors (Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Relaix et al., 2006).
Myotome compartmentalization: determination and regulation of myogenesis

Myotome formation has been intensely studied (Denetclaw and Ordahl, 2000; Denetclaw et al., 2001; Gros et al., 2004; Kahane et al., 2001; Venters et al., 1999) starting forming at about E8.5-E8.75 in the mouse (Biressi et al., 2007; Hadchouel et al., 2000; Venters et al., 1999). Delamination of dermomyotomal cells starts in its medial region to form the epaxial myotome, from which all the deep back muscles arise (Venters et al., 1999; Gros et al., 2004; Kalcheim and Ben-Yair, 2005, Venters and Ordahl, 2002). A myotomal basement membrane, which separates the myotome from the sclerome cells is then formed (Duband et al., 1987; Tosney et al., 1994, Bajanca et al., 2006). The MPCs that enter the myotome become myoblasts which express the myogenic regulatory factors (see below) and then differentiate into mononucleated, postmitotic myocytes which stretch bidirectionally parallel to the axis of the embryo until they reach the rostral and caudal somitic borders (Denetclaw and Ordahl, 2000; Gros et al., 2004; Hollway and Currie, 2005). Later on, MPCs enter the myotome from all four dermomyotome lips contributing to the growth of the epaxial myotome in both length and width. The hypaxial myotome population arises from the translocation of MPCs from the lateral dermomyotome and contributes to abdominal and intercostal muscle formation (Gros et al., 2004; Sporle, 2001). Later on, at around E10.5 in mice, cells from the central part of the dermomyotome expressing Pax3 and Pax7 enter the myotome, forming a new pool of proliferating myogenic precursors, contributing

Figure 3: Most recent model for myotome formation in chick A- Myotome is formed by subsequent waves of myogenic precursor cells, first from the dorsomedial lip of the dermomyotome (red), then from the caudal and rostral borders (green) and finally from the ventro-lateral lip (blue) B- Different myogenic cellular populations within the myotome domain. Abbreviations: DML- dorsomedial lip; VLL- Ventro-lateral lip; NT- Neural tube; No- Notochord. Gros et al., 2004.
to the growth of the myotome (Ben-Yair and Kalcheim, 2005; Gros et al, 2005; Kassar-Duchossoy et al, 2005; Relaix et al 2005).

Despite sharing the same somitic origin, trunk and limb muscles are formed by different regulatory mechanisms. Limb muscle masses are formed by the delamination and migration of MPCs expressing Pax3 and Pax7, from the hypaxial and central regions of the dermomyotome, respectively. These cells migrate to the limb bud where they form the limb muscle masses (Buckingham et al., 2003). Finally, head muscles are formed from the mesodermal core of the branchial arches and from prechordal mesoderm (Buckingham and Vincent, 2009; Noden and Trainor, 2005). Thus, during vertebrate morphogenesis we can find different muscle patterning pathways that underlies the myogenic program, depending on the axial level of the embryo.

At later stages, the dermomyotome progressively dissociates. This dissociation starts in the dermomyotome central region at E10.5 in the mouse and at HH18 in the avian embryos and is marked by an increase of asymmetric cell divisions (Buckingham, 2006; Kalcheim and Ben-Yair, 2005; Thorsteinsdóttir et al., 2011). Then, it spreads towards the epaxial and hypaxial lips that remain epithelial for a time before they get disintegrated (Venters and Ordahl, 2002).

### The myogenic regulatory factors

Myogenic regulatory factors (MRFs) are transcription factors which activate the skeletal muscle differentiation program. In vertebrates, four different MRFs: Myf5, MyoD, Mrf4 and myogenin, play a role in this process (Pownall et al., 2002; Buckingham et al., 2003). Myf5 and MyoD were identified as being the major regulators of muscle specification in vertebrates (Pownall et al., 2002). Mrf4 acts in the maintenance of myogenic cell populations being considered a muscle differentiation regulator (Kassar-Duchossoy et al., 2004). Myogenin modulates the action of several genes, especially the ones that are involved in fiber differentiation and regeneration, being used as a marker to detect differentiating skeletal muscle cells (Pownall et al., 2002). Myogenin acts to control myoblast differentiation, inducing the expression of the terminal skeletal muscle phenotype (Berges and Tapscott, 2005; Hinis et al., 2009).

The differentiation program activity will then induce the expression of muscle-specific structural proteins, such as desmin and myosin, in the myotome.

### Myotome translocation

In mammals and birds, the myotome is a transient structure that gradually transforms into new complex muscle masses. This constitutes a necessary event for muscle adaptation to terrestrial life (Deries et al., 2008). In the mouse it occurs at E11.5, at the time when the myotomal myocytes start to fuse with each other and become multinucleated (Venters et al., 1999). Gradually, the segmented structure of the myotome disappears and the complex axial
muscle masses form. This transformation of muscle fibers leads to a displacement of the myotomal cells, supporting the formation of epaxial muscles (Derries et al., 2010). The same occurs hypaxially to form the pre-vertebral, intercostal and abdominal muscles (Evans et al., 2006).

Remarkably, this transition occurs rapidly during embryogenesis and is poorly understood. Epaxial morphogenesis involves extensive movements of differentiated cells were the multinucleated myocytes elongate and change their orientation. The most dorsal-ones form a tilt at the rostral tip. From E12.5 developmental stage onwards, the myotomal shape changes progressively to a more complex organization to form the axial muscle masses, while the myocytes become multinucleated (Derries et al., 2010; Thorsteinsdóttir et al., 2011). Thus, the epaxial myotome domain is gradually replaced by the adult deep back muscles, which can be divided in three main muscle groups: the tranverso spinalis, longissimus and iliocostalis muscles (Derries et al., 2010). All these muscles attach to the vertebrae and have an extensor function (Derries et al., 2010).

However, it is also known that muscle translocation is not an epaxial musculature-specific event. Extra-ocular muscles are formed by a massive migration of myogenic cells from the neural crest-mesoderm interface, which in turn, folds and reaches a periocular microenvironment, achieving their extra-ocular terminal promordia (Noden and Francis-West, 2006). Moreover, it was recently discovered, in the mouse, that perineal muscles arise through movements of differentiated cells (Valasek et al., 2005).

This abrupt transition of the embryonic segmented myotome into a complex muscular adult morphology may be influenced by the surrounding connective tissue or extracellular matrix (ECM). ECM has a tight spatio-temporal synchrony with musculoskeletal morphogenesis (Evans et al., 2006). Despite of this close relationship, until nowadays, the role of the ECM upon the skeletal muscle development remains unclear. Thus, the study of cell-ECM interactions during myogenic development is important to unveil if the ECM directly influences the final destination of differentiated cells by leaving footprint-like clues during the translocation phase. Understanding how muscles are formed it is important to develop therapies to treat muscular diseases.
The extracellular matrix

All cells of multicellular organisms are surrounded by and interact with an ECM throughout all life stages. The ECM is a complex and dynamic network composed of a large variety of macromolecules: the protein-rich molecules, which include collagens, laminins, tenascins and fibronectin, and the polysaccharide-rich family where glycosaminoglycans and proteoglycans are included (Frantz et al., 2010; Rozario and DeSimone., 2010; Thorsteinsdóttir et al., 2011). The interstitial ECM fills the spaces between tissues and is the matrix of connective tissue, while pericellular ECMs, such as basement membranes, are in close contact with cells (Goody and Henry, 2010; Frantz et al., 2010; Rozario and DeSimone., 2010; Thorsteinsdóttir et al., 2011). ECM molecules are produced by cells and are assembled into highly organised tridimensional (3D) matrices. ECMs have a wide range of different functions depending on ECM structure and composition. The ECM acts as a scaffold, providing an adhesive substrate, defining physical and mechanical proprieties to cells and can also have the ability to sequester and store several signaling molecules such as growth factors (Frantz et al., 2010; Rozario and DeSimone., 2010).

Fibronectin

Fibronectin (FN) is a glycoprotein, containing binding sites for cell surface receptors and ECM molecules, including FN itself to allow FN matrix assembly (Mao and Schwarzbauer, 2005). FN matrix assembly is a cell-dependent process, in which cells secrete the FN dimer in a compact form. These dimers are engaged by integrin receptors on the cell surface, where they are unfolded, exposing fibronectin-fibronectin binding sites in the N-terminal assembly domain. Fibronectin molecules bind to each other through these assembly domains and progressively give rise to a stable fibrillar FN network (figure 5).

