Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Extracellular matrix and integrins influence in the regulation of myogenic precursor cells behaviour

Raquel Rodrigues Vaz

Mestrado em Biologia Molecular Humana

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Dissertação orientada por Professora Doutora Gabriela Rodrigues

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Abstract

Myogenesis is the process by which undifferentiated dermomyotomal cells are specified for myogenesis, move towards the myotome where they differentiate into skeletal muscle cells that fuse into myotubes and later in development form myofibers which will constitute the skeletal muscles of the adult. The muscle precursor cells arise from the dermomyotome, an epithelial-like structure that is the source for skeletal muscle and dorsal dermis cells.

Some cells, called satellite cells, go throughout part of this differentiation process but remain in a quiescent undifferentiated state (although committed to skeletal muscle fate). These cells are activated in the adult in case of muscle injury or enhanced exercise, for example.

In this work we used a satellite cell-derived cell line, C2C12, and the mouse embryo to study the extracellular matrix (ECM) and integrins influence in myogenic determination and differentiation. Integrins are heterodimeric ECM receptors constituted by an α and a β subunit that can induce, for example, migration or differentiation. The integrin ligand specificity is acquired by the combination of both subunits.

Our studies have addressed that laminin-α6β1 integrin interaction may be coordinating with Notch signaling the maintenance of undifferentiated dermomyotomal cells. By inhibiting Notch signaling, we observed precocious myogenic differentiation of dermomyotomal cells (by Myf5 expression) and the assembly of a laminin matrix around these cells. This result suggests that Myf5 induces laminin assembly.

In vitro, fibronectin enhances C2C12 myoblasts alignment and migration. When we observed the myotubes of cells grown on fibronectin, we believe that the enhanced cell alignment imposed by fibronectin-α5β1 integrin interaction will facilitate cell fusion. *In vivo*, we found that fibronectin is important for dermomyotome epithelial-integrity, especially through the polarization of N-cadherin, and that α5β1 integrin signaling may also contribute to myogenic repression in the dermomyotome.

These observations show that the ECM and integrins are of paramount importance in myoblast cell behaviour.

Key words: myogenesis, laminin, fibronectin, integrins, C2C12

Resumo

A miogénese é o processo através do qual as células precursoras miogénicas são especificadas, se diferenciam em mioblastos e posteriormente em miócitos que se fundem formando miotubos que vão constituir as fibras musculares do organismo adulto. Ao longo deste processo são também especificadas as células satélite, células que se mantêm indiferenciadas e quiescentes, sendo apenas activadas em caso de lesão muscular ou exercício intenso (Grounds & McGeachie, 1987; Bischoff & Heintz, 1994).

As células precursoras músculo-esqueléticas do tronco são especificadas ainda no dermamiótomo, estrutura derivada da porção dorsal do sómito e que se mantém epitelial, mais concretamente no lábio epaxial do dermamiótomo, correspondente à porção dorso-medial do dermamiótomo (Christ *et al*, 1998). No embrião de ratinho, o inicio da diferenciação destas células é detectado pela expressão do factor de transcrição Myf5, seguido de outros factores de regulação miogénicos (FRM) como Miogenina, criando uma espécie de cascata de FRM importantes para a formação correcta dos músculos esqueléticos (revisto por Cossu *et al.*, 1996). No lábio oposto, denominado hipaxial (porção mais lateral do dermamiótomo), são definidas as células que vão dar origem aos músculos dos membros, mas também à musculatura da parede ventral do corpo (Ordahl *et al.,* 1992).

Além de serem a fonte de células precursoras de músculo esquelético, as células do dermamiótomo também se diferenciam em células da derme dorsal (Brent *et al.*, 2002). A especificação dos diferentes tipos celulares é possível pois as células do dermamiótomo recebem diferentes sinais provenientes de diferentes tecidos adjacentes, como tubo neural, notocorda, ectoderme ou mesoderme lateral, que permitem a "padronização" do dermamiótomo (Cossu *et al.*, 1996; Cossu *et al.*, 2000; Yusuf *et al.*, 2006). Nesta tese estivemos particularmente interessados na especificação e diferenciação das células precursoras de músculo esquelético, células provenientes do lábio epaxial do dermamiótomo. Mais precisamente, pretendemos entender um pouco mais sobre a influência da matriz extracelular (MEC) e das integrinas na miogénese, tanto na diferenciação das células precursoras miogénicas como no seu comportamento.

A MEC corresponde a uma rede altamente dinâmica e complexa constituída por diferentes moléculas como glicoproteínas, proteoglicanos ou colagénios, que são sintetizadas e organizadas pelas próprias células. A MEC pode estar organizada na forma de matriz intersticial, uma estrutura porosa como a do tecido conjuntivo, ou na forma de matriz pericelular, como a membrana ou lâmina basal, presente por exemplo a rodear o dermamiótomo (Yurchenco *et al.*, 2004; Schwarzbauer *et al.*, 1999). Estas moléculas interagem com as células através de receptores específicos, dos quais se destacam as integrinas, heterodímeros constituídos pela combinação de uma subunidade α (de 18

existentes) e uma β (de 8). Da combinação das duas subunidades surge a especificidade para o ligando.

Nesta tese focámo-nos apenas na fibronectina e na laminina, duas glicoproteínas da MEC, e nas integrinas que interagem com estas moléculas, principalmente α5β1 e α6β1, respectivamente.

Como modelos de estudo foram usados embriões de ratinho (*Mus musculus*) e a linha celular C2C12. Esta linha celular foi estabelecida a partir de células satélite de ratinho adulto em que a diferenciação destas células em miócitos e miotubos passa pela mesma cascata de FRM que a miogénese embrionária (Parker *et al.,* 2003) Assim sendo, estas células são um bom modelo para estudos preliminares de miogénese (Burattini *et al.,* 2004).

Os primeiros estudos realizados nesta tese pretendiam perceber qual o efeito da matriz de laminina na diferenciação dos mioblastos C2C12. Ao cultivar estas células ao longo de vários dias em meio de crescimento e de diferenciação sobre laminina e gelatina (controlo), não observámos qualquer diferença na expressão de diferentes FRM, o que sugere que a laminina não terá influência na diferenciação destas células.

Estudos anteriores descreveram que, *in vivo*, a matriz de laminina e a sua interacção com a integrina α6β1 são importantes para a manutenção do estado não diferenciado das células do dermamiótomo, e também como barreira que impede a dispersão das células precursoras musculares (Bajanca *et al*., 2006). No seguimento deste estudo, tentámos perceber um pouco mais da regulação do estado indiferenciado das células do dermamiótomo. Assim, questionámos se a via de sinalização Notch estaria envolvida nesta regulação.

A importância da via de sinalização Notch, iniciada pela ligação de Notch a Delta e conduzida intracelularmente através de um domínio de Notch que é clivado, *Notch Intracellular Domain* (NICD), tem sido documentada em vários processos, como por exemplo na diferenciação neuronal ou hematopioese (Wakamatsu, *et al*, 1999; Weber & Calvi, 2009). Outros estudos têm relacionado Notch e miogénese. A grande maioria dos estudos tem sido feita em sistemas *in vitro*, no entanto alguns estudos têm sido feitos também *in vivo*. Em ambos os casos é consensual que a sinalização Notch impede a diferenciação miogénica, mais especificamente inibindo MyoD (um FRM importante na determinação miogénica; Braun, *et al*, 1994), no entanto pouco se sabe sobre quais são os intervenientes celulares importantes para esta inibição (e.g. Sasai, *et al*., 1992; Hirsinger *et al*., 2001; Kopan, Nye, & Weintraub, 1994; Buas, *et al*., 2009).

Ao cultivarmos embriões de ratinho com DAPT, um inibidor da γ-secretase, enzima que é responsável pela clivagem do NICD, observámos a presença de Myf5 nas células do dermamiótomo. Este resultado confirma que Notch é importante para reprimir a miogénese, já que o bloqueio da sua sinalização permite que as células do dermamiótomo activem o

programa de diferenciação miogénica. Este efeito foi semelhante ao observado quando é inibida a ligação laminina-integrina α6β1 (Bajanca *et al.,* 2006).

Tentando perceber se a inibição da sinalização Notch e consequente diferenciação miogénica no dermamiótomo teria algum efeito na matriz de laminina, foram feitas imunofluorescências em embriões cultivados com DAPT (e controlos) e observou-se a montagem de laminina numa matriz pontilhada onde normalmente não existe, à volta das células do dermamiótomo, além da lâmina basal. Assim sendo, confirmam-se resultados anteriores que descrevem que Myf5 será necessário para a montagem da matriz de laminina, já que embriões mutantes para Myf5 não conseguem fazer a montagem da laminina numa matriz (Bajanca *et al.*, 2006).

Quando C2C12 foram cultivadas sobre lamelas de fibronectina, cedo notámos que as células alinhavam precocemente, mas que no entanto esta característica não estava associada a diferenciação precoce. Ao analisarmos a forma dos núcleos de células cultivadas sobre fibronectina e sobre gelatina (controlo), confirmámos que as células na primeira condição estão de facto mais alinhadas, já que os núcleos destas células são mais elípticos que os núcleos das células cultivadas sobre gelatina.

Para percebermos melhor as diferenças na dinâmica celular nas diferentes matrizes, colocámos as células numa caixa incubadora associada a uma lupa, na qual eram adquiridas imagens sequenciais em *time-lapse*, as quais foram conjugadas num vídeo. Ao analisar o movimento de várias células em cada vídeo, concluímos que a fibronectina induz a migração das C2C12, já que sobre a matriz de fibronectina estas células movem-se mais e a distância entre o ponto inicial e o final no vídeo é significativamente maior. Após verificar que a integrina α5 é expressa nestas células e ao impedir a ligação destas células á fibronectina (através de um péptido inibidor da ligação fibronectina-integrina α5β1, o RGD; Takahashi *et al.*, 2007), deduzimos que as células necessitam das moléculas de fibronectina solúveis no meio de cultura para conseguirem eficazmente aderir ao substrato e concluímos que uma matriz de fibronectina induz o alinhamento. Para este alinhamento ser possível, o citoesqueleto é reorganizado e pensamos que a N-caderina (molécula de adesão célula-célula) seja também importante para o correcto alinhamento das células. Este alinhamento parece ser importante quando os miócitos começam a fundir entre si, pois sobre fibronectina parece haver mais miotubos do que sobre laminina ou gelatina (para o mesmo tempo de cultura).

Tendo em conta os resultados obtidos com as C2C12, tentámos perceber se, *in vivo*, a fibronectina seria necessária tanto para o alinhamento dos precursores musculares após especificação e nas fases iniciais de diferenciação (embriões mais precoces, com cerca de 9 dias embrionários – E9.0), mas também na altura em que ocorre a fusão dos miócitos

(E12.5), fase em que apenas estão presentes integrinas que interagem com a fibronectina (Cachaço *et al*, 2005). Neste caso não conseguimos detectar diferenças na orientação das células.
No entanto, ao inibir a montagem da matriz de fibronectina com o fragmento de 70kDa (McKeown-Longo *et al.*, 1985; Rifes *et al.*, 2007), observámos que a polarização da N-caderina no dermamiótomo estava perturbada, já que estava presente homogeneamente nas células, em vez de estar apenas na porção apical das mesmas (revisitando os resultados obtidos na somitogénese de galinha pelo nosso grupo; Martins *et al.*, *in press*). Por outro lado, ao cultivar embriões com RGD durante 12 horas observámos a presença de algumas células positivas para Myf5 no dermamiótomo. Este resultado sugere que também a fibronectina, mediada pela interacção com a integrina α5β1, poderá ser importante na inibição da diferenciação precoce no dermamiótomo.

Nesta tese conseguimos descrever um pouco mais da influência da MEC e das integrinas na miogénese, nomeadamente na regulação da diferenciação das células do dermamiótomo em precursores miogénicos. Estabelecemos uma relação entre a laminina e a sinalização Notch, mas também observámos a influência da fibronectina no alinhamento, migração e na "eficiência" da fusão de mioblastos *in vitro* e *in vivo* e na manutenção das características epiteliais do dermamiótomo.

Palavras-chave: miogénese, laminina, fibronectina, integrinas, C2C12

1. Introduction

1.1 Myogenesis

1.1.1 Vertebrate myogenesis

Embryonic myogenesis is the process by which cells differentiate into skeletal muscle cells. If this highly coordinated process does not proceed properly, several defects may decrease newborn survival and adult health. Skeletal muscle cells begin their differentiation when they leave the dermomyotome and move to the myotome. The dermomyotome is a transient structure that arise from somites, blocks of epithelial cells that segment on both sides of the neural tube from the pre-somitic mesoderm and differentiate in a rostro-caudal gradient (figure 1; reviewed by Christ *et al.*, 1998; Pourquié, 2001).

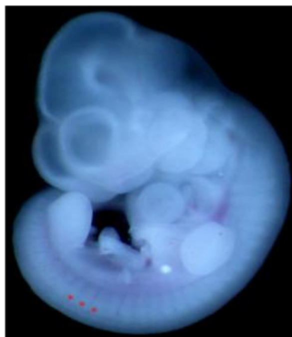


Figure 1: Embryonic day (E) 10.5 mouse embryo where differentiating somites can be identified (e.g. asterisks).

Influenced by signals from surrounding tissues, somites mature and give rise to cells with the ability to differentiate in different tissues. The first process consists in the differentiation of the sclerotome, which gives rise to the axial skeleton. The dorso-medial portion of the somite remains epithelial, named dermomyotome, which is the source for myotomal and dorsal dermis cells. The myotome (ventrally to the dermomyotome) contains the differentiating precursors of the skeletal muscle cells (Brent *et al.*, 2002).