Figure 5: Fibronectin fibril assembly steps. First, FN soluble dimers binds to specific integrins (A) and other receptors (pink bars in B). Finally, FN-FN interactions form the FN fibrillar matrix (C). Blue molecule indicate the RGD motif. Mao and Schwarzbauer, 2005.

FN is expressed early during vertebrate development (Wartiowaara et al., 1979; Perkinson and Norton, 1997; Trinh et al., 2004; Zhou et al., 2010). In chick embryo, it is produced by the ectoderm (Rifes et al., 2007) and surrounds the rostral PSM and the early formed somites (Duband et al., 1987; Ostrovsky et al., 1988).
FN-null mouse embryos exhibit severe mesodermal and vascular defects, lack of somites, and a shortened antero-posterior axis, resulting in developmental arrest and death (George et al., 1993; Georges-Labouesse et al., 1996). The role of ECM in myogenic tissues has received significant attention, due to the recognition of several congenital muscle dystrophies (Ringelmann et al., 1999). However, FN pattern during skeletal muscle morphogenesis and how it influences the behavior of muscular cells is still to be studied.

**ECM receptors**

All cells interact with the ECM through several types of surface receptors. Among these receptors, the integrins are the best studied (ffrench-Constant and Colognato, 2004; Hynes, 2002; Streuli, 2009; Barczyk et al., 2010). Integrins are heterodimeric glycoproteins receptors composing by an α chain associated non-covalently to a β chain, forming an extracellular head, a transmembrane and a cytoplasmatic domain. The combination of these subunits defines the integrin specificity. In mammals, 18 α and 8 β subunits have been described forming a total of 24 different integrins (figure 6).

Integrins can exhibit different conformational states. The balance and combination between the nature of the ECM ligands and the intracellular proteins that are associated with the integrin cytoplasmatic domain regulates integrin activation and state, in a given tissue. Once integrins are linked to cytoskeleton, the integrin-mediated assembly triggers a large variety of downstream signaling pathways and transductions events (ffrench-Constant and Colognato, 2004; Hynes, 2002). Therefore, integrin based-adhesions act as an extracellular environment sensitive tool and provide a dynamic feedback to the cells (Barczyk et al., 2010; Danen and Sonnenberg, 2003). Thus, integrin activity is essential in the majority of biological process, where cell behavior and cell-fate determination are constantly regulated (e.g. gene expression, polarity, proliferation, mitosis, apoptosis, motility, migration, differentiation and cell shape), which modulates tissue development, function and disease.

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**Figure 6:** Integrins heterodimers family in vertebrates. Barczyk et al., 2010.
Integrins in myogenesis

ECM-integrin interactions play an important role in muscle development (Thorsteinsdóttir et al., 2011). Previous studies have described the expression of several integrin subunits (receptors to laminin, fibronectin and tenascin) during skeletal muscle development (Bajanca and Thorsteinsdóttir, 2002; Bajanca et al., 2004, Cachaço et al. 2005; Fässler et al. 1996; Hirsch et al. 1994). Here, we will only focus on the integrins that specifically attach to fibronectin and which are known to be expressed during myotome development. Eight integrins (α4β1, α5β1, α8β1, αvβ1, αvβ3, αvβ6, αvβ8 and αIIbβ3) bind to FN (table 1). Until date, only the α4β1, α5β1 and αv-containing integrins are known to be present during epaxial muscle morphogenesis (Thorsteinsdóttir et al., 2011).

The α5β1 integrin is believed to be a key receptor of FN. It is involved in a large range of cellular processes being highly expressed during embryogenesis (Taverna et al., 1998). mRNA for the α5 integrin subunit (Itga5) starts to be expressed in the PSM, where FN matrix is assembled (Bajanca et al., 2004; Rifes et al., 2007). At E10.5, Itga5 is expressed by the myotomal myocytes and by MPCs migrating to the limbs (Bajanca and Thorsteinsdóttir, 2002; Bajanca et al., 2004). At E12.5, Itga5 is expressed within the trunk and forelimb muscles masses (Cachaço et al. 2005). Mouse Itga5-null embryos die before myogenesis (Goh et al., 1997; Yang et al., 1993), but in chimeric embryos, several alterations in skeletal muscle formation are observed, with a delay in muscle development and an increase of muscular dystrophy (Taverna et al., 1998).

The α4β1 integrin binds both fibronectin and VCAM-1 (Rosen et al., 1992). In the mouse, α4 is present in the dermomyotome at E9.5 and is later upregulated in the myotome (Bajanca et al., 2004). At E12.5 it is detected in trunk and between E12.5-E14.5, it is also present in the forelimb muscle masses, having a wider expression pattern than α5 subunit (Cachaço et al., 2005). In the mouse, absence of α4 subunit (Itga4) leads to cardio-vascular defects and death between E11.0 and E14.0 (Yang et al., 1995), and chimeric α4-null embryos and cultured α4-deficient cells exhibit no significant morphological skeletal muscle defects (Yang et al., 1996).

αv subunit-containing integrins can bind fibronectin, tenascin, vitronectin, among others (table 1). In skeletal muscle, αv is first detected in E10.5 in myotomes and at E12.5 it is present in limb and trunk muscle masses, being enriched in myotubes extremities (Hirsch et al., 1994). Ablation of αv leads to a neonatally death caused by severe intracerebral and gastrointestinal hemorrhages with no muscle defects reported (Bader et al., 1998; Thorsteinsdóttir et al., 2011).

Despite these studies, many gaps remain in our knowledge to understand how cell-ECM interactions are involved in skeletal muscle morphogenesis.
### Table 1: Integrins and their ECM ligands. Abbreviations: Col-Collagen; Fn-Fibronectin, Ln-Laminin; Nn-Nephroneectin, Opn-Osteopontin; Tn-Tenascin; Vn-Vitonectin. Adapted from Thorsteinsdóttir et al., 2011.

<table>
<thead>
<tr>
<th>Integrin</th>
<th>α4β1</th>
<th>α5β1</th>
<th>α8β1</th>
<th>αvβ1</th>
<th>αvβ3</th>
<th>αvβ6</th>
<th>αvβ8</th>
<th>αIIbβ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM ligand</td>
<td>Fn</td>
<td>Fn</td>
<td>Fn, Tn, Vn, Ln</td>
<td>Fn, Vn, Opn</td>
<td>Vn, Fn, Tn, Ln (α1 chain), Opn</td>
<td>Fn, Tn</td>
<td>Vn, CoIV, Fn, Ln (α1 chain)</td>
<td>Fn, Vn</td>
</tr>
</tbody>
</table>

### Aims of this thesis

We aimed to study the relationship between the fibronectin matrix and the myogenic cells during early axial myogenesis in mouse embryos. First, we analyzed the distribution pattern of fibronectin to understand the 3D organization of this matrix during the skeletal muscle morphogenesis. Then, we examined the spatial profile of the integrin αv subunit, a potential fibronectin receptor. Finally, we studied the effect of fibronectin matrix assembly inhibition and the interruption of cell-ECM interactions during the development of the epaxial muscle masses, through the analyses of their morphology and the expression of myogenic regulatory factors.
Materials and Methods

Embryo collection

Wild-type adult mice (Charles-River CD1; Harlan Interfauna Iberica, SA) were crossed and the day of the plug designated as E0.5. Pregnant females were sacrificed by cervical dislocation and the embryos were removed from the uterus in cold phosphate buffered saline (PBS; annex I). Developmental ages of the collected embryos range between E9.5 and E12.5 (this project has the approval of Direcção Geral de Veterinária).

Fluorescent immunohistochemistry

On cryosections

Immunohistochemistry on sections was performed as in Bajanca et al., 2004. Embryos were fixed overnight (ON) at 4°C, in 0.2% paraformaldehyde (PFA) in solution 1 (annex I). Then, they were washed in solution 1 and 2 both ON at 4°C (annex I). After these incubations, the specimens were incubated in a gelatin and sucrose solution (solution 3; annex I) for 1 hour at 37°C and frozen immediately in isopentane chilled with dry ice. Embryos were stored at -80 ºC until use. 30µm thick sections were made in a Bright Clinicut cryostat and placed on SuperFrost Ultra Plus microscope slides (Menzel-Gläser). Sections were air dried during 1 hour before being washed 3x5min in PBS. The specimen were then blocked for 30 min in a blocking solution (1% bovine serum albumin (BSA; Sigma) in PBS) and incubated with primary antibodies (table 2) ON at 4°C. The next day, samples were washed 3x10min in PBS, blocked for 30 min in the blocking solution and stained with secondary antibodies (with TO-PRO3, Invitrogen 1:500 and RNase 10µg/ml, Sigma) (table 2) for 2h at room temperature (RT). Sections were then washed 3x5min in a 4 strength PBS solution (4xPBS; higher salt concentration is more efficient to remove non-specific bindings). Stained sections were fixed for 45min in 2% PFA in PBS and washed again 3x20 min in PBS. Samples were mounted in propyl-galate-PBS-glycerol (annex I), sealed with nail varnish and stored in the dark at 4ºC until viewed on the confocal microscope. All antibodies were diluted in the blocking solution.

Whole mounts

For whole mount embryo immunohistochemistry we used two different methods. The DMSO/Methanol method uses DMSO as the primary permeabilizing agent and is efficient to stain the ECM (Deries al., 2008). However, this method does not preserve membrane receptor.
In addition, this method requires that embryos be stored in DMSO/Methanol for 3 weeks before the immunostaining. In second method, the Triton method uses Triton-X100 as a permeabilizing agent and the embryos can be stained just after fixation (Venters et al., 1999). This protocol preserves membrane receptors and is much shorter to perform.

**DMSO/Methanol method**

Embryos were fixed in 2% PFA in PBS ON at 4°C, washed 3x1h in PBS, 1h in a 0.1M glycine diluted in PBS to remove the aldehyde radicals of the PFA, which create fluorescent background and 1h again in PBS. Specimens were then, progressively dehydrated in increasing methanol solutions, 15 min each one (20%, 50%, 80% and 100%, respectively), permeabilised with a solution of DMSO:methanol (1:4) for three weeks at 4°C and stored in methanol 100% at -20°C until use. After rehydration and several washes in PBS, samples were dissected as appropriate (table 3) and incubated with primary and secondary antibodies (including TO-PRO3 1:300 and RNase 10µg/ml, 1:100) in PBS containing 1% BSA (Sigma) and 20% DMSO solution (annex I), ON at 4°C. After each antibody application, embryos were extensively washed in 4xPBS for two days, where the 4xPBS was changed every 2h. After immunolabeling, embryos were dehydrated in methanol and stored at -20°C until use or transparentized in methylsalicylate (Martins et al., 2009) and mounted in glass coverslips sealed with paraffin to analyze with confocal microscopy.

**Triton method**

Embryos were fixed in 0.2% PFA in PBS ON at 4°C. Then they were dissected as appropriate (table 3) and washed 2x1h in PBS, permeabilized in a 0.2% ID solution (annex I) for 4h and stained with primary antibodies diluted in 0.2% ID, at 4°C for 3 days (table 2). Embryos were washed all day in 4xPBS (changed every 2h) with agitation at RT and left in the same solution ON at 4°C. Secondary antibodies diluted in 0.2% ID were added (with TO-PRO3 1:300 and RNase 10µg/ml, 1:100) and incubated for 2 days at 4°C (table 2). One more time, embryos were washed all day. After the washing period, stained samples were fixed 2h in 2% PFA in PBS and a 0.1M glycine solution diluted in PBS was added for 1h. Samples were then washed in 4xPBS for the rest of the day. Finally, embryos were progressively dehydrated in methanol and mounted as described above. To visualize myogenic cells, we used either a cocktail of three mouse antibodies (anti-myogenin, anti-desmin and anti-MHC) or, particularly at later stages, only the anti-MHC antibody.
Explant s cultures

For culture experiments, embryos were collected, as described above, and immediately dissected as appropriate (table 3) in Dulbecco’s Modified Eagle Medium/F12 GlutaMax medium (DMEM/F12) (Invitrogen) supplemented with 10mM HEPES (Sigma; annex I), 1mM sodium pyruvate (Invitrogen), and 100U/ml penicillin and streptomycin antibiotics (Invitrogen). After dissection, embryos were divided into different experimental groups.

In one set of experiments, the culture medium of experimental explants was supplemented with 100µg/ml of a 70kDa fibronectin fragment (Sigma), whereas control explants were incubated with 100 µg/ml of BSA (table 4). The 70kDa fibronectin fragment inhibits FN matrix assembly by competing with native FN for the FN-FN binding sites (McKeown-Longo and Mosher, 1985; Rifes et al., 2007).

In another set of experiments, experimental explants were incubated in culture medium supplemented with 4.5 mM of RGD peptide (Sigma), whereas control explants were incubated in the same culture medium but with 4.5 mM of DGR peptide (Sigma; table 4). The RGD peptide acts by blocking the RGD recognition site on integrins which use this site to bind to their ECM ligand (figure 5), thus blocking cell-ECM binding through those integrins. All cultured embryos were positioned on 0.8µm polycarbonate Millipore filters (Isopore™) and incubated for 12h, at 37 ºC with a 5% carbon dioxide humified atmosphere.

After the time of the culture, explants were processed for whole mount immunohistochemistry (Triton method) and analysed by confocal microscopy (figure 7).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reference</th>
<th>Company</th>
<th>Dilutions used (on sections; whole mounts or cultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Desmin</td>
<td>D3</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>Light meromyosin</td>
<td>MF20 asc</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>Anti-Human Fibronectin</td>
<td>F-3648</td>
<td>Sigma</td>
<td>1:400; 1:100</td>
</tr>
<tr>
<td>Anti-Tenascin</td>
<td>LAT-2</td>
<td>A.Sonnenberg</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>Anti-Myogenin</td>
<td>F5D</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>Anti-α4 integrin</td>
<td>PS/2</td>
<td>Serotec</td>
<td>1:50; 1:50</td>
</tr>
<tr>
<td>Anti-Myosin Heavy Chain</td>
<td>F59</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>Anti-Pax7</td>
<td>Pax7</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
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<tr>
<td>Anti-Pax3</td>
<td>Pax3</td>
<td>D.S.H.B.</td>
<td>1:100; 1:50</td>
</tr>
<tr>
<td>CD49d endotoxin low</td>
<td>MCA1230EL</td>
<td>Serotec</td>
<td>1:50; 1:50</td>
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<tr>
<td>Anti-Myogenin</td>
<td>SC-576</td>
<td>Santa Cruz</td>
<td>1:100; 1:50</td>
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<td>Anti-αv integrin</td>
<td>CD51</td>
<td>Enzo</td>
<td>1:500; 1:250</td>
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<td>Mol. Probes</td>
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<td>Goat Anti-Rat 488</td>
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<td>Mol. Probes</td>
<td>1:1000; 1:1000</td>
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<td>TO-PRO3</td>
<td>T3605</td>
<td>Mol. Probes</td>
<td>1:500; 1:300</td>
</tr>
</tbody>
</table>

Table 2: Antibody list
Figure 7: Scheme of the explant cultures protocol (in the previous page) After their collection the embryos were split into different groups. T0 embryos were immediately fixed. Tested explants (E11.5 and E12.0) were cultured for 6h or 12h and fixed. The experimental explants (incubated with 70kDa fragment, BSA, RGD or DGR) were incubated during 12h. All embryos or explants were stained by immunohistochemistry after fixation and each staining is indicated. The developmental stages of the explants after culture are also indicated. Embryo stage images were reproduced from http://www.emouseatlas.org/emap/ema/theiler_stages/StageDefinition/stagecriteria.html
Table 3: Description of methods for embryo dissection

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Embryo processing description</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5</td>
<td>Remove head and heart</td>
</tr>
<tr>
<td></td>
<td>Small incision in the cranial edge of the neural tube</td>
</tr>
<tr>
<td>E10.5-E12.5</td>
<td>Remove head and internal organs</td>
</tr>
<tr>
<td></td>
<td>Split down across the midline</td>
</tr>
<tr>
<td></td>
<td>Remove limbs and neural tube</td>
</tr>
</tbody>
</table>

Table 4: Inhibitory drugs used in explant cultures experiments

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Catalog</th>
<th>Use</th>
<th>Concentration</th>
<th>References</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>70kDa fragment</td>
<td>Sigma</td>
<td>F0257</td>
<td>Inhibits FN fibrillogenesis</td>
<td>100 µg/ml</td>
<td>McKeown-Longo and Mosher, 1985; Rifes et al., 2007</td>
<td>BSA</td>
</tr>
<tr>
<td>RGD peptide</td>
<td>Sigma</td>
<td>G4391</td>
<td>Block FN α5 and αv integrin binding</td>
<td>4.5 mM</td>
<td>Takahashi et al., 2007</td>
<td>DGR peptide (ref. S3371)</td>
</tr>
</tbody>
</table>