As the dermomyotome is established, in both the dorso-medial (DML) and the ventro-lateral (VLL) regions two particular structures are defined: the epaxial and hypaxial lips, respectively. In these lips, cells are presumed to divide, delaminate and move towards the myotome and become committed to a skeletal muscle fate (Denetclaw, *et al.*, 1997; Denetclaw & Ordahl, 2000), coupled with myogenic regulatory factors (MRFs) expression. Cells derived from the epaxial lip will give rise to trunk muscles and hypaxial cells will give rise to limb muscles and ventral body wall muscles, depending on their location in the anterior-posterior axis (Ordahl & Le Douarin, 1992; Denetclaw, Christ, & Ordahl, 1997).

1.1.2 Genetic regulation of myogenesis

Several transcription factors have been described as necessary for somite patterning and cell commitment. The paired box (Pax) family of transcription factors appears to be expressed first and define the somite patterning, namely distinguishing the ventral and the dorsal portions of somites. The ventral portion that will give rise to the sclerotome is defined early by Pax1 expression (Wallin *et al.*, 1994), while the dorsal somite expresses Pax3 and Pax7 (Jostes *et al.*, 1990; Williams *et al.*, 1994). Probably due to Pax expression,

dermomyotomal cells are apoptosis-protected and do not express differentiating factors (Kassar-Duchossoy *et al.*, 2005; Relaix *et al.*, 2005).

After the establishment of the dermomyotome and the lips, cells that will differentiate in skeletal muscle are induced to express the beta helix-loop-helix myogenic class of MRFs Myf5 (first in mouse), MyoD (first in chicken), Mrf4 and Myogenin (Cossu *et al.*, 1996). Later in myoblast differentiation, Desmin (intermediate filament) and Myosin will be expressed, defining the myocytes.

The first MRF to be expressed in the mouse embryo is Myf5, whose mRNA is detected on the dorso-medial quadrant of E8.0 embryos most anterior somites. From E11.5 stages on its expression decreases being undetectable at E14, suggesting that Myf5 is mostly related to early muscle determination (Ott *et al.*, 1991).

In vivo studies showed that MyoD is activated in response to dorsal ectoderm and axial structures signals (Cossu *et al.,* 1996). This MRF, the major responsible for hypaxial myogenic precursors (Kablar *et al.*, 1997), is expressed in muscle progenitors and mature myofibers.

It has been described that MRF4 is strongly expressed in embryonic myotome after Myf5 and Myogenin expression and earlier than MyoD, being maintained in adult muscles at high levels, which suggests that MRF4 might be needed to regulate maturation and maintenance of adult muscle phenotype (Bober *et al.*, 1991; Hinterberger *et al.*, 1991).

Myogenin is detected in E8.5 embryos in the most anterior somites, when myocytes appear, being associated with fusion and differentiation (Smith *et al.*, 1994). In Myogenin knock-out mice, a normal number of myoblasts was observed but myofibers were absent, suggesting that this MRF is expressed in cells entering the terminal differentiation program (Nabeshima *et al.,* 1993; Hasty *et al.*, 1993).

Desmin is the muscle specific intermediate filament that is present in muscles (for example in the Z-disk of striated muscles) (reviewed by Paulin & Li, 2004). This protein appears in myotomes of E9.0 mouse embryos (Schaart *et al.*, 1989), especially in differentiated myotubes (Kaufman *et al.*, 1988). Although Desmin-null mutants survive and muscle fibers maturation does not appear to be affected, these animals suffer from myopathies and have less tolerance to exercise. This means that muscle formation is not compromised but myofibers are severely disorganized and their function is compromised (reviewed by Paulin & Li, 2004).

Similarly to Desmin, Myosin is first detected between E9.0 and E10.0 in the rostralmost myotomes, being implicated in myoblast fusion and present in myofibers. Myosin is maintained in adult skeletal muscles (Lyons *et al.*, 1990).

1.2 Satellite cells

As described above during muscle development, some cells, called satellite cells, remain undifferentiated and quiescent, lying in contact with the basal lamina of muscle fibers (Schultz *et al.*, 1978; Bischoff & Heintz, 1994). In post-natal life and throughout adulthood, in case of muscle injury or enhanced exercise, mononucleated satellite cells are activated, proliferate and are induced to differentiate (Grounds & McGeachie, 1987; Schultz & McCormick, 1994). The fact that these cells divide asymmetrically, one of which differentiates and the other remains in the undifferentiated niche connected to the basal lamina (Cossu *et al.*, 2007), led to the idea that these cells represent a type of stem cells (Cossu & Tajbakhsh, 2007; Zammit *et al.*, 2006). Satellite cells are characterized *in vivo* by the expression of Pax7 and, in many muscle masses, Pax3 (reviewed by Buckingham, 2007) as well as Myf5, although some heterogeneity is detected (reviewed by Kuang & Rudnicki, 2008). Once activated, satellite cells differentiate similarly to embryonic myogenesis (Parker *et al.*, 2003), namely expressing the characteristics MRFs and then they fuse with pre-existing fibers or with themselves creating new ones.

In order to study several processes concerning satellite cells, three major approaches are possible: 1) *in vivo* manipulation; 2) removal of skeletal-muscles satellite cells and *ex vivo* culture or 3) *in vitro* studies with C2C12 cell line. The third approach is frequently used, as C2C12 is an immortalized satellite cell line, being easy to culture and maintain, and allows several useful studies (Burattini *et al.*, 2004).

1.3 Notch signaling

The Notch signaling pathway has been extensively studied in many different models. When Notch interacts with Delta, Serrate or Lag2 (DSL) ligands, it undergoes a proteolytic cleavage by γ-secretases or others proteinases, releasing the Notch intracellular domain (NICD) to the cytoplasm. As the NICD is translocated to the nucleus, it associates with the CBF1, Su(H) or LAG-1 (CSL) transcription factors, modulating a broad range of signaling pathways. Most studies concerning Notch signaling in cell differentiation in development have been performed in *Drosophila melanogaster* nervous system and muscle development (Corbin *et al.*, 1991; Ruiz Gómez & Bate, 1997; Roegiers & Jan, 2004), although in vertebrates some studies have addressed a role of Notch in myogenic regulation, as well as in other systems such as neuronal differentiation, eritropoiesis and hematopoiesis (Wakamatsu *et al.*, 1999; Cheng *et al.*, 2008; Weber & Calvi, 2009). This signaling pathway has also been implicated in systems approaching stem cells maintenance or differentiation. In stem cell niches Notch signaling inhibits differentiation and, in some cases, inducing proliferation (reviewed by Lathia *et al.*, 2007).

1.3.1 Notch signaling in myogenesis

Notch signaling pathway has been implicated as being important to prevent myogenic differentiation, even in committed myoblasts (Kopan *et al.*, 1994) or avian embryos (Hirsinger *et al.*, 2001). Several transcription factors have been associated with the Notch intracellular signaling, but all studies describe that Notch signaling antagonizes or represses MyoD (e.g. (Sasai *et al.*, 1992; Shawber *et al.*, 1996; Kuroda *et al.*, 1999). Knock-out experiments showed that in Notch1 null-embryos the somitogenesis timing is perturbed, although epithelial somites form and the muscle differentiation markers are present (Conlon *et al.*, 1995).

1.4 Extracellular matrix and integrins

1.4.1 Extracellular matrix

Cells within all multicellular organisms are surrounded by a multi-component structure, called the extracellular matrix (ECM). The ECM is a dynamic network composed of several proteins (glycoproteins; collagens; proteoglicans like perlecan and others) synthesized and organized by the cells. This network can exist in different forms: as intersticial matrices (like the one of the connective tissue, a porous structure that allows cell movements and support) and as pericellular matrices (like the basement membrane, also known as the basal lamina, a sheet-like structure that serves as a barrier), providing support to cells and tissues (Schwarzbauer, 1999; Yurchenco *et al.*, 2004). As the ECM interacts with cells directly and serves as a reservoir for growth factors, it gives the positional and environmental information needed for cells to coordinate their (own) behaviour. Several studies have been describing the ECM as a key player in embryogenesis (reviewed by Zagris, 2001). From the large variety of ECM molecules, laminin and fibronectin glycoproteins are the most studied so far, and those are the ones studied in this thesis.

1.4.1.1 Laminin

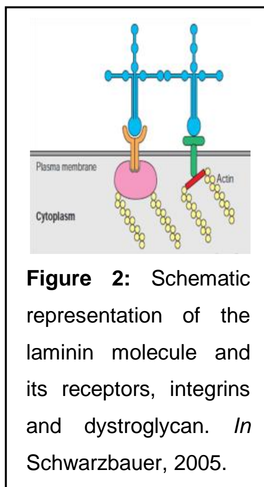


Figure 2: Schematic representation of the laminin molecule and its receptors, integrins and dystroglycan. *In* Schwarzbauer, 2005.

Functional laminins (LN) are heterotrimers composed of a combination of one α, one β and one γ chain (figure 2). These glycoproteins, important components of basement membranes, can form a large variety of laminins, depending on the combination of the chains (Colognato and Yurchenco, 2000), although not all possible combinations exist. Studies of specific laminin-deficient embryos show that laminin is important in muscle formation (Sunada & Yamadas, 1994; Hynes, 1996).

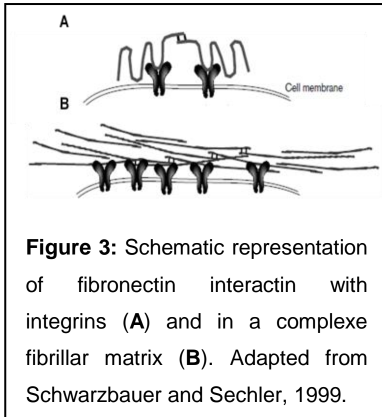


Figure 3: Schematic representation of fibronectin interactin with integrins (**A**) and in a complexe fibrillar matrix (**B**). Adapted from Schwarzbauer and Sechler, 1999.

1.4.1.2 Fibronectin

Fibronectin (FN) is a large glycoprotein that usually forms a fibrillar network, by fibronectin-fibronectin binding. This glycoprotein is secreted as a dimer (figure 3A), which each subunit containing several domains that mediate the interaction with cells and other ECM molecules (Mao *et al.*, 2005). Fibronectin mutants show mesodermal and vascular defects, as well as no somite formation (reviewed by Hynes, 1996).

1.4.1.3 Extracellular matrix in myogenesis

Not surprisingly, the ECM seems to be important in myogenesis, as myogenic precursor cells are in close contact with this network. As somites form, they are surrounded by a basement membrane that remains in the dermomyotome basal side (Duband *et al.*, 1987; Zagris *et al.*, 2000) and is produced between the myotome and sclerotome when the first forms, being mostly composed of laminin and collagen. When the myotome disappears and muscle cells reorganize, the ECM disaggregates and fibronectin and laminin are present in a dotty pattern (Cachaço *et al.*, 2005). Later in myotube maturation, a basement membrane containing laminin is formed (Cachaço *et al.*, 2005; Patton, 2000). Nevertheless, there are several open questions regarding the influence of laminin and fibronectin in myogenesis, as some studies seem to indicate that fibronectin promotes myoblast proliferation and laminin promotes their differentiation (von Der Mark *et al.*, 1989), and others indicate that fibronectin is important for myoblast differentiation inhibition (Sastry *et al.*, 1996).

1.4.2 Integrins

The ECM interacts with the cells by ECM-receptors, most commonly integrins, that directly affect cell fate (Hynes, 1992; van der Flier & Sonnenberg, 2001). Functional integrins are heterodimers composed by the combination of one α and one β subunit. Several α and β subunits have been described, namely 18α and 8β in mammals (figure 4). The combination of these subunits define the ligand specificity (table 1).

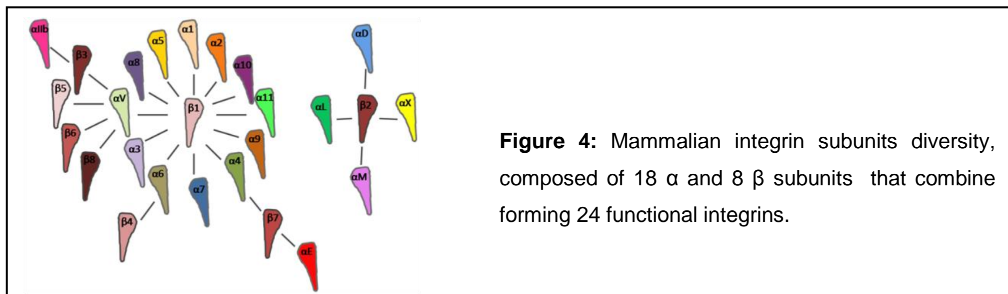


Figure 4: Mammalian integrin subunits diversity, composed of 18 α and 8 β subunits that combine forming 24 functional integrins.

Integrin	α1β1	α2β1	α3β1	α4β1	α5β1	α6β1	α7β1	α8β1	α9β1	α10β1	α11β1	αVβ1
Ligand	Col I/IV; LN, Ten	Col, LN, Ten	LN, Col, FN	FN	FN, Ten	LN	LN	FN, Ten, VN	Col I, LN, Ten	Col	Col	Col, FN, VN

Table 1: Integrins and their ECM ligands. Abbreviations: Col, collagen, LN, laminin; FN, fibronectin; VN, vitronectin; Ten, tenascin.

When these transmembrane receptors interact with the EMC, they transduce the signal to adapter proteins (as paxillin), kinases (as integrin-linked kinase or focal adhesion kinase) and other effectors present in the cytoplasm that influence a variety of signaling pathways. As cytoplasmic proteins and the cytoskeleton are reorganized, integrins themselves modulate and organize the ECM, forming clusters (reviewed by Giancotti & Ruoslahti, 1999). This duality of functions allows cells to signal both outside-in and inside-out. Depending on the EMC molecule, the integrin involved in the interaction and the cell type, cells may be induced to migrate, differentiate, proliferate or undergo apoptosis (figure 5).

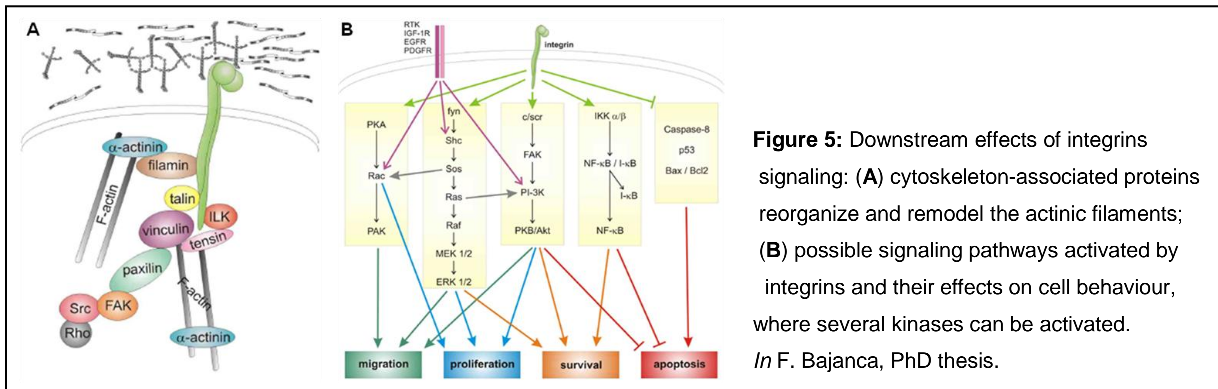


Figure 5: Downstream effects of integrins signaling: (**A**) cytoskeleton-associated proteins reorganize and remodel the actinic filaments; (**B**) possible signaling pathways activated by integrins and their effects on cell behaviour, where several kinases can be activated. *In* F. Bajanca, PhD thesis.

<u>1.4.2.1 Integrins in myogenesis</u>

Several α integrin subunits that combine with β1 subunit are expressed during skeletal myogenesis, being important in myogenic cell migration, myoblast fusion, muscle fiber maturation or in maintenance of muscle integrity (Jones & Walker, 1999; Darribère *et al.*, 2000). The β1 subunit was shown to be important for myogenic precursor cells migration in chick embryos and myoblast fusion *in vitro* (Jaffredo *et al.*, 1988) as well as primary and secondary mouse embryo myogenesis (Cachaço *et al.*, 2003). Due to the difficultly of studying integrins influence *in vivo*, several studies have been performed *in vitro* or *ex vivo*, where α5β1 integrin seems to be important in myoblast proliferation and inhibiting their differentiation and α6Aβ1 in myoblast proliferation inhibition (Boettiger *et al.*, 1995; Sastry *et al.*, 1996). α4β1 appears to be involved in primary myotube fusion (Rosen *et al.*, 1992). *In vivo*, several expression patterns have been determined for some α subunits in dermomyotome maturation as well as in limb skeletal muscle precursors specification. In the mouse dermomyotomes and myotomes, several α subunits expression were detected and their patterns were described from E8.5 to E10.5: α6β1 colocalizes with Myf5 in the dorso-medial lip, being proposed as an epaxial-specific integrin; α1 and α7 subunits are first

detected in E10.5 myotomes, by the time that α6 is downregulated, being associated with a new myogenic differentiation phase; α4β1 integrin is absent in the epaxial myotome but present in the hypaxial one. Both α4 and α5 integrin subunits appear to be specific for differentiating myoblasts (Bajanca *et al.*, 2004).

Although several α subunits mutant mice have been produced, only α5 and α7 mutants showed muscle defects, namely dystrophies (Mayer *et al.*, 1997; Taverna *et al.*, 1998). However, this does not mean that other subunits are not important. Some mutants have early lethality, being thus impossible to be analyzed and others do not have a dramatic defect in skeletal muscle possibly due to compensation by other subunits (Hynes, 1996). Another way of studying the ECM and integrins influence *in vivo* is by perturbing their interaction. When laminin-α6β1 integrin interaction is blocked, the results suggested a dual role for laminin-α6β1 integrin interaction in myogenesis *in vivo*, as it inhibits myogenic precursor cells dispersion and precocious differentiation (Bajanca *et al.*, 2006).

1.5 Aims of this thesis

Several studies have addressed the importance of the ECM in skeletal muscle formation, although unfortunately this issue is not as studied as other molecules involved in this process. Moreover, as the ECM seems to be in close interaction with other signaling pathways in the cell, its importance in myogenesis, as well as in other systems, is evident.

In this thesis we wanted to understand further about the influence of the ECM and integrins in myogenesis. We studied in more detail the importance of the laminin-α6β1 integrin and the fibronectin-α5β1 integrin interactions in regulating myogenic precursor cells behaviour.

For this purpose we used an *in vitro* model, C2C12 myoblasts, as well as mouse embryos for *in vivo* studies.

2. Materials and Methods

2.1 *In vitro* studies

2.1.1 Cell culture

C2C12 myoblasts were maintained in culture in DMEM GlutaMax (ref. 31966, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (ref. 10500, Invitrogen) and 100U/mL of streptomycin and penicillin antibiotics (ref. 15140, Invitrogen) in a humidified atmosphere of 5% CO_2 and 37ºC. Cells were detached from the culture flasks when strictly subconfluent (≅80% confluency) using 0,05% Tripsin-EDTA (ref. 25300, Invitrogen) to maintain their differentiation potential.

2.1.2 Extracellular matrix preparation

Laminin: Laminin (ref. L2020, Sigma) diluted at 5µg/mL in 1X PBS (137mM NaCl; 2,68mM KCl; 8,1mM Na_2HPO_4; 1,47mM KH_2PO_4; pH 7,3 in deionized water) was placed on sterile glass coverslips and incubated for 1 hour at 37ºC for coating. Next, the excess was removed, washed with 1X PBS and the laminin-coated coverslips were ready to use.
Fibronectin: 2D fibronectin pre-coated coverslips were obtained from BD Biocoat Cellware® (ref. 354088).
Gelatin: Sterile 1% gelatin was placed on sterile glass coverslips, left for one hour at 37ºC for polymerization coating and the excess removed. This matrix was used as control.

2.1.3 Immunofluorescence

C2C12 cells were passaged onto 6- or 12-well plates with the pre-coated fibronectin coverslips and sterilized coverslips coated with laminin and gelatin, as described above.
After cells reached the desired confluence, they were washed twice briefly with 1X PBS fixed with 1% paraformaldehyde (PFA) in 1X PBS for 30 minutes (or 2% PFA in differentiated cells), washed with 1X PBS and permeabilized with 0,5% Triton X-100 in 1X PBS for 5 minutes. After washing briefly with 1X PBS, blocking was performed with 2% bovine serum albumin (BSA) in 1X PBS solution for 30 minutes. Coverslips with cells were transferred to parafilm-coated dishes where they were incubated with primary antibodies in blocking solution for 2 hours at room temperature (RT). Before and after incubation with secondary antibodies for 2 hours (with nucleic acid stain ToPro3 and RNase in blocking solution), cells were washed 3x10min with 1X PBS. In the case nuclei staining was performed with DAPT, ToPro3 and 1:100 RNase were not used and cells were incubated with 5µg/mL of DAPI for 1 minute and then washed 3x10min with 1X PBS. Finally they were mounted on microscope slides with propyl gallate-PBS-glycerol and sealed with nail varnish.

2.2 *In vivo* studies

2.2.1 Mouse embryo collection and culture

Charles-River mice were used to generate embryos. The morning plug was counted as E0.5 and females were sacrificed by cervical dislocation to collect embryos at the desired stage, which ranged from E9 to E12.5. For culture, embryos were dissected in Dulbecco's Modified Eagle Medium/F12 GlutaMax medium (DMEM/F12) (ref. 31331, Invitrogen) supplemented with 10mM HEPES (organic buffer ideal for maintain physiological pH), 1mM sodium pyruvate (ref. 11360-039, Invitrogen), penicillin and streptomycin antibiotics (100U/mL) and cultured on 0,8µm Milipore size filter (ref. ATTPo2500, Isopore^{TM}) floating on culture medium (dissection medium without additional HEPES) at 37ºC and 5% CO_2 humidified atmosphere for the desired time.

2.2.2 Immunofluorescence

Mouse embryos were fixed in 0,2% PFA ON at 4ºC, dehydrated in an increasing glucose series until included in gelatin solution and then frozen in dry ice-chilled liquid isopentane and stored at -80ºC. Embryos were sectioned in 30 thick slices using a cryostat (Bright Clinicut) and placed on SuperFrost Ultra Plus miscroscope slides (Menzel-Gläser).

The embryo transversal sections (and coronal sections in E12.0 and E12.5 cultured embryos) were washed 3x5min with 1X PBS, permeabilized for 40 minutes in 0,2% Triton X-100 in 1X PBS, washed again 3x5min and blocked for 1 hour in 1% BSA in 1X PBS. Primary antibodies in blocking solution were added and left ON at 4ºC. Then, embryo sections were washed 3x10min and incubation with secondary antibodies (in blocking solution, with ToPro3 and 1:100 RNase) was performed for at least 2 hours at RT. Embryo sections were washed with 4X PBS for 20 minutes and 3x10min with 1X PBS to eliminate non-specific antibody binding and finally mounted with glass coverslips on propyl gallate-PBS-glycerol, sealed with nail varnish and kept in the dark at 4ºC.

2.3 Experimental studies

Drug/Antibody	Cat., Company	Use	Concentration	References	Control
70kDa Fragment	F0287, Sigma	Inhibit FN fibrillogenesis	100µg/mL	McKeown-Longo & Mosher, 1985; Rifes *et al.*, 2007	BSA
RGD	G4391, Sigma	Block FN-α5β1 integrin binding	0,9mM	Takahashi *et al.*, 2007	DGR (S3771)
GoH3	A. Sonnenberg	Block LN-α6β1 integrin binding	10µg/mL pure and 1:10 supernatant	Sonnenberg *et al.*, 1987; Bajanca *et al.*, 2006	Anti- α7 (Ann Sutherland)
DAPT	565784, Calbiochem	γ-secretase inhibitor	0,1mM	Gibb *et al.*, 2009	DMSO (D2650, Sigma)
N-cadherin antibody	MNCD2, D.S.H.B.	Block N-cadherin	10µg/mL	Linask *et al.*, 1998	(without a specific control)

Table 2: List of drugs and blocking antibodies used diluted in the embryo culture medium.

2.4 Antibodies

	Antibody	Reference, Company	Dilution
Primary antibody	Anti-LN	L-9393, Sigma	1:100
	Anti-FN	F-3648, Sigma	1:100
	Anti-α5	AB 1928, Chemicon	1:100
	Anti-N-cadherin	C-70320, BD Biosciences	1:100
	Anti-ZO-I	40-2200, Zymed	1:100
	Anti-Myf5	SC-302, Santa Cruz Biotec.	1:100
	Anti-Myogenin	F5D, D.S.H.B.	1:100
	Anti-Desmin	D3, D.S.H.B.	1:100
	Anti-MHC	F59, D.S.H.B.	1:100
	Anti-Myosin	MF20 asc., D.S.H.B.	1:100
	Anti-MyoD	MyoD, John Harris	1:100
	Anti-Pax3	Pax3, D.S.H.B.	1:100
	Anti-pH3	H3, Upstate	1:100
	Anti-cleaved caspase3	9669, Cell Signaling	1:100
	Anti-Notch1 (NICD)	SC-6014-R, Santa Cruz Biotec.	1:50
Secondary antibody	Anti-Rabbit Alexa488	A-11070, Mol. Probes	1:1000
	Anti-Rabbit Alexa568	A-21069, Mol. Probes	1:1000
	ToPro3	T3605, Mol. Probes	1:1000
	Phalloidin-Alexa488	A-12379, Mol. Probes	1:400

Table 3: List of the antibodies used in immunofluorescence.

2.5 Imaging

2.5.1 Wide-field image acquisition

For nuclear quantifications and myogenic markers expression throughout C2C12 differentiation studies, cells were imaged using an Olympus BX60 microscope coupled to an Olympus DP50 digital camera.

2.5.2 Time-lapse image acquisition

C2C12 myoblasts were passaged to the desired condition and before time-lapse video was performed, cell medium was replaced by growth medium supplemented with 20mM of HEPES. Then, the Petri dish containing these cells was sealed with parafilm and placed in an incubation chamber prepared to maintain the proper temperature. Time-lapse images were acquired each 3 minutes using an automated Zeiss stereo LUMAR Stereoscope and an Axiocam cooled CCD camera.

2.5.3 Confocal image acquisition

Immunostained embryo sections and cells were imaged using a Leica SPE confocal laser scanning microscope and 20x 0.7NA, 40x 1.3NA, 63x 1.4NA lens, acquiring either single or z-stacked images of 1024x1024 pixels at 8bit grayscale levels.

Images and videos were then treated and analyzed using ImageJ, Amira v4.2 and Imaris v5.7.2 software.