Image acquisition and 3D reconstruction

Confocal stacks (embryo sections, whole mounts and explants) were acquired on a Leica SPE laser scanning confocal microscope with a 20x 0.7NA and a 40x ACS APO 1.15 oil-immersion lens. Whole mounts and cultured explants stacks were 3D reconstructed and analyzed using the Amira v5.3.3 software (Visage Inc.). One individual segment and its associated matrix were isolated by manual contouring of confocal sections (i.e. other cells and the matrix from surrounding tissues were “digitally erased”). Embryo cryosections stacks were treated and analysed on Image J v1.44p to produce Z-projections images.
Results

Fibronectin dynamics along early skeletal muscle development

To better understand the relationship between the surrounding interstitial ECM and the dermomyotomal and myotomal cells, we described the precise 3D distribution pattern of fibronectin, within and around the dermomyotome/myotome, during the beginning of the myogenic program until the translocation of the myotomal myocytes (E9.5-E12.5). Whole mount embryos were stained by immunofluorescence to visualize myogenic cells and the fibronectin matrix. Confocal images were rendered to obtain a 3D reconstruction of the fibronectin matrix with the myogenic cells (materials and methods). Figures 8-11 show isolated segments of each stage with myogenic cells in red, the whole fibronectin in white and transparentized to be able to see inside the segment and the fibronectin inside the segment (i.e. excluding dermomyotomal and intersegmental fibronectin matrix) in yellow. As the fibronectin staining inside the segment was weaker, we increased the exposure time to values above the average to allow its visualization. Moreover, cryosections were also immunolabelled with the same staining for each stage showing myogenic cells in red and fibronectin in green.

Fibronectin is enriched at intersegmental borders and runs along the medial side of the myotome

At E9.5 hindlimb level, the somites are still epithelial with fibronectin surrounding them (Ostrovsky et al., 1988) and the dermomyotome is not yet morphologically distinguishable from the presumptive sclerotome. Myotome is only identified, with fully differentiated cells expressing MHC, at interlimb level (figure 8A-E). At this stage, a fibronectin matrix fills all the interstitial space on the lateral side of the dermomyotome, i.e. the space between the ectoderm and the dermomyotome (figure 8A, C, E, F, H and J). Fibronectin is highly abundant in the intersomitic borders, as well as around all somites, separating them from each other and surrounding tissues, at all levels (figure 8A, B, F, G, D, and white arrows in I). It is also possible to observe that epaxially, the fibronectin matrix is organized in small bundles on the medial side of the myotome and between the myocytes (figure 8B, D, G and green arrows in G and I). At forelimb level (figure 8F-J), when the dermomyotome and the myotome are more mature (note that the number of myocytes have increased) the fibronectin labeling forms small protrusions that starts to run along the myocytes into the myotome and on the lateral side of the myotome, between the dermomyotome and the myotome (Figure 8H and arrow in J).
Figure 8: Fibronectin distribution in a E9.5 mouse embryo.

Immunohistochemistry on whole mounts (A-D and F-I) and cryosections (E, J) of E9.5 embryos at interlimb (IL, A-E) and forelimb levels (FL, F-J) stained for fibronectin and myogenic cells. The whole mount were reconstructed in 3D, myogenic cells are in red, fibronectin in transparent white and the fibronectin inside the segment in yellow. On sections, myogenic cells are in red and fibronectin in green. The epaxial (Ep) side upwards and the hypaxial (Hyp) side downwards. In lateral views, rostral is to the left and to the right on medial views. Transversal (C and H) and sagittal slabs (D and I) were obtained from the 3D reconstructions. Fibronectin is very abundant through all stages in intersegmental borders, (white arrows in I) bundles run along myocytes on the medial side of the myotome (B and green arrows in G) and invading the myotome (green arrows in I). Some bundles of fibronectin are also seen between the myotome and the dermomyotome on the lateral side of the myotome. (arrow in J). NT- Neural tube; D-Dorsal; V-Ventral; M-Medial; L-Lateral. Scale bars: 150µm on whole mount reconstructions and 100µm on sections.
**Fibronectin matrix occupies the space between the dermomyotome and myotome and elongates into the muscle masses**

As myogenesis progresses, at E10.5, the number of myocytes increases and these myocytes elongate (figure 9). In fact the myotomal myocytes now span the whole segment and start to penetrate the ECM at the intersegmental borders (figure 9D, I and N). As the segments mature, the fibronectin matrix becomes denser on the lateral side of the dermomyotome and on the medial side of the myotome (figure 9, compare hindlimb, A-E, interlimb F-J, and forelimb level, K-O). Bundles of fibronectin elongate form the intersegmental border into the segment and it seems that fibronectin matrix increases its involvement with the myocytes, as more and more fibrils penetrate into the muscle masses (figure 9, green arrows in A, D, G and K, F, white arrow in I). Interestingly, most of these bundles have the same orientation as the myotomal myocytes (figure 9A, B, F, G, K and L). The fibronectin matrix which occupies the space between the dermomyotome and the myotome has increased (figure 9C, E, H, J, M and O, white arrows) and it is interesting to notice that this augmentation coincides with the entry of muscle progenitor cells (MPCs) into the myotome, originating from the central part of the dermomyotome (Relaix et al 2005, Kassar-Duchossoy et al 2005).

**As the dermomyotome begins to dissociate, fibronectin invade the all the dermomyotomal space and enshets the myotome**

At E11.5, fibronectin is strongly present around the myotome. The matrix is generally thicker and the fibronectin bundles are denser than at previous stages (figure 10). On whole mount reconstruction, it seems that fibronectin is present more dorsolaterally, in an epaxial to hypaxial gradient, both on the lateral and medial sides (figure 10A, B, F, G, K and L). However, this pattern was not observed in immunofluorescence experiments on sections (figure 10E, J and O). This differences registered may be due to a problem in the antibody penetration, since in whole-mount embryos the tissues are thicker than in sections. Fibronectin has also invaded the dermomyotomal space that has de-epithelialised (figure 10C, E, H, J, M and O, arrows) but the area occupied by the epithelial epaxial dermomyotome lip, remains free of fibronectin (figure 10J, arrows). At forelimb level when this epaxial lip has de-epithelialised, fibronectin invades all tissues surrounding the myotome, including the forming dermis and the space left by the de-epithelialisation of the dermomyotome and becomes tightly associated with the muscle cells around and within the myotome (figure 10C, arrows in E, H and M). The interstitial fibronectin matrix remains dense and continues to be present during the beginning of the transformation of the myotome.
Figure 9: Fibronectin distribution in a E10.5 mouse embryo.

Immunohistochemistry on whole mount (A-D, F-I and K-N) and cryosections (E, J, O) of E10.5 embryos at hindlimb level (HL, A-E), interlimb (IL, F-J) and forelimb levels (FL, K-P) stained for fibronectin and myogenic cells. The whole mount were reconstructed in 3D with myogenic cells in red, fibronectin in transparent white and the fibronectin inside the segment in yellow. On sections, myogenic cells are in red and fibronectin in green. The epaxial (Ep) side upwards and the hypaxial (Hyp) side downwards. In lateral views, rostral is to the left and to the right on medial views. Transversal (C, H and M) and sagittal slabs (D, I and N) were obtained from the 3D reconstructions. At E10.5 myocytes elongate into the intersegmental fibronectin matrix (arrows in A, G and K). Fibronectin matrix occupies the space between the dermomyotome and myotome (arrows in C, E, H, J, M and O) and some fibrils elongate along the muscle masses (arrow in D and I). NT-Neural tube; D-Dorsal; V-Ventral; L-Lateral. Scale bars: 150µm on whole mount reconstructions and 100µm on sections.
Figure 10: Fibronectin distribution in a E11.5 mouse embryo. Immunohistochemistry on whole mount (A-D, F-I and K-N) and cryosections (E, J, O) of E11.5 embryos at hindlimb (HL, A-E), interlimb (IL, F-J) and forelimb levels (FL, K-P) stained for fibronectin and myogenic cells. The whole mounts were reconstructed in 3D with myogenic cells in red, fibronectin in transparent white and the fibronectin inside the segment in yellow. On sections, myogenic cells are in red and fibronectin in green. The epaxial (Ep) side is upwards and the hypaxial (Hyp) side downwards. In lateral views, rostral is to the left and to the right on medial views. Transversal (C, H and M) and sagittal slabs (D, I and N) were obtained from the 3D reconstructions. Fibronectin fibrils are thick and elongated within the muscle masses (A, B, F, G, K and L). At hindlimb and interlimb (A-J) the central part of the dermomyotome has de-epithelialised (arrow in J) and fibronectin has invaded the newly mesenchymal space (H and J). At forelimb level the whole dermomyotome is de-epithelialised and fibronectin seems to be present in every part of the tissue, even the muscle mass. (arrows in C, E, H, J, M and O). NT-Neural tube; D-Dorsal; V-Ventral; M-Medial; L-Lateral. Scale bars: 150µm on whole mount reconstructions and 100µm on sections.
At later stages fibronectin continues to be present around and within the muscle masses