2.6 Image analysis and quantifications

2.6.1 *In vitro* studies

2.6.1.1 Quantification of nuclear shape

In order to be able to count cells and estimate their shape, we applied a *Median filter* of 4.0µm Radius to reduce noise and better define the contour of the nuclei. Then, nuclei between 100-500µm^2 of area were selected (rejecting specs and overlapping nuclei) and analysed using the *Analyse particles* function in ImageJ.

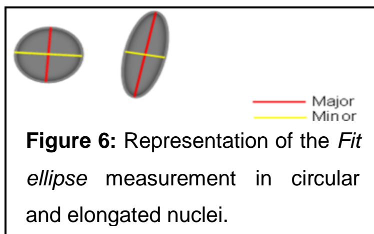


Figure 6: Representation of the *Fit ellipse* measurement in circular and elongated nuclei.

To compare the nuclear shape of cells growing on fibronectin and gelatin, we used the *Fit ellipse* measurement of ImageJ that gives the major and minor measurements that correspond to the primary and secondary axis of the best fitting ellipse for the nuclei (figure 6). The ratio between these measurements gives an estimate of the elongated shape of the nuclei. Elongated, spindle shaped cells have elongated nuclei, whereas cells with a more symmetrical distribution of cytoplasm tend to have more round nuclei. A ratio of Major/Minor =1 was interpreted as belonging to a cell that is not elongated and a Major/Minor >1 as a cells that was elongated.

2.6.1.2 Cell tracking from time-lapse movies

To study cell movements, we had to correct the drift from the time-lapse image sequences using the *Align Slices* function of the Amira software. Image contrast was enhanced by dividing each image of the time-lapse sequence by a copy of that same image processed using a 25.0 Sigma (Radius) Gaussian Blur filter. This corrected for uneven illumination and out-of-focus blurred image contaminants. Afterwards, the Imaris software was used to manually track the movements of individual cells. Since we used bright-field images, the software could not do automatic cell identification and tracking, therefore we manually tracked the cells. To randomize the cell sampling we applied a grid with 100µm squares to the time-lapse image sequence and tracked the cells present at the intersections of the grid at the beginning of the sequence as represented in figure 7, as red dots.

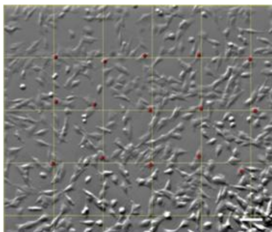


Figure 7: First frame of a time-lapse movie, in which cells are growing on gelatin in subconfluency (around 60-70% confluency). Each red spot represents a cell whose movements were tracked. Fifteen cells per movie were analysed. Bar: 100µm.

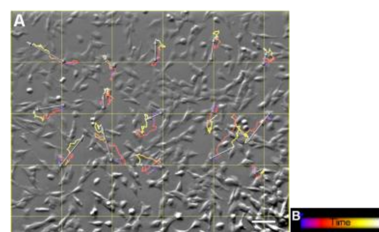


Figure 8: Tracks obtained for the movie of cells seeded on gelatin at subconfluency (**A**). The track colours sequence represents time (**B**). Bar: 100µm.

Each chosen cell was followed during the whole time-lapse sequence by placing a spot in the center of the cell body at each time-point. The tracks were then created, analysed and compared for some parameters that Imaris calculates, the cell displacement and track length. The tracks were then calculated by the software (figure 8), which also calculated the displacement (linear distance between beginning and ending position) and full track length.

2.6.2 *In vivo* studies

2.6.2.1 Quantification of dermomyotome fluorescence

To compare the expression of Myf5 in dermomyotomes we measured the average fluorescence intensity in images of embryo sections immunostained as described above.

Quantifications were standardized by measuring the average fluorescence levels in the central dermomyotome and dividing it by the average fluorescence intensity of the neural tube (figure 9). The neural tube was chosen as the “background control” as both tissues have a similar cell density but the neural tube had no specific immunoreactivity for Myf5 (as in Bajanca *et al.*, 2006). Only the central dermomyotome excluding lips fluorescence was measured because some cells of the lips normally express Myf5, a fact that could mask the differences from the experimental and control situations.

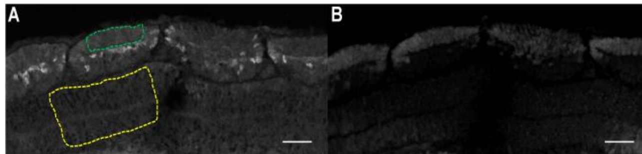


Figure 9: (**A**) Representation of the Myf5 average fluorescence measurements in the central dermomyotome (green dashed line) and the neural tube (yellow dashed line); (**B**) Pax3 was used to delimit the dermomyotome. Bar: 50µm.

2.7 Statistical analysis

To be possible to test differences with Student’s t-test or ANOVA, the tests requirements needed to be verified. To determine if the samples had a normal distribution, SPSS v17.0 software was used. In the cases where homogeneity were not statistically significant, both groups of measurements were transformed using the $Logarithm_{10}$ or the Square Root (discriminated on the respective analysis, in section 3) and then tested for homoscedasticity with Levene test, performed with STATISTICA 8.0 (Stat soft, Inc.) software. In the cases where both requirements for statistical test were verified, we could test our measurements for differences using t-test for independent variables or groups and ANOVA. When normal distribution could not be achieved, we used non-parametric Mann-Whitney and Kruskal-Wallis test. We further confirmed our non-parametric test results with t-test. Both parametric (Student’s t-test and ANOVA) and non-parametric tests (Mann-Whitney and Kruskal-Wallis) were performed in STATISTICA software.

All our comparison tests were performed using a 95% confidence interval.

3. Results

3.1 Laminin

3.1.1 Laminin influence in *in vitro* myogenesis

To study the effect of a laminin matrix on myogenic differentiation, we cultured C2C12 cells on laminin- or gelatin-coated coverslips and compared the dynamics of MRFs expression throughout the activation and development of the myogenic program performing immunofluorescence in cells maintained over several days in growth and differentiation medium. In the first 8 days of C2C12 differentiation, we could not observe any difference in MRFs, namely Myf5 and Myogenin (figure S1, section 6) nor in Myosin expression and the correspondent myotubes (figure S2, section 6).

3.1.2 Laminin influence in *in vivo* myogenesis

It has been previously shown that the laminin matrix is necessary to inhibit myogenic differentiation in the dermomyotomal cells (Bajanca *et al.*, 2006). One possibility is that it could be related to the Notch signaling pathway.

3.1.2.1 Laminin-α6β1 interaction maintains dermomyotomal cells undifferentiated

In a first approach, we tried to reproduce the laminin-α6β1 interaction blocking experiments performed in Bajanca, 2006, but it was very difficult to achieve it fruitfully. When E9.0 mouse embryos were cultured for 24 hours with GoH3 (blocking antibody) we observed in some embryos a slight increase of Myf5 protein in the dermomyotome as compared with the control embryos. However, when the fluorescence measurements were compared with the t-test, after transformation with Log_{10}, the difference was not statistically significant ($t=1.2505$, $n_{control}=49$, $n_{GoH3}=53$, $p=0.214$) (figure S3, section 6).

3.1.2.2 Notch signaling is necessary for inhibition of dermomyotomal cells myogenic differentiation

To test the Notch signaling pathway involvement in myogenic differentiation, we blocked the γ-secretase activity with DAPT thus inhibiting the nuclear translocation of the NICD.

Our first approach was to confirm that in embryos cultured with DAPT, the NICD is more retained in the membrane, in a non-cleaved form, than in control embryos. In the neural tube of DAPT-cultured embryos (figure 10 C and D), where the difference can be seen easier, the NICD is almost exclusively present at the membrane level (figure 10 C, arrows) than in control embryos (figure 10 A).

When E9.0 mouse embryos were culture for 14 hours with 0,1mM of DAPT, we observed the presence of Myf5-positive cells in the entire dermomyotome (figure 11 E and G). The

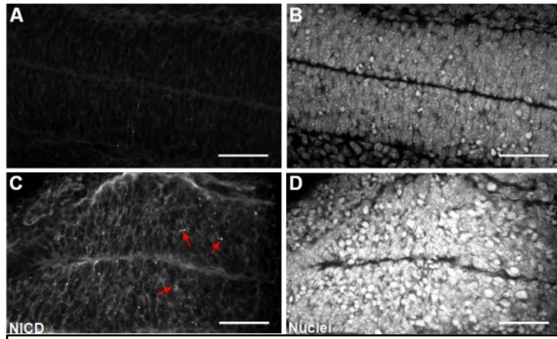
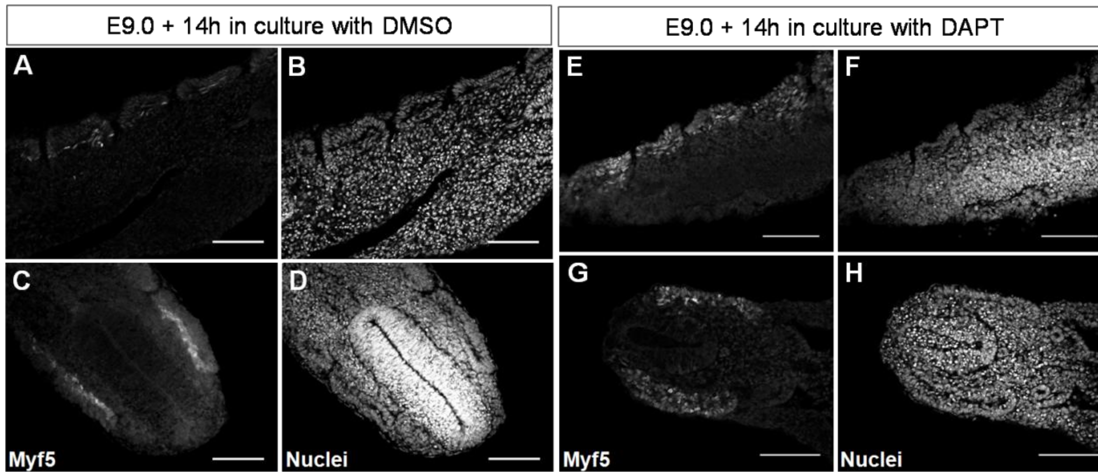


Figure 10: In 14 hours cultured embryos with DAPT (**C** and **D**), NICD is mostly present at the membrane level (arrows) than in the control embryos (**A** and **B**). Bar: 50µm.

difference between Log_{10}-transformed DMSO and DAPT-cultured embryos dermomyotome fluorescence is statistically significant, with a higher Myf5 expression in embryos cultured with DAPT (figure 11 I; t=0.6007, n_{DMSO}=62, n_{DAPT}=59, p= 0.00017).



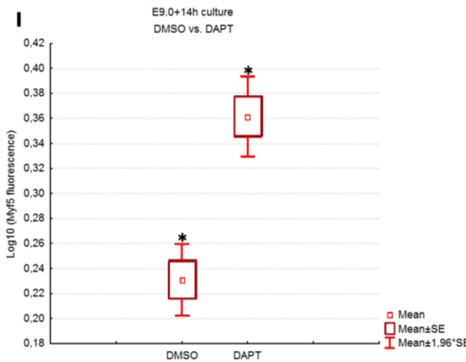


Figure 11: E9.0 embryos cultured for 14 hours with 0,1mM of DAPT (**E - H**) have an increase in Myf5 expression on dermomyotomal cells (**E, G**) when compared with the control culture (**A, C**). The difference between Log_{10}-transformed Myf5 fluorescence intensity in the dermomyotome is shown in **I**. Bar: 100µm.

3.1.2.3 Notch inhibition and Myf5 expression in the dermomyotome may be inducing an enhancement of laminin matrix assembly

Considering the data above, we tried to observe if any differences in the laminin matrix were present when γ-secretase activity was blocked. In normal embryos, laminin is present around the basal side of the dermomyotome (integrating a basal lamina) and in the dermomyomtome-myotome transition, in a patched-like matrix that develops to a basal lamina-like matrix with time and myotome maturation (figure 12 A-C).

When we compared the laminin assembly between embryos cultured with DAPT and DMSO, in DAPT-cultured embryos laminin is present in the basal lamina but also surrounding the dermomyotomal cells (figure 12 G and J, arrows), similar to small patches, which are not found in the control embryos (figure 12 D), or at least not as pronounced.