At E12.5, myotomal myogenic cells are in the translocation phase to give rise to the adult epaxial muscle masses (introduction). Unfortunately, whole mount immunohistochemistry at this stage was unsuccessful, probably due to incomplete fibronectin antibody penetration (materials and methods). Therefore, immunohistochemistry on sections was the only efficient method to visualize the distribution pattern at this stage. A fibronectin matrix is present around and within the muscle masses at all levels (figures 11 arrows in D, H and L). The fibronectin matrix seems to be less fibrillar than at previous stages. However, it is difficult to assess the exact 3D shape of the matrix as we are only seeing stained cryosections. It is possible that the fibrils are smaller because they have less space within the more dense muscle masses (figure 11, G and K).

Figure 11: Fibronectin distribution in a E12.5 embryo.
Cryosections of E12.5 embryos were labeled by immunohistochemistry with antibodies against MHC (B, F, J and red in D, H, L) and fibronectin (C, G, K and green in D, H, L) at hindlimb (B-D, HL), interlimb (F-H; IL) and forelimb (J-L) levels. The orientations of each section are indicated by a dashed line on the embryo images (A, E and I). Fibronectin is highly present around and within the muscle masses (arrows in D, H and L). Images of the embryos were taken at [http://mouseatlas.caltech.edu/gallery.html](http://mouseatlas.caltech.edu/gallery.html). D-Dorsal; V-Ventral; M-Medial; L-Lateral. Scale bars: 100µm.
Cell- ECM interactions during myogenic morphogenesis

To test the possible relationships between the fibronectin matrix and the MPCs entering the myotome from the central part of the dermomyotome, immunohistochemistry on cryosections was done using mouse embryos at stages from E9.5 to E11.5. Sections were labelled against fibronectin (figures 12B, F, K, O and S) and with an antibody combination against Pax3 and Pax7 to mark the MPCs (Relaix et al 2005; Kassar-Duchossoy et al. 2005; figures 12C, G, L, P and T).

A fibronectin matrix is in a position to aid MPC entry into the myotome

Pax3 is expressed in the somite and then becomes restricted to the dorsal part of the somite, the dermomyotome. Subsequently, Pax7 is also expressed in the dermomyotome (Buckingham, 2006). At these early developmental stages (figure 12A-D), a fibronectin matrix is abundant all around the somites and in the intersegmental borders as seen on whole-mount embryos at the same stage (see above, figure 8A, D, F and I). At E9.5 forelimb level, the first MPCs delaminating from the hypaxial dermomyotome lip starting to migrate towards the limb bud, are also positive for Pax3 (Goulding et al., 1994; figure 12C). It is interesting to note that small patches of fibronectin surround these migrating MPCs (figure 12, arrow in D). Fibronectin matrix staining labels the ectoderm and all the interstitial space ventral to the dermomyotome (figure 12B).

At E10.5, the first Pax3/ Pax7-positive cells start to leave the central part of the dermomyotome to enter the myotomal space. These cells are the pool of MPCs which will eventually contribute to the growth of the myotome and the axial muscles. (Relaix et al., 2005 and Kassar-Duchossoy et al., 2005, figure 12, yellow arrows in G and L). Concomitantly, fibronectin bundles increase in number in the area between the dermomyotome and myotome (figure 12, yellow arrows in F and K, H and white arrow in M). A close-up view of an oblique section shows that cells that are entering the myotome are in close contact with these fibronectin fibrils (figure12, arrows in I). This relationship between the Pax3/Pax7-positive cells and the fibronectin is not seen at earlier stages when the cells are still in the dermomyotome (shown at E9.5 IL figures12B-D). At all levels E11.5, when the dermomyotome dissociates completely and release the MPCs on its medial side (Kalcheim and Ben-Yair 2005), the fibronectin matrix between the dermomyotome and the myotome becomes even thicker (figure 12 yellow arrows in O and S, white arrows in Q and U) than at the previous stages (figure 12F-H and K-M). As seen on a longitudinal (figure 11O-Q) and on a transverse section (figure 12S-U), fibronectin fibrils spread from the intersegmental borders, through the myotome area and Pax3/Pax7-positive MPCs seem to be completely surrounded by the fibrils (figure 12 arrows in Q and U). Together, these results suggest that the fibronectin matrix may help mediate the entry of Pax3/Pax7-positive cells into the myotome.
Figure 12: Fibronectin matrix and MPCs relationship during myogenic development.

Cryosections of E9.5 (B-D), E10.5 (F-I and K-M) and E11.5 mouse embryos (O-Q and S-U) at different levels and orientation indicated by dashed lines in A, E, J, N, R. Sections were immunostained for fibronectin (B, F, K, O and S; green in D, H, M, Q, and U) and Pax3 and Pax7 (C, G, L, F and T; red in D, H, M, Q, and U) at interlimb (IL, F-H, O-Q), and forelimb levels (FL, B-D, K-M, S-U). At early stages, the dermomyotome expresses Pax3 and Pax7 and is surrounded by fibronectin on its lateral side (B and D). Fibronectin is abundant around the somites and in the intersegmental borders (B and D) and surrounds the first delaminating MPCs from the hypaxial dermomyotome (arrows in D). At E10.5, Pax3 and Pax7 expressing cells start enter the myotomal space from the central part of the dermomyotome (arrows in G and L) and fibronectin matrix occupies the space between the dermomyotome and myotome (arrows in F, I, K and M) with thick fibrils which are in close contact with the Pax3/ Pax7 positive cells (arrows in H and M). At E11.5, a patchy line of fibronectin delimits the dermomyotomal from the myotomal space (O, Q, S and U). Fibronectin fibrils spread through the dermomyotome and myotome areas and surrounds the MPCs (arrows in O, Q, S and U). Embryo images were reproduced from http://mouseatlas.caltech.edu/gallery.html. NT-Neural tube; D-Dorsal; V-Ventral; M-Medial; L-Lateral. Scale bars: 100µm.
α4 and α5 integrins subunits immunofluorescence experiments reveal a lack of immunoreactivity quality

We were also interested in characterising the distribution of the integrins that are known to specifically interact with fibronectin matrix. For that purpose we performed co-immunolabelling experiments on sections for α4, α5 and αv integrins subunits on E9.5 to E12.5 mouse embryos to determine whether any of these integrins are in a position to mediate interactions between the myogenic cells and fibronectin (see material and methods). Unfortunately, the batches of two different antibodies, against α4 integrin subunits (table 2) that we received did not give as good results as the batches used previously in the lab (Bajanca et al., 2004 and Cachaço et al., 2005) with the same protocol and embryo stages (compare figure 13 arrows in A and B). The antibody that was used against the α5 integrin proved to cause unspecific staining, and the signal was present in all tissues (figure 13 C and E). Thus, we were unable to draw a detailed description of the distribution pattern of these two proteins.