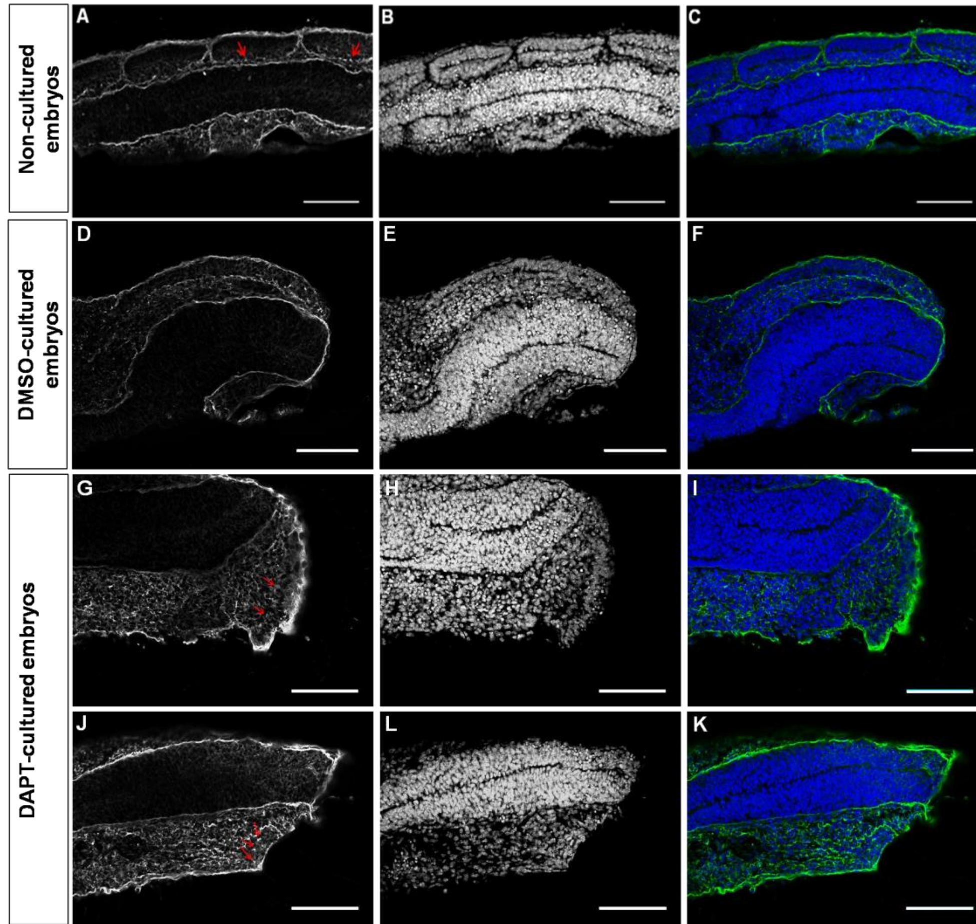


Figure 12: Non-cultured embryos (**A** - **C**), DMSO-cultured embryos (**D** – **F**) and DAPT-cultured embryos (**G** - **K**) immunostained for laminin (**A**, **D**, **G** and **J**) and nuclei (**B**, **E**, **H** and **L**). With DAPT, laminin seems to be present in places where in control embryos it is not, around of dermomyotomal cells (arrows). Bar: 100µm.

3.2 Fibronectin

3.2.1 Fibronectin influence in *in vitro* myogenesis

3.2.1.1 Fibronectin is important for C2C12 myoblasts alignment

When C2C12 cells were cultured on fibronectin pre-coated coverslips, we observed that they were aligning parallel to each other earlier (figure 13 B), even forming streams of cells (figure 13 B'), than cells cultured for the same time on gelatin (figure 13 A).

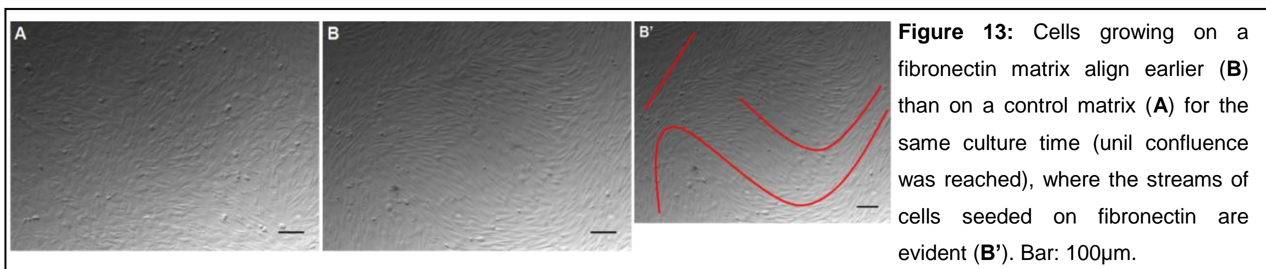


Figure 13: Cells growing on a fibronectin matrix align earlier (**B**) than on a control matrix (**A**) for the same culture time (unil confluence was reached), where the streams of cells seeded on fibronectin are evident (**B'**). Bar: 100µm.

Using nuclear staining we were able to easily detect a difference in the nuclear shape (figure 14), which is representative of the differences observed in whole cells (figure 13). When we compared the nuclei of cells seeded on fibronectin with those growing on gelatin, maintained in growth medium until they reached confluency (more than 90% confluency), we found that the first ones are more elliptical that the control ones, which are more round. After determining the ratio of the major and minor distances of the ellipse that fits the nuclei (see section 2.5.1.1 for details), the Mann-Whitney test was performed and we found a statistically significant difference between them ($U= 563258$, $n_{gelatin}=1383$, $n_{fibronectin}=1337$, $p<0.0001$) (figure 15). This result confirms that cells cultured on fibronectin are more elongated than cells growing on gelatin.

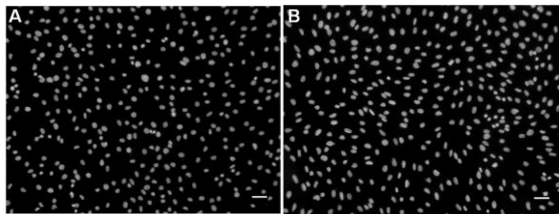


Figure 14: Example of nuclear staining of cells seeded on gelatin (**A**) and fibronectin (**B**) in growth medium. Note that the nuclei of cells seeded on fibronectin seem much more organized and elongated. Bar: 100µm.

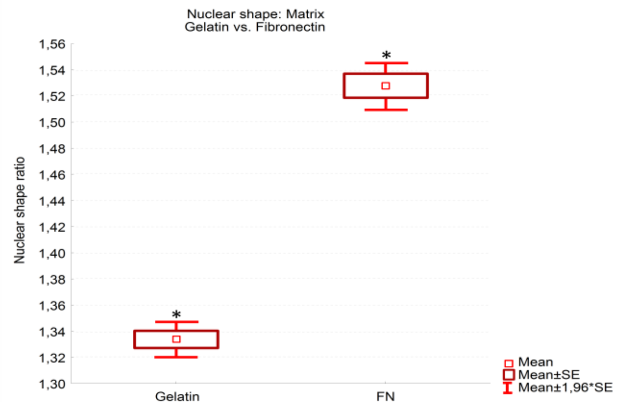


Figure 15: Graphic representation of the nuclear shape (major/minor measurements ratio) differences between cells growing on gelatin and fibronectin. Cells on fibronectin have more elongated nuclei.

In order to understand more about the C2C12 dynamics in culture when growing on fibronectin or gelatin (control), we performed time-lapse image sequences with a stereomicroscope. To have a more broad analysis, we used cells on both matrices at the time they were subconfluent (less than 60% confluency) and already confluent (more than 90% confflueny). Afterwards, we tracked cells during the movie, analysed their tracks (figure 16; supplementary video1-4; section 6) and compared several parameters (supplementary table 1, section 6).

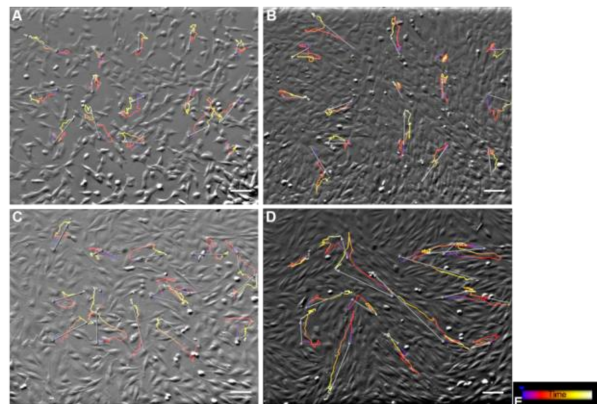


Figure 16: Cell tracks from 15 cells growing on gelatin (**A** and **B**) and fibronectin (**C** and **D**), both starting in subconfluency (<70% confluency) (**A** and **C**) or confluency (**B** and **D**). Time is represented by the colours sequence (**E**).

When comparing the track length and the cell displacement measurements, gathering in the same group the subconfluent and confluent conditions since they are not significantly different (figure S4 and S5, section 6), we observed that cells on fibronectin move significantly more and go further away from their starting position than cells on gelatin (figure

17; t=2.5135, $n_{gelatin}$=30, $n_{fibronectin}$=30, p=0.002 and t=3.214, $n_{gelatin}$=30, $n_{fibronectin}$=30, p= 0.013, respectively).

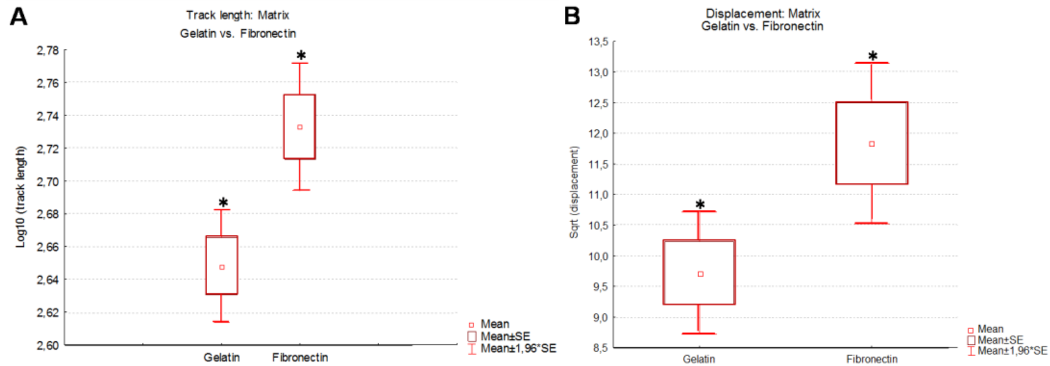


Figure 17: Graphic representation of gelatin-fibronectin comparisons for track length (**A**) and cell displacement (**B**), where cells on fibronectin move more and end up further away from the initial point. The track length and displacement measurements were transformed with Log_{10} and square root functions, respectively, to allow the t-test comparison.

Furthermore, the Imaris software calculated an estimate of the predictability of the movements of each cell tracked (the ARMean parameter). This parameter estimates the overall probability of each movement continuing the trend of the previous movement. If the value calculated for a determined time-point approaches 1, then the speed and direction of the cell is maintained from the previous movement, while if the value approaches 0 then the direction taken by the cell is different; cells with a random “walk” will tend to have ARMean values closer to 0, while cells migrating on a linear path tend to have more predictable movements (*i. e.,* ARMean values closer to 1). When we compared this parameter in cells cultured on gelatin and fibronectin, we found that cells on fibronectin had a more predictable movement directionality then cells cultured on gelatin (figure 18; U=216,5, $n_{gelatin}$=30, $n_{fibronectin}$=30, p=0.0006).

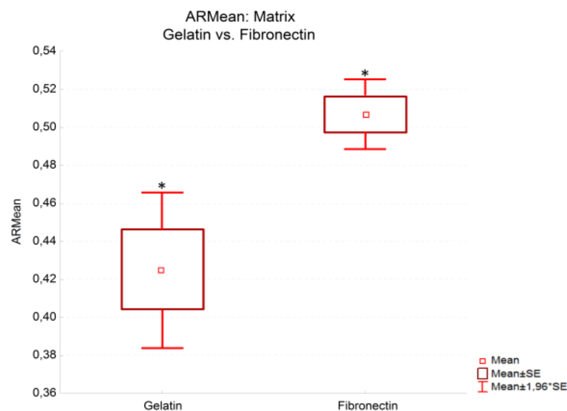


Figure 18: Graphic representation of ARMean difference of cells seeded on gelatin and fibronectin, where it can be seen that on fibronectin cells have a more predictable path than on gelatin.

3.2.1.2 Fibronectin-α5β1 integrin interaction appears to be responsible for *in vitro* cultured myoblasts alignment

In order to understand which integrin could be interacting with fibronectin and possibly influencing cell alignment, we performed immunostaining for several α integrin subunits that are known to bind to fibronectin, namely α4, α5 and αV. Only the anti-α5 antibody worked on C2C12, limiting our results. Early in culture (one day after seeding 1/10 of cells at 80% confluency) cells express α5 integrin

subunit, both on gelatin and fibronectin matrices (figure 19 A and B) being maintained throughout the culture, until confluency is reached (figure 19 C and D). We also noticed that the actin cytoskeleton of the cells on fibronectin is particularly organized, possibly allowing cells to acquire the elongated shape (figure 20 F), and that N-cadherin is present along the cell-cell contact surface (figure 20 G).

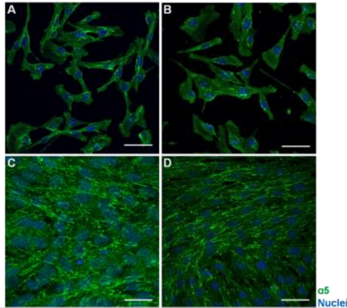


Figure 19: Immunostaining for α5 integrin subunit show that cells cultured on gelatin (**A** and **C**) and fibronectin (**B** and **D**) express this integrin in confluency (**C** and **D**), but also in subconfluency (**A** and **B**).
Bar: 50µm.

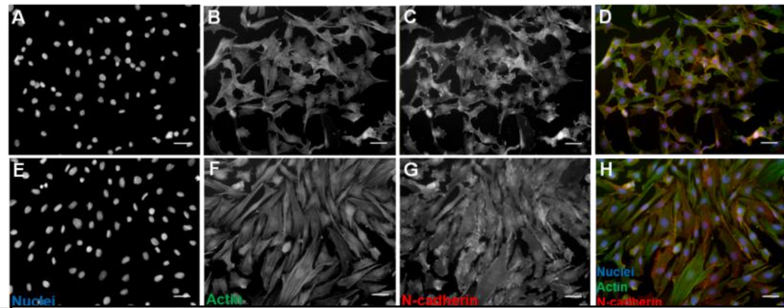


Figure 20: Actin (**B** and **F**) and N-cadherin (**C** and **G**) expression on cells growing on gelatin (**A** - **D**) and on fibronectin (**E** - **H**), showing the cell shape differences between them. Bar: 40µm.