Figure 13: α5 and α4 integrins antibodies have a lower quality of immunofluorescence. A and B: transverse sections at hindlimb level in a E10.5 embryo (A) and in a caudal myotome at E11.5 (B) immunolabelled with the α4 integrin current batch (arrows in A), and one used earlier (arrows in B). Image B adapted from Cachaço et al., 2005. NT-Neural tube; D-Dorsal; V-Ventral; L-Lateral; M-Medial. C-E: Transverse section of E9.5 mouse embryos at interlimb level immunolabelled for α5 (green) and DNA (TO-PRO3, blue). Images adapted from Raquel Vaz, Master Thesis, University of Lisbon, Portugal. Scale bars: 100µm.
αv integrin subunit is enriched in the tips of epaxial myocytes

We also analyzed the expression pattern of the αv integrin subunit at stages between E9.5 and E12.5 by immunohistochemistry on sections, stained with antibodies against myocytes (red) and αv integrin subunit (green; E9.5 and E11.5 are shown in figure 14). Surprisingly, αv is already detected in the myotomal myocytes at E9.5 (figure 14 B-D,) which is earlier than what had been reported before (Hirsch et al., 1994). It is present on all myocytes and is especially enriched at the tips (figure 14, arrows in B and D). The same distribution pattern is seen in all subsequent stages shown here at E11.5 on a longitudinal (figure 14 F-H, arrows in F and H) and on a transverse section (figures 14 J-L, arrows in J-L). Thus, αv integrin subunit seems to have a constant distribution pattern during myogenesis and it is restricted to differentiated, MHC-positive myogenic cells. It should be noted that we did not detect αv expression in the dermomyotome cells or in MPCs inside the myotome.

Figure 14: αv integrin subunit is especially enriched at the tips of epaxial myocytes. Cryosections of mouse embryos at stage E9.5, interlimb level (A-D, IL), E11.5 hindlimb level (E-H, HL) and E11.5 forelimb level (I-L, FL) at different orientations indicated in A, E and I. Sections were stained by immunohistochemistry for αv integrin subunit (green; B, F and J) and desmin and MHC (C) or just MHC (G and K). αv integrin subunit is expressed from early developmental stages (A-D) and enriched at the tips of myocytes (B and D, arrows). This expression pattern remains constant during the myogenic development, as αv integrin subunit is still mainly expressed on the tips of the epaxial myocytes (arrows in F, H, J and L). Embryo images were reproduced from http://mouseatlas.caltech.edu/gallery.html. NT-Neural tube; D-Dorsal; V-Ventral; M-Medial; L-Lateral; R-Rostral; C-Caudal Scale bars: 100µm.
Functional study of the role of the fibronectin matrix assembly and cell-matrix interactions during myocyte translocation

Cultured embryo explants are viable and develop normally until 12h of culture

Since the translocation of the myotome is characterized by extensive cellular reorganization, and because the receptors of fibronectin are present throughout these events (Cachaço et al 2005 and results shown above), we hypothesized that fibronectin could have a role during the morphogenesis of the epaxial muscles. Therefore, we aimed to assess how the attachment and interaction of the epaxial myocytes with the fibronectin matrix affects the myogenic cells during the translocation of the myotome. To answer this question we wanted to perturb the fibronectin assembly and the interaction of the cells with the fibronectin matrix in cultured explants.

Our first major concern was to select the desired developmental stage to culture the explants and to assess whether the culture affects the morphology and the development of the explants. Ideally, the beginning of the culture would be when epaxial-most myocytes initiate the translocation phase and we should capture this translocation during the time of the culture. Two different stages were selected: E11.5 and E12.0 embryos. These embryos were cultured (figure 7) for six or twelve hours to optimize the time of the culture. The control group called time zero (T0) was represented by the explants that were not cultured, but immediately fixed. For both developmental stages, immunohistochemistry were performed on whole mount explants which were stained with antibodies against MHC, fibronectin and TO-PRO3 for nuclear staining (table 3) to see the morphology of the muscle masses and the ECM state.

Explants from both E11.5 and E12.0 embryos have a normal morphology with no apparent abnormalities to report after 6h and 12h of culture (shown for E11.5 in figure 15), when compared with the T0 control embryos (figure 14A, B, E, F, I and J). In fact, in E11.5 explants cultured for 12h, epaxial myotomal myocytes are completely elongated from the intersegmental borders and they begin to form the characteristic tilt of the translocation process (figure 14, white arrow in I). This normal development of the myogenic cell masses indicates that the tissues are healthy and develop well. In accordance with this, the analysis of TO-PRO3 labeling did not reveal a big number of picnotic nuclei, characteristic of cell death (compare figures 15D, H and L).

In parallel, the fibronectin matrix has a normal distribution pattern, as it is present around the whole segment, in the intersegmental borders, separating segments from each other (figure 15B, F and J) and among the muscle cells medially and laterally (figure 15, yellow arrows in C, G I and K). Moreover, the fibronectin matrix appears to keep its fibrillar conformation, at all
levels (figure 15A, E and I). Since we wanted a time of culture as long as possible to get as much as possible the translocation process during the culture, 12h was the time chosen to culture our experimental explants.

However, the developmental time window of neither of these embryo stages was satisfying: E11.5 explants were too young with not enough translocation happening during the time of culture and E12.0 embryos were too old with already too many muscle fibres re-orientated along the axis levels. Therefore, we chose to use an intermediate stage (E11.75) between the first two selected developmental stages to perform the experimental cultures. Table 6 summarizes the stage of development before and after culture of all the stages and time of cultured used in this study.

**Inhibition of fibronectin fibrillogenesis induces severe defects in skeletal muscle development**

Experiments were then performed on the chosen developmental stage E11.75 embryos cultured for 12h. As explained above, to see the culture effects, the developmental changes through time and to ascertain their morphology before culture, we created a group of selected embryos that were not cultured (T0). Two sets of experiments were performed on the explants cultured for 12h. One set of explants was incubated with a 70KDa amino terminal fragment or BSA (a protein with the same molecular weight) to analyze the influence of fibronectin assembly on the development of the epaxial muscles during translocation of the myotome. The other set, was incubated with a peptide containing a RGD motif which blocks the binding of RGD-containing integrins to fibronectin, or its control, the DGR peptide (figure 7). All the embryos and explants were stained by whole mount immunohistochemistry.

Unexpectedly, all the explants cultured with the DGR peptide died during the cultures (n=9) Indeed after the culture the explants were opaque white and fragile characteristic of massive cell death instead of being translucide and elastic. This death could be due to an abnormal acidification of the medium as during the experiments, we noticed a change in the color of the culture medium (pink into orange) denoting an acidification of the medium. This medium acidification with GDR, which was also verified by other laboratory colleagues (Pedro Rifes, personal communication), was not observed in the other explant culture media. Another hypothesis to explain the acidification would be a contamination. Therefore, it was impossible to analyze these explants and the explants cultured with RGD do not have a specific control group. The RGE peptide would be more appropriate and will be incubated as control in future experiments (Danen et al., 2002).

We first analyzed the muscle morphology of the explants by staining the myocytes using the anti-MHC antibody. First of all, it is important to note that the control cultured explants incubated with BSA (n=8) have a normal morphology (compare figures 15A and B with E and F
and 16A and B with E and F) and that they have developed normally compared to T0 control embryos (n=4). The dorsal-most myocytes of the T0 control embryos have changed their orientation and are no longer parallel to the embryo axis (figure 16A and B, yellow arrow in A; 17A and B). In the explants cultured in BSA the complex structure of the epaxial muscle masses begins to stand out. In fact, it is now possible to identify the separation of the different muscle masses (figures 16E and F; 17E and F). Besides, when nuclei are analyzed, no massive cell death can be identified (compare figure 16D with H and 17D with H).

Muscle masses of explants cultured with RGD (n=10) (figure 16 M-O; 17J-L) exhibit a normal phenotype through all levels and equal to the T0 control and BSA cultured explants. Again, myocytes are elongated through the whole segment, parallel to each other and the most epaxial ones have started to change orientation to give rise the adult muscle masses. Moreover, as in the control incubated with BSA, the separation between the different muscle masses becomes visible (figures 16M and N; 17M and N). This normal development is supported by a normal shape of the nuclei as well (figures 16P; 17P).

The explants cultured with the 70KDa fragment (n=10) showed dramatic defects in the muscle mass morphology (figures 16I and J; 17I and J). When fibronectin fibrillogenesis is blocked, myocytes are shrunken and condensed. They also seem collapsed and misaligned on lateral and medial views, at all levels when compared to the control explants. However, the muscle masses did not exhibit massive cell death, as only few picnotic nuclei are present (figures 15L; 16L). Interestingly, the ventral myocytes are more stretched and elongated than the dorsal ones, which could indicate that the fragment has a dorsal to ventral effect (figures 16, arrow in I; 17 arrows in G).

![Figure 15](next page): Morphological effect on explants development during culture. 3D reconstructions of interlimb level (A-C, E-G and I-K) of an E11.5 embryo fixed before culture (A-C; T0 culture), embryo explants cultured for 6h (E-G; 6h culture) and for 12h (I-K; 12h culture) and optical sections stained with TO-PRO3 of each experimental group (D; T0 culture; H; 6h culture and L; 12h culture, respectively). Explants were immunostained for MHC (red) and fibronectin (green). The most anterior side of the explants is to the left in lateral views and to the right in the medial views. The epaxial (Ep) side is upwards and the hypaxial (Hyp) side downwards. Transversal (C, G and K) were also stained from 3D reconstructions. 6h and 12h cultured explants muscle masses developed normally with no apparent abnormalities (A, B, D, E, F, H and white arrow in I). Fibronectin ECM is present in the intersegmental borders, ectoderm and surround the muscle masses (yellow arrows in C, G, I and K). D-Dorsal; V-Ventral; R-Rostral; C-Caudal. Scale bars: 150µm on whole mount reconstructions and 100µm on sections.
Inhibition of fibronectin assembly influences the tenascin matrix

Previous studies showed that tenascin (TN), another component of the interstitial matrix, is present during muscle development. It colocalizes with fibronectin at the intersegmental borders and along the myotome and it is considered to be a tendon marker (Wang et al., 1995, Deries et al., 2011). In this study, we were interested to analyze the influence of fibronectin assembly and ECM - cell interactions via RGD on tenascin within the
developing tendons. To study this, explants whole mounts were stained with antibodies against TN matrix and MHC.

Tenascin matrix had a similar normal phenotype at all levels of the control T0 explants (n=3; figure 16A-C) and explants incubated in BSA (n=6; figure 16E-G). Tenascin is strong at the intersegmental borders and bundles of tenascin run along the myocytes on the lateral and the medial side of the muscle masses (figure 16A, white arrow, B, arrows in C, E, F and arrows in G). Interestingly, explants muscle masses cultured with the 70KDa fragment (n=8) have completely lost their TN staining when compared with the T0 control embryos (figure 16I-J), suggesting that the disturbance of the fibronectin assembly perturbed the TN matrix. In contrast, TN matrix of the explants incubated with the RGD peptide (n=8) seemed to be normal, showing a similar phenotype as the T0 control. It is present at the intersegmental borders and between the muscle masses as well (figure 16M, N and arrows in O). Blocking the interaction of the cells with the matrix with the RGD peptide does not seem to have an effect on the TN matrix.

**Blocking fibronectin assembly cell-ECM interactions has a direct role on the myogenic differentiation program**

In order to study the influence of fibronectin assembly and interactions with the myogenic cells on the differentiation of the epaxial muscles, during translocation, we stained whole mount explants using antibodies against myogenin (table2, green; figure 17), to label the differentiating myogenic cells. T0 embryos have myogenin cells spread in the epaxial muscle masses and especially concentrated in the epaxial most part of the myotome. The differentiation program does not seem to be affected in explants cultured with BSA (n=2; figure 17D-F), when compared with the T0 control explants (n=1; figure 17A-C). Myogenin positive cells are seen, intermingled with the myocytes and more numerous at the epaxial most side (figure 17, arrows in C and F). Myogenin positive cells were absent in both explants cultured with the 70 kDa fragment (n=2; figure 17 G-I) and the RGD peptide (n=2; figure 17J-L). Therefore, we conclude that the perturbation of fibronectin fibrillogenesis by 70kDa and blocking the interaction between cells and ECM with the RGD peptide pertubs the myogenic program.
Figure 16: Inhibition of fibronectin assembly influences the interstitial ECM. 3D reconstructions of interlimb level of E11.75 embryos fixed before culture (A-C; T0 culture), explants cultured 12h with BSA (E-G; 12h BSA), explants cultured 12h with a 70KDa fragment (I-K; 12h 70KDa fragment) and explants culture 12h with the RGD peptide (M-O; 12h RGD). Optical sections show nuclei labelled with TO-PRO3 of each experimental group (D; T0 culture; H; 12h BSA; L; 12h 70KDa fragment and P; 12h RGD, respectively). Explants were immunostained with antibodies against MHC (red) and tenasin (green). The rostral side is to the left in lateral views and to the right in the medial views. The epaxial (Ep) side is upwards and the hypaxial (Hyp) side downwards. Transversal slabs (C, G, K and O) were obtained from 3D reconstructions. Explants cultured with BSA and RGD show a normal developed muscle masses morphology and tenascin labelling. The myocytes span from the borders and elongate and the most dorsal ones have started to change their orientation (arrow in A). Tenascin is strong at the intersegmental borders and bundles of tenascin run along the myocytes on the lateral and the medial side of the muscle masses (A, white arrow, B, C, arrows; E, F, G, arrows). In explants cultured with the 70KDa fragment, the myocytes are collapsed and this effect seems to be less severe on the most ventral side of the muscle masses (arrow in I). Tenascin matrix is not visible (I-K). D-Dorsal; V-Ventral; R-Rostral; C-Caudal. Scale bars: 150µm on whole mount reconstructions and 100µm on sections.
Figure 17: Blocking fibronectin assembly cell-ECM interactions has a direct role on the myogenic differentiation program. 3D reconstructions of interlimb level of E11.75 embryos fixed before culture (A-C; T0 culture), explants cultured 12h with BSA (E-G; 12h BSA), explants cultured 12h with a 70KDa fragment (I-K; 12h 70KDa fragment) and explants culture 12h with the RGD peptide (M-O; 12h RGD). Explants were immunostaining for MHC (red) and myogenin (green). The rostral side of the explants is to the left in lateral views and to the right in the medial views. The epaxial (Ep) side is upwards and the hypaxial (Hyp) side downwards. Transversal (C, G, K and O) were obtained from 3D reconstructions. Muscle masses developed normally in explants cultured with BSA and RGD (A, B, E, F, M and N). However, the explants incubated with the 70KDa exhibit shortened and collapsed (I-K) myocytes. This effect is not as strong on the ventral part of the muscle mass (arrows in I). Myogenin positive cells are seen in the T0 and BSA cultured explants (arrows in C and G). However, both in 70KDa and RGD cultured explants, no cells are positive for myogenin staining (I-K and M-O). Scale bars: 150µm on whole mount reconstructions and 50µm on sections.
Discussion

Fibronectin matrix as a tendon-like structure

We showed that at early stages, fibronectin is especially enriched in the intersegmental boundaries and all around the somites. These observations are in agreement with previous studies (Ostrovsky et al., 1988; Georges et al., 1993), which indicate that the epithelial somitic organization is dependent on the surrounding interstitial fibronectin matrix, as has been shown for the chick (Rifes et al., 2007; Martins et al., 2009). As the myotome forms, myocytes span the whole segment, elongate and penetrate into the fibronectin matrix at the intersegmental boundaries. Besides, fibronectin also spreads into the myotome from these intersegmental borders, suggesting multiple interactions between the matrix at intersegmental boundaries and muscle cells, but also indicating a constant adhesive and structural support, maintaining their morphology. In fact, it was proved in zebrafish that interactions between muscle fibers and fibronectin at somite boundaries mediate embryo segmentation (Henry et al., 2005). Moreover, zebrafish fibronectin mutants have disrupted myogenesis, due to disorganization and misalignment of myotomal cells (Snow et al., 2008). This suggests that fibronectin acts as a tendon-like structure. Our data support this hypothesis. In addition we have shown that the αv integrin subunit is detected on the tips of the myocytes, from early stages and remains especially enriched in the tips of the epaxial myocytes in the myotome during its development as well as during the morphogenesis of the epaxial muscle masses. Thus, αv integrin subunit, working in synchrony with α4β1 and α5β1 integrins which are also expressed in the myotome (Bajanca et al., 2004; Cachaço et al., 2005) is in position to promote the anchoring of myotomal cells to the ECM. Taking this into account, we conclude that fibronectin is a prominent interstitial matrix, acting as a tendon-like structure, and possibly promoting the anchoring and consequent elongation of the differentiated myocytes.