The next step was to block fibronectin-α5β1 interaction with RDG. RGD (Arginine-Glycine-Aspartic Acid peptide) corresponds to the fibronectin molecule domain that interacts with α5β1 and αVβ3 integrins (figure 21; Takahashi *et al.*, 2007).

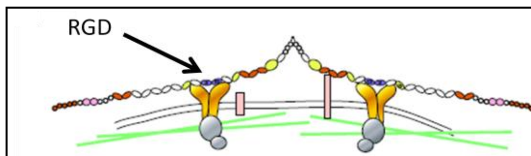


Figure 21: Illustration of the fibronectin secreted molecule showing the RGD domain, the responsible for interaction with integrins. *Modified from* Mao and Schwarzbauer, 2005.

When cells were cultured on fibronectin with RGD, we noticed that fewer cells would attach to the matrix and proliferate (figure 22 C) although they were not as aligned as control cells (figure 22 D). To quantify this observation, we stained the nuclei of cells cultured on fibronectin coverslips with growth medium supplemented with 0,9mM of RGD and the control peptide, DGR. The RGD and the control peptide were added at the moment of seeding the cells (day 0) or RGD was added one day after seeding (day 1). When we compared the major/minor ratios with Kruskal-Wallis test, we found a significant difference between these three conditions ($H=2$, $n_{control}=1657$, $n_{RGD\ day\ 2}=1675$, $n_{RGD\ day\ 1}=1675$, $p<0.0001$). Then, we performed a *Multiple Comparisons p value ratios* (to detect which conditions were statistically different) and found that only the measurements of cells cultured with RGD added at day 0 had a statistically significant difference in nuclear shape when compared with the other situations (figure 23). The nuclei of C2C12 myoblasts cultured with RGD by the time they are seeded are less elliptical than those cultured with DGR (*) and with RGD added after one day of culture (*), confirming the differences in cell shape described previously (figure 14).

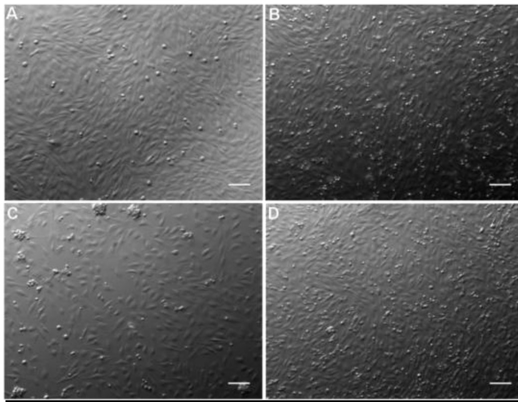


Figure 22: Cells cultured with RGD (**C** and **D**) and with the control peptide (DGR) (**A** and **B**) after 1 (**A** and **C**) and 3 days (**B** and **D**) of culture. Cells were passaged to be around 80% confluency at day 1. Bar: 100µm.

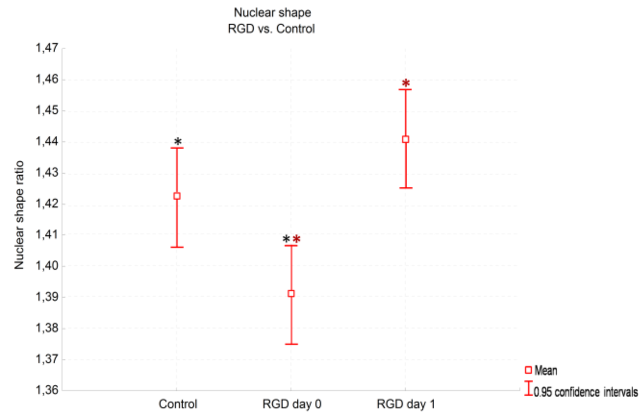


Figure 23: Graphic representation of C2C12 nuclear shape with RGD added at seeding (RGD day 0) and one day after (RGD day 1), compared with control (DGR added at seeding). RGD seems to interferes with cell and nuclear shape, as cells cultured with RGD added at seeding are more round than cells with DGR (control, *) and cells with RGD added one day after seeding (*).

3.2.1.3 N-cadherin appears to be necessary for *in vitro* cultured myoblasts alignment

To investigate whether N-cadherin could have a role in C2C12 behaviour on fibronectin, we used MNCD2, an antibody known to block N-cadherin interaction (Linask *et al.*, 1998).

When MNCD2 was added to the culture medium at the same time cells were seeded on the fibronectin-coated coverslips (figure 24 C and D), cells could not align as in the control situation (figure 24 A and B). In this experiment and in the previous one (cells cultured with RGD) cells were passaged to be 80% confluent at day 1, so it would be easier to look for differences.

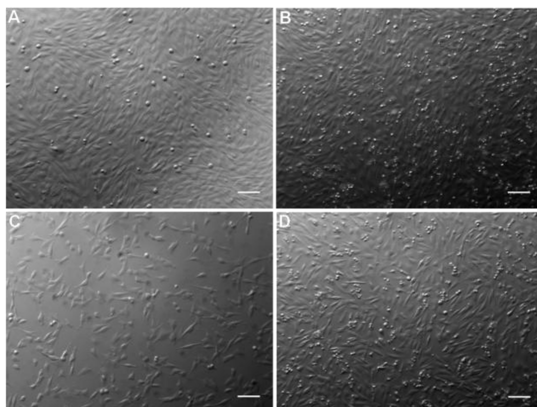


Figure 24: Cells cultured on a fibronectin matrix with N-cadherin blocking antibody (**C** and **D**) and the control (**A** and **B**). N-cadherin seems important to cell alignment. Cells were passaged to be around 80% confluency at day 1. Bar: 100µm.

3.2.2 Fibronectin influence in *in vivo* myogenesis

3.2.2.1 Fibronectin and α5β1 integrin are present in the myotome

In normal embryos, fibronectin is present in the dermomyotome basal lamina, as is the case of laminin, and surrounds the myotome as well (figure 25 A). α5 integrin is expressed on cells of both dermomyotome and myotome (figure 25 D).

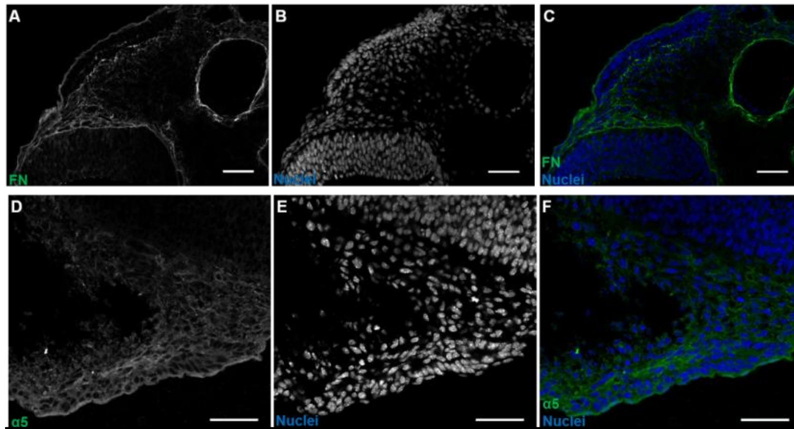


Figure 25: E9.5 embryos have fibronectin around the dermomyotome and myotome (**A** - **C**) and α5 integrin is present in both dermomyotomal and myotomal cells (**D** - **F**). Bar: 50μm.

3.2.2.2 Fibronectin matrix seems to be important for N-cadherin polarization

When E9.0 embryos were cultured for 6 hours with the 70kDa fragment, a fragment which impairs fibronectin fibrillogenesis (McKeown-Longo *et al.,* 1985; Rifes *et al.,* 2007), we could see that, instead of being concentrated on the apical side of dermomyotomal cells (figure 26 A and D) as normally, N-cadherin appears to be distributed more evenly throughout the cell membrane (figure 26 G and J). In addition, it appears that the distribution pattern of *Zonula Occludens*-I (ZO-I), one protein component of the tight junctions (González-Mariscal *et al.*, 2003), seems to be slightly perturbed (figure 26 H and K), although the difference is not as obvious as for N-cadherin.

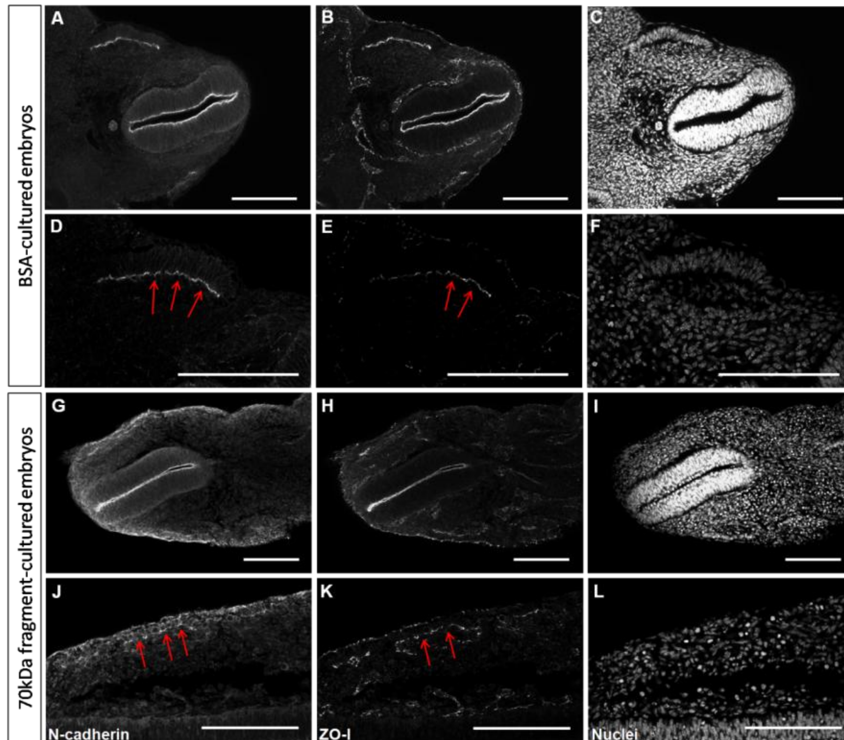


Figure 26: Control embryos (**A** - **F**) and 70kDa-cultured embryos (**G** - **L**) for 6 hours immunostained for N-cadherin (**A**, **D**, **G**, **J**), ZO-I (**B**, **E**, **H**, **K**) and nuclear staining (**C**, **F**, **I**, **L**). Arrows highlight the differences between control and experimental embryos, where the major effect is N-cadherin localization. Bar: 100μm.

3.2.2.3 Fibronectin matrix-α5β1 integrin interaction might be important in cell alignment

Using E9.0 and E10.0 embryos cultured for 6 hours with 70kDa fragment or RGD (block α5β1-fibronectin interaction) we investigated if any perturbation occurred in the myocytes.

We could not see any obvious change in myoblasts alignment and orientation (figure S6, section 6).

It has been previously shown that in E12.5 embryos only fibronectin and fibronectin-binding integrins are present in primary myocytes as they begin to fuse into myofibers (Cachaço *et al.*, 2005). Therefore, we questioned if fibronectin is important for proper myocytes alignment and fusion in E12.5 staged embryos. E12.0 mouse embryos were cultured for 6 hours with the 70kDa fragment or RGD and we did not find any difference in myotome derived myocytes of both cultures (figure S7, section 6). We cultured E12.5 embryos for 14 hours and could not see a difference in cell alignment as well (data not shown).

3.2.2.4 Fibronectin-α5β1 interaction maintains dermomyotomal cells undifferentiated

During these experiments we observed that, in some sections of the E9.0 embryos cultured for 12 hours with RGD, Myf5 protein was present in the dermomyotome, similar to what we saw when embryos were cultured with DAPT (see section 3.1.2.2) (figure 27 D).

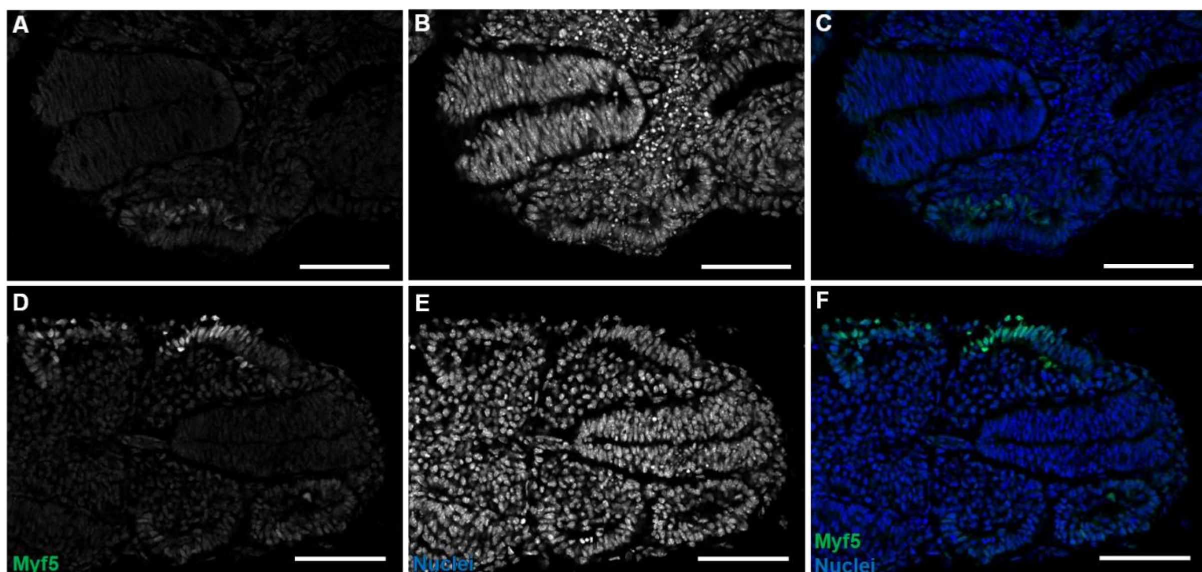


Figure 27: E9.0 embryos cultured with RGD for 12 hours (**D** - **F**), where in some sections we could detect Myf5 expression in the dermomyotome (**D**), never detected on control cultured-embryos (**A** - **C**). Bar: 100µm.