Fibronectin as a route for entry of MPCs into the myotome

We also observed from early stages (being most clear at E10.5) that fibronectin matrix is present in the most lateral side of the myotome, i.e between the dermomyotome and myotome. Previously, we also showed that fibronectin matrix becomes denser inside the myotome. Interestingly, this developmental stage coincides with the beginning of the entry of MPCs from the central domain of the dermomyotome (Buckingham, 2006; Buckingham and Relaix, 2007; Relaix et al., 2005, Kassar-Duchossoy et al 2005). Furthermore, α4β1 integrin is known to be expressed in the dermomyotome (Bajanca et al., 2004) and, although it is not clear whether α5β1 in expressed on MPCs within the myotome, it is expressed by migrating limb MPCs.
(Bajanca and Thorsteinsdóttir, 2002). In fact, we see that myotomal MPCs have close relationship with the fibronectin matrix. To clarify this issue further, it would be interesting extend this study to include a tridimensional approach, in which the spatial relationships between the MPCs and the fibronectin matrix is easier to analyze.

At approximately at E11.5 in the mouse, the dermomyotome begins to de-epithelialise and eventually disappears and at the same time, we recorded a massive invasion of fibronectin matrix into the dermomyotome space. In parallel, the number of the fibronectin fibrils increases as the muscle masses reach their full maturation, and, globally, the fibronectin matrix complexity increases as well. It is possible that fibronectin palys a role in guiding the MPC population, which is released upon the dissociation of the myotome, into the myotome at this stage as well. We suggest that Pax3/Pax7 positive cells use the fibronectin matrix to enter into the myotome, where they then proliferate and/or eventually differentiate. Fibronectin may act as a guiding cue, providing mechanical, physical or even a chemical extracellular environment (a particular micro-niche), in which MPCs can orient themselves, via an ECM-mediated mechanism.

An intact fibronectin matrix is essential for normal myocyte morphology, tenasin matrix accumulation and myocyte translocation.

At the stage of experimental cultures, the myotomal myocytes change their orientation and translocate to the final maturation location to give rise the adult epaxial musculature (Deries et al., 2010). Furthermore, the number of fibronectin fibrils increases. When the muscle masses start to translocate, fibronectin continues to be detected around and within of those muscle masses. Taking all these results into account, fibronectin seems to be the primary candidate to promote reorientation of myogenic cells during the translocation phase, and possibly also their fusion, thus being a candidate regulator of skeletal muscle morphogenesis.

We used an amino terminal 70kDa fragment to inhibit the fibronectin assembly (McKeown-Longo and Mosher, 1985). When fibronectin assembly is blocked, myocyte morphology is severely affected, especially in the most epaxial domain. Indeed, muscle cells were collapsed and shrunken. This cell morphology could suggest cell death. However, the nuclear shape of the cells within the tissues apparently seemed normal, without an abnormally high number of picnotic nucleus, which suggests that the disruption of fibronectin assembly and its consequences do not induce cell death. However, the picnotic phenotype appears at the last stage of apoptosis when the nucleus condenses and get fragmented (Kroemer et al., 2009). Therefore, it is possible that myogenic cells have entered apoptosis but have not advanced enough to show picnotic nuclei. If that was the case, it would show that fibronectin has a role in the survival of myogenic cells. To verify this possibility, it would be interesting to stain the cells by immunohistochemistry with an antibody against caspase3, a protein that has been shown to
be involved in the early steps of apoptosis. The absence of tenascin matrix in these explants was constant at all levels. It is known in cancer that tenascin needs fibronectin to be able to assemble (Jones and Jones, 2000). Moreover, in the early embryo, fibronectin is present before tenascin (Crossin et al., 1986; Ostrovsky et al., 1988). In these experiments, fibronectin fibrillogenesis was completely inhibited and it apparently leads to the complete disappearance of tenascin. The dramatic phenotype of the myocytes, being shortened may be explained by the fact that the muscle cells no longer have the support of fibronectin nor tenascin.

The data obtained highlights the importance of the fibronectin as a major and crucial component of the interstitial matrix, which constitutes an adhesive substrate for the support, elongation and translocation of myocytes within the muscle masses.

### Blocking cell-ECM interactions through the RGD motif does not perturb myocyte morphology, tenascin matrix formation or myocyte translocation.

The RGD peptide blocks the binding of RGD-containing integrins to fibronectin, in particular the α5β1 and αv-containing integrins (Takahashi et al., 2007). Muscle of explants cultures incubated with RGD exhibit a normal morphology. This could indicate that the interaction between cells and fibronectin via RGD motif is not essential for the morphology of the epaxial muscles during their development. However, it could also mean that the interaction of other integrins with fibronectin, such as the α4β1 integrin, which is a non-RGD-dependent fibronectin receptor (Sechler et al., 2000) and expressed during the stage under study (Cachaço et al. 2005), is sufficient to support the myotocytes when interaction through the RGD motif is impaired. It is also possible that other ECM molecules, for example collagens (Thorsteindóttir et al., 2011), act in concert with fibronectin and compensate the interactive interruption between cells and the fibronectin and tenascin matrices. To test this hypothesis, additional studies, where the interactions between α4β1 integrin and fibronectin are specifically inhibited, could be conducted.

### An intact fibronectin matrix and cell-ECM interactions through RGD are essential for the entry of MPCs into the myogenic programme.

Surprisingly, myogenin labeling is completely absent in explants incubated with the 70kDa fragment showing that the differentiation program was affected. These results suggest that the assembly of fibronectin could have an effect upon the expression of myogenin. Several explanations could be drawn to explain the fibronectin functions in myogenesis. The use of the
70kDa fragment prevents fibronectin fibrillogenesis. Thus the state of the fibronectin matrix could influence the cellular fate. Interestingly, explants cultured with the RGD peptide exhibit the same phenotype as the explants incubated with the fragment. These results suggest that during muscle development MPCs within the muscle masses need to be in permanent contact with the fibronectin matrix through the RGD motif to proceed in their differentiation programme. Inhibiting these interactions may have caused the cells to block the myogenic gene expression, such as myogenin. This interesting hypothesis implies that the interaction of myogenic cells with the ECM through the RGD motif would have outside-in signaling role (Hynes, 2002) on the myogenic program. If this hypothesis is correct, cell-ECM binding through RGD may target integrin signaling to the nucleus.
Conclusion

In summary, the data presented in this thesis highlights that fibronectin matrix is a very dynamic matrix *in vivo*, and could be a key molecule in the regulation of myogenic development, by instructing different morphogenetic cellular behaviors. During embryogenesis, the ECM is consistently being remodeled, adjusting itself as the embryo proceeds in its intrinsic development. In this study, we highlighted that the nature and architecture of fibronectin matrix directly affects and are related with characteristic phenomena of muscle differentiation and that interferes with the muscle masses organization. Knowing that the fibronectin matrix assembly is a cellular event (Mao and Schwarzbauer, 2005) it means that cells create their own extracellular environment, which is later necessary for their development, thus influencing their own fate. Therefore, studying the architecture of ECM components, the cell-ECM adhesive properties help to understand the ECM dimension not only for skeletal muscle development, but may also provide useful knowledge on other important morphogenetic events.
References


Relaix F, Polimeni M, Rocancourt D, Ponzetto C, Schäfer BW, Buckingham M. 2003. The transcriptional activator PAX3-FKHR rescues the defects of Pax3 mutant mice but induces a
myogenic gain-of-function phenotype with ligand-independent activation of Met signaling in vivo. Genes Dev 17:2950-2965.


Annex I

Recipes

10x Phosphate Buffer Saline (PBS) – 1370 mM NaCl
   268 mM KCl
   81 mM Na₂HPO₄·H₂O
   147 mM KH₂PO₄
   Fill with distilled water

20% Paraformaldehyde (PFA) – 200 g PFA
   Fill with distilled water (1L)

Sucrose Solutions for cryosectioning

Solution 1 – 40 g sucrose
   120 µl CaCl₂ (dissolve in water first)
   385 ml Na₂HPO₄
   115 ml NaH₂PO₄
   Fill with distilled water (1L)

Solution 2 – 150 g sucrose
   5 ml Phosphate Buffer 1
   Fill with distilled water (1L)

Solution 3 – 150 g sucrose
   75 g gelatin
   500 ml Phosphate Buffer 2

0.2% ID – 0.2% Triton (v/v)
   1% BSA (v/v)
   Fulfill with PBS
Blocking Solution – 1% BSA (v/v)
    20% DMSO (v/v)
    75% PBS (v/v)

HEPES (1M) – 11.915g HEPES
    50 ml destilled water

Propyl-galate-PBS-Glycerol – 4% N-Propyl-galate (v/v)
    90% Glycerol (v/v)
    10% 10x PBS (v/v)