4. Discussion and Concluding Remarks

The major goal of this thesis was to investigate in more detail the influence of the extracellular matrix in myogenesis, namely in myogenic specification of dermomyotomal cells, their translocation to the myotome and myoblasts behaviour in this “compartment”.
Our first approach was to study C2C12 myoblasts followed by mouse embryos to address the ECM influence in specific processes in myogenesis.

4.1 Laminin

Several studies have addressed different functions for laminin in both *in vivo* and *in vitro* systems: in mouse stem cells, laminin induces cell differentiation (Hayashi *et al.,* 2007) and lamimin-5 coating (α3β3γ2 laminin) prevents chondrogenic differentiation of human mesenchymal stem cells (Hashimoto *et al.*, 2006); in C2C12 myoblasts, mechanically stimulation through laminin receptors show enhanced differentiation (Grossi *et al.*, 2007). *In vivo*, for example, laminin is important for cerebral cortex formation (by α3β1 and α6β1 integrins) and axonal regeneration (by α7β1 integrin interaction) (Danen *et al.,* 2003) and for myotendinous junction adhesion and differentiation (Patton 2000).
Our studies with C2C12 myoblasts growing on a laminin matrix showed that laminin does not enhance myogenic differentiation, at least until they start to fuse into myotubes. Although C2C12 cells are an excellent model to study myogenesis, this cell line was obtained from adult skeletal muscle stem cells, named satellite cells. Therefore, even though we can use these cells to infer for mechanisms *in vivo*, specifically in mouse embryonic myogenesis, satellite cells are different from dermomyotomal and myotomal cells. Mainly, satellite cells have already established their fate, myogenesis, while dermomyotomal cells are undifferentiated “bipotent” cells (they can either follow myogenic or dorsal dermis fate). Another difference is that the surrounding environment where satellite cells and embryonic skeletal muscle precursors are (signaling factors present,…) is most certainly different. Nevertheless, we can try to establish some comparisons between models, since they display the same MRFs expression cascade (reviewed by Seale *et al.*, 2001).
In mouse embryo myogenesis, the interaction between laminin and α6β1 integrin is necessary to inhibit precocious myogenic differentiation in the dermomyotome (Bajanca *et al.*, 2006). Therefore, we tried to unveil more about laminin influence in myogenesis *in vivo*, especially in myogenic differentiation. Our hypothesis was that Notch signaling pathway could be related with laminin-integrin signaling. Our first approach was to try to repeat the experiments performed by Bajanca. Although we could see a slight increase in Myf5 fluorescence in the dermomyotome, it was not a significant difference. The difficulties in

reproducing Bajanca's results may be related to deficient conditions of the blocking antibodies.

In vitro studies showed that when Delta binds Notch, the Notch intracellular domain (NICD) is cleaved and translocated into the nucleus where it represses myogenesis by inhibiting MyoD activity (Kopan *et al.*, 1996; Kuroda *et al.*, 1999). In these studies several transcription factors downstream of Notch have been addressed as important for this process, although the exact inhibition pathway is not known (Shawber *et al.*, 1996; Nofziger *et al.*, 1999; Buas *et al.*, 2009). *In vivo*, most studies have been performed in chicken embryos by overexpression assays of several components of the Notch pathway, where it is clear that Notch controls MyoD activation (Delfini *et al.*, 2000; Hirsinger *et al.*, 2001). When we cultured E9.0 mouse embryos for 14 hours with DAPT, a γ-secretase inhibitor, we detected $Myf5^+$ cells in the dermomyotome. This result is in accordance with our predictions: if Notch is repressing myogenesis in the dermomyotome, the inhibition of Notch signaling would promote precocious differentiation.

Furthermore, we looked for possible differences in laminin matrix in DAPT-cultured embryos. Myf5-null embryos do not have a laminin matrix in the dermomyotome: although laminin is produced and secreted, the assembly does not occur (Bajanca *et al.*, 2006). We, therefore, hypothesized that if Notch signaling inhibition "forced" dermomyotomal cells to follow a myogenic fate by Myf5 expression, maybe we could be enhancing laminin assembly in places it normally does not assemble. In mouse embryos cultured with DAPT, we observed the maintenance of the laminin basal lamina and, as expected, laminin was present between dermomyotomal cells, in a patched-like matrix. We can now conclude that Myf5 is important for laminin assembly, namely enhancing the process.

We therefore propose a preliminary model in which laminin-α6β1 integrin interaction would somehow influence Notch signaling in dermomyotomal cells, namely maintaining the translocation of the NICD into the nuclei. At the epaxial lip, dermomyotomal cells would detach from the laminin matrix, move towards the myotome, activate Myf5 expression and assemble a new laminin matrix (this has been the model proposed for this process in our group). But the question concerning the relation between laminin, mediated by α6β1 integrin interaction, and Notch remains. As Notch expression appears in both avian dermomyotome and myotome (Hirsinger *et al.*, 2001), it seems necessary that a repressor exists to allow cell differentiation. Our hypothesis is that a previously described Notch repressor, Numb, may be involved in this process.

Numb has been described as a blocker of the NICD nuclear translocation (Frise *et al.*, 1996; Guo *et al.*, 1996; Wakamatsu *et al.*, 1999) and an inducer of its endocytosis for degradation (Santolini *et al.*, 2000; Berdnik *et al.*, 2002). Furthermore, it has been shown that Numb overexpression promotes differentiation of skeletal muscle progenitors (Conboy *et al.*, 2002).

Previous studies revealed that, in the chick embryo, Numb is asymmetrically localized in the dermomyotome, being accumulated on the basal site of the epaxial lip cells and present uniformly in the myotome. Moreover, when cells divide asymmetrically in the epaxial lip Numb is asymmetrically inherited, which suggests that the $Numb^+$ daughter cell will be the myogenic committed one (Venters & Ordahl, 2005; Holowacz *et al.*, 2006). These studies revealed more about myogenic differentiation regulation, namely describing what appears to be a “key” pathway in the regulation of cell differentiation. Numb has also been implicated in other models and organisms as Drosophila, for example in neuronal differentiation (Petersen *et al.*, 2004; Ruiz Gómez & Bate, 1997; Wakamatsu *et al.*, 1999).

Considering our proposed model described above, we hypothesize that in the mouse embryo, Numb may be present in the apical side of dermomyotomal cells, being asymmetrically inherited in asymmetrical cell divisions at the epaxial lip. We used an anti-Numb antibody (a kind gift of Dr. Yoshio Wakamatsu), but the results were not clear, so our model is yet to be clarified (figure 28).

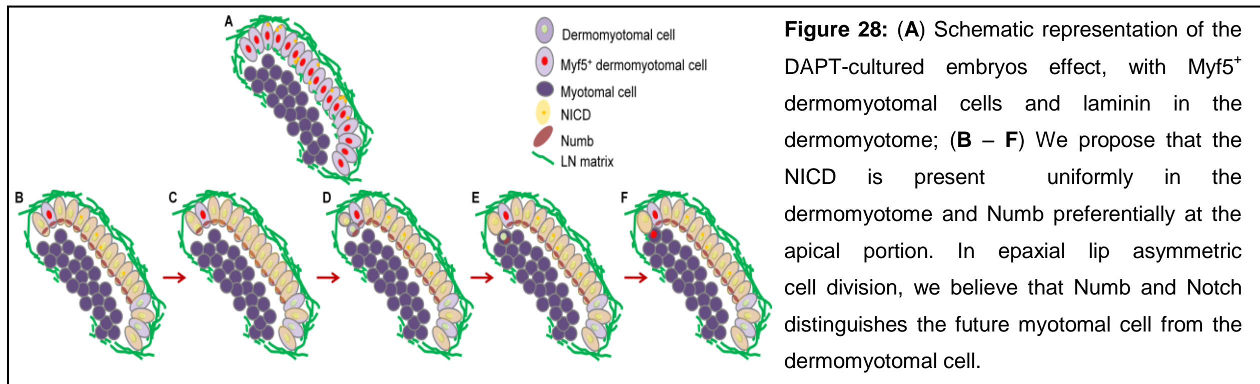


Figure 28: (**A**) Schematic representation of the DAPT-cultured embryos effect, with $Myf5^+$ dermomyotomal cells and laminin in the dermomyotome; (**B** – **F**) We propose that the NICD is present uniformly in the dermomyotome and Numb preferentially at the apical portion. In epaxial lip asymmetric cell division, we believe that Numb and Notch distinguishes the future myotomal cell from the dermomyotomal cell.

4.2 Fibronectin

Several studies approaching fibronectin influence in myogenesis have been performed, and most of them associate fibronectin to myoblast proliferation (von Der Mark & Ocalan, 1989; Grossi *et al.*, 2007). In quail myoblasts studies with α5 subunit ectopic expression showed increased proliferation and differentiation inhibition even in confluent cells, although they concluded that the surrounding environment (growth factors present,…) is important as well (Sastry *et al.*, 1996).

When C2C12 myoblasts were cultured on fibronectin pre-coated coverslips, cells acquired a more elongated shape and aligned with each other more that cells seeded on gelatin-coated coverslips. In what concerns myoblast differentiation, after maintaining C2C12 for several days in culture we saw no difference in MFRs expression (data not shown). Nevertheless, after 8 days of culture in growth medium, it seemed that more myotubes were present in fibronectin-coated coverslips than in the control. Therefore, we believe that fibronectin could

enhance cell alignment to prepare them for fusion, although no enhanced differentiation is observed.

When the nuclear shape was compared, we found that the nuclei of cells seeded on fibronectin were more elliptical than those of cells seeded on gelatin. Using the time-lapse image acquisition we found that fibronectin promotes cell migration and that cells on fibronectin have a more predictable path than cells seeded on gelatine. All these results made us hypothesize that fibronectin is responsible for early myoblasts alignment but also for cell migration.

We then investigated which integrins, from those which interact with fibronectin, were present in cells growing on fibronectin and could be responsible for this effect. Several studies have been performed to understand if fibronectin is important for the migratory behaviour. Depending on the cells and the experimental methodology used, in some cases α5 integrin subunit is not important for cell migration or invasiveness, in other cells the addition of anti-α5 or anti-β1 blocking antibodies do indeed affect cell migration (reviewed by Akiyama *et al.*, 1995; Truong & Danen, 2009). Our results revealed that early in culture α5 integrin subunit is present in cells growing on both a fibronectin and a gelatin matrix and that it is maintained throughout the culture time. Our hypothesis so far was that fibronectin, mediated by α5β1 integrin interaction, would make cells elongate and align with each other. This interaction would possibly remodel the actin cytoskeleton and increasing the N-cadherin in the membrane (Huttenlocher *et al.,* 1998). To test if α5β1 integrin was indeed important to the alignment, we used the RGD fragment known to block fibronectin-α5β1 integrin interaction (Takahashi *et al.*, 2007) on cells seeded on fibronectin pre-coated coverslips. Although RGD blocks fibronectin interaction with α5β1 integrin and αV integrin subunit, it is known that fibronectin has another binding site for αV (Takahashi *et al.*, 2007). The FN-α4β1 integrin interaction is not affected because this integrin binds to fibronectin in another domain of the ECM molecule (IIICS domain) (Sechler *et al.*, 2000). Therefore, by adding RGD to the culture medium, we are affecting only FN-α5β1 interaction. When we cultured cells on fibronectin with RGD at the moment of seeding, we noticed that only a few cells would effectively attach to the coverslip and those those that could attach were not elongated as the control cells. After 2 days of culture, we observed that those cells managed to proliferate but still they were not as aligned as those seeded with the control peptide. We believe that this could be due to a change in integrins that were expressed, so they could attach to the surface (fibronectin matrix) by other receptors. When we compared the nuclear Major/Minor ratios between them, we found a statistically significant difference between nuclei cultured with RGD and the control, with the first ones being more rounded that the control nuclei. In this experiment we also added RGD one day after culture, when cells were around 80% confluent, but in this case we did not perturb cell elongation. In fact, the nuclei of these cells were not different

from the control ones. When we seeded cells with RGD on gelatin, we found an interesting effect: cells would not attach at all (data not shown). The fact that gelatin is believed to capture the fibronectin molecules present in the culture medium seems to indicate that the cells need to bind to soluble fibronectin and then attach to the coverslip. The addition of RGD in the medium, inhibiting this interaction, would unable cells to attach and induce new matrix assembly. This model is in agreement with the fact that cells seeded on gelatin with DGR, can indeed attach and grow. We hypothesize that cells bind to the fibronectin molecules present in the culture medium serum, attach to the coverslips and, in the case of cells cultured on gelatin, produce and assemble a fibronectin matrix *de novo* or remodel the pre-existing matrix in the case of cell on a fibronectin matrix and eventually produce and assemble new matrix as well. In another experiment we wanted to see what happens to cells cultured on fibronectin with MNCD2, an N-cadherin blocking antibody (Linask *et al.,* 1998). When we seeded cells with this antibody, we noticed that cells were more polygonal than control cells. Even in confluency, they could not align as control cells. This result suggests that N-cadherin is important for the cell alignment/elongation, maybe mediating cell-cell contact that allows cells to align with each other. To have a more complete understanding of N-cadherin possible influence in cell migration, we should have performed time-lapse image acquisitions on cells seeded on fibronectin with MNCD2 and compared the parameters described above and look for possible differences.

When we gathered all these results, we drew a model where soluble fibronectin, present in the culture medium, mediated by α5β1 integrin interaction is initially necessary for cell attachment and a fibronectin matrix is important for inducing changes in cell shape. This may be happening by actin cytoskeleton remodeling and maybe increasing N-cadherin at the membrane, making cells align and promoting cell migration (figure 29).

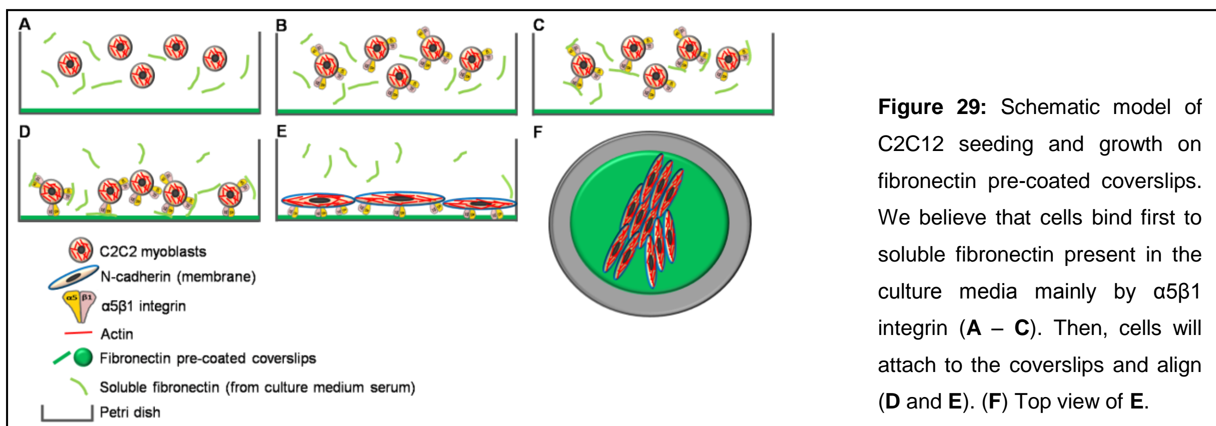


Figure 29: Schematic model of C2C12 seeding and growth on fibronectin pre-coated coverslips. We believe that cells bind first to soluble fibronectin present in the culture media mainly by α5β1 integrin (**A** – **C**). Then, cells will attach to the coverslips and align (**D** and **E**). (**F**) Top view of **E**.

Because fibronectin is present in the basal lamina of the dermomyotome and in the myotome and α5β1 in myotomal cells, we wanted to see if fibronectin is also involved in myoblast alignment in mouse embryos, as in C2C12 myoblasts. *In vivo*, when the myoblasts are

specified and move towards the medial portion of the myotome, they align along the antero-posterior axis. When we cultured E9.0 and E10.0 mouse embryos for 6 hours with both the 70kDa fragment and RGD, we could not see any difference in myoblasts orientation. This result is, in fact, not as surprising as it looked initially because myoblasts at this stage are surrounded by a complex matrix (like laminin, fibronectin, collagen and perlecan; Adams & Watt, 1993). We also cultured embryos in these developmental stages for 12 hours but 70kDa fragment-cultured embryos showed higher apoptosis levels so they were not used for analysis. Nevertheless, in embryos cultured 6 hours with the 70kDa fragment we observed that N-cadherin had lost its polarized location, *i.e.*, it would be present evenly in dermomyotomal cells instead of being restricted to the apical domain. Furthermore, immunostaining for ZO-1 showed that the localization of this tight junction protein was slightly perturbed although not as much as N-cadherin, because it is still present in the apical portion of the dermomyotomal but forming a more discontinuous line as compared to control embryos. This result is similar to the effect of the 70kDa fragment on chick somitogenesis, where the fragment inhibits new somites formation and has an effect on the distribution of N-cadherin (Martins *et al*, *in press*). The loss of N-cadherin polarization in the 70kDa cultured embryos may indicate that fibronectin is important for epithelia maintenance.

We then used mouse embryos in later stages of development, E12 and E12.5, cultured for 6 and 14 hours respectively, since it was previously reported that, at E12.5, trunk myocytes downregulate laminin-receptors and only express α1, α4, α5 and αV integrin subunits. It was suggested that fibronectin could be important for myocytes correct alignment for fusion, the following process in myogenesis (Cachaço *et al.*, 2005). As before, we could not find an effect in myocytes alignment. We analysed myotome-derived pre-muscle masses, those derived from dermomyotomal cells that differentiated into skeletal muscle precursors and occupied the myotome. In these cultures we could not find a visible difference in myocytes alignment and orientation as well. This could be due to the fact that we used the same concentration of the 70kDa fragment and RGD as in younger embryos which may by insufficient because E12.0 mouse embryos have a much more complex structure and cell density. In future experiments will definitely include the increase of the fragment and peptide concentration in E12.0 or E12.5 cultured embryos.

Nevertheless, when we cultured E9.0 for 12 hours with RGD, we detected in a few sections of each RGD-cultured embryo Myf5 protein in the dermomyotome. We hypothesize that fibronectin, mediated by α5β1 integrin interaction, may be important in the maintenance of undifferentiated dermomyotomal cells.

In summary, in this work we were able to unveil a little more about the extracellular matrix influence in myogenesis, namely in myogenic precursor cells determination or specification

and behaviour. Using C2C12 myoblasts we found that laminin does not enhance cell differentiation and *in vivo* we descibed that Notch signaling pathway inhibition leads to myogenic differentiation of dermomyotomal cells, seen by Myf5 expression. Now we hypothesize that Notch signaling may be interacting with laminin-α6β1 signaling, probably mediated by Numb. Furthermore, we found that α5β1 integrin, mediated by fibronectin interaction, may be important in myogenic differentiation, namely in the maintenance of undifferentiated dermomyotomal cells.

Fibronectin seems to be essential for C2C12 myoblasts alignment, mediated by α5β1 integrin interaction. Furthermore, this ECM molecule is important for myoblast migration. Although we did not detect any differences in MRFs expression, it seems that fibronectin, by making cells align, may be conferring cells the physical properties necessary to fuse, as we saw more myotubes on cells seeded on fibronectin than in cells seeded on laminin or gelatin, after 8 days of culture with growth medium. We also tried to see if fibronectin is important in similar process *in vivo*, in myoblasts and myocytes alignment and consequently fusion. Perturbing both fibronectin fibrillogenesis and fibronectin-α5β1 integrin interaction, we could not find an effect in cell alignment but we described that fibronectin is important for N-cadherin polarization and general epithelial maintenance, namely in the dermomyotome.

5. References

Adams, J. C., and Watt, F. M. (1993). Regulation of development and differentiation by the extracellular matrix. Development (Cambridge, England) 117, 1183-98.
Akiyama, S. K., Olden, K., and Yamada, K. M. (1995). Fibronectin and integrins in invasion and metastasis. Cancer metastasis reviews 14, 173-89.
Bajanca, F., Luz, M., Duxson, M. J., and Thorsteinsdóttir, S. (2004). Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Developmental dynamics : an official publication of the American Association of Anatomists 231, 402-15.
Bajanca, F., Luz, M., Raymond, K., Martins, G. G., Sonnenberg, A., Tajbakhsh, S., Buckingham, M., and Thorsteinsdóttir, S. (2006). Integrin alpha6beta1-laminin interactions regulate early myotome formation in the mouse embryo. Development (Cambridge, England) 133, 1635-44.
Berdnik, D., Török, T., González-Gaitán, M., and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Developmental cell 3, 221-31.
Bischoff, R., and Heintz, C. (1994). Enhancement of skeletal muscle regeneration. Developmental dynamics : an official publication of the American Association of Anatomists 201, 41-54.
Bober, E., Lyons, G. E., Braun, T., Cossu, G., Buckingham, M., and Arnold, H. H. (1991). The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. The Journal of cell biology 113, 1255-65.
Boettiger, D., Enomoto-Iwamoto, M., Yoon, H. Y., Hofer, U., Menko, A. S., and Chiquet-Ehrismann, R. (1995). Regulation of integrin alpha 5 beta 1 affinity during myogenic differentiation. Developmental biology 169, 261-72.
Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R., and Arnold, H. H. (1994). MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. Development (Cambridge, England) 120, 3083-92.
Brent, A. E., and Tabin, C. J. (2002). Developmental regulation of somite derivatives: muscle, cartilage and tendon. Current opinion in genetics & development 12, 548-57.
Buas, M. F., Kabak, S., and Kadesch, T. (2009). Inhibition of myogenesis by Notch: evidence for multiple pathways. Journal of cellular physiology 218, 84-93.
Buckingham, M. (2007). Skeletal muscle progenitor cells and the role of Pax genes. Comptes rendus biologies 330, 530-3.
Burattini, S., Ferri, P., Battistelli, M., Curci, R., Luchetti, F., and Falcieri, E. (2004). C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. European journal of histochemistry : EJH 48, 223-33.
Cachaço, A. S., Chuva De Sousa Lopes, S. M., Kuikman, I., Bajanca, F., Abe, K., Baudoin, C., Sonnenberg, A., Mummery, C. L., and Thorsteinsdóttir, S. (2003). Knock-in of integrin beta 1D affects primary but not secondary myogenesis in mice. Development (Cambridge, England) 130, 1659-71.
Cachaço, A. S., Pereira, C. S., Pardal, R. G., Bajanca, F., and Thorsteinsdóttir, S. (2005). Integrin repertoire on myogenic cells changes during the course of primary myogenesis in the mouse. Developmental dynamics : an official publication of the American Association of Anatomists 232, 1069-78.
Cheng, X., Huber, T. L., Chen, V. C., Gadue, P., and Keller, G. M. (2008). Numb mediates the interaction between Wnt and Notch to modulate primitive erythropoietic specification from the hemangioblast. Development (Cambridge, England) 135, 3447-58.
Christ, B., Schmidt, C., Huang, R., Wilting, J., and Brand-Saberi, B. (1998). Segmentation of the vertebrate body. Anatomy and embryology 197, 1-8.
Conboy, I. M., and Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Developmental cell 3, 397-409.
Conlon, R. a., Reaume, a. G., and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. Development (Cambridge, England) 121, 1533-45.
Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T., and Young, M. W. (1991). A role for the Drosophila neurogenic genes in mesoderm differentiation. Cell 67, 311-23.
Cossu, G. *et al.* (2000). Determination, diversification and multipotency of mammalian myogenic cells. The International journal of developmental biology 44, 699-706.
Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. Development (Cambridge, England) 122, 429-37.
Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996). How is myogenesis initiated in the embryo? Trends in genetics : TIG 12, 218-23.
Cossu, G., and Tajbakhsh, S. (2007). Oriented cell divisions and muscle satellite cell heterogeneity. Cell 129, 859-61.
Danen, E. H., and Sonnenberg, A. (2003). Integrins in regulation of tissue development and function. The Journal of pathology 201, 632-41.
Darribère, T., Skalski, M., Cousin, H. L., Gaultier, A., Montmory, C., and Alfandari, D. (2000). Integrins: regulators of embryogenesis. Biology of the cell / under the auspices of the European Cell Biology Organization 92, 5-25.
Delfini, M. C., Hirsinger, E., Pourquié, O., and Duprez, D. (2000). Delta 1-activated notch inhibits muscle differentiation without affecting Myf5 and Pax3 expression in chick limb myogenesis. Development (Cambridge, England) 127, 5213-24.
Denetclaw, W. F., Christ, B., and Ordahl, C. P. (1997). Location and growth of epaxial myotome precursor cells. Development (Cambridge, England) 124, 1601-10.
Denetclaw, W. F., and Ordahl, C. P. (2000). The growth of the dermomyotome and formation of early myotome lineages in thoracolumbar somites of chicken embryos. Development (Cambridge, England) 127, 893-905.
Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M., and Thiery, J. P. (1987). Adhesion molecules during somitogenesis in the avian embryo. The Journal of cell biology 104, 1361-74.
Frise, E., Knoblich, J. a., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996). The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. Proceedings of the National Academy of Sciences of the United States of America 93, 11925-32.
Giancotti, F. G., and Ruoslahti, E. (1999). Integrin signaling. Science (New York, N.Y.) 285, 1028-32.
Gibb, S., Zagorska, A., Melton, K., Tenin, G., Vacca, I., Trainor, P., Maroto, M., and Dale, J. K. (2009). Interfering with Wnt signalling alters the periodicity of the segmentation clock. Developmental biology 330, 21-31.
González-Mariscal, L., Betanzos, a., Nava, P., and Jaramillo, B. E. (2003). Tight junction proteins. Progress in biophysics and molecular biology 81, 1-44.

Grossi, A., Yadav, K., and Lawson, M. a. (2007). Mechanical stimulation increases proliferation, differentiation and protein expression in culture: stimulation effects are substrate dependent. Journal of biomechanics 40, 3354-62.
Grounds, M. D., and McGeachie, J. K. (1987). A model of myogenesis in vivo, derived from detailed autoradiographic studies of regenerating skeletal muscle, challenges the concept of quantal mitosis. Cell and tissue research 250, 563-9.
Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17, 27-41.
Hashimoto, J., Kariya, Y., and Miyazaki, K. (2006). Regulation of proliferation and chondrogenic differentiation of human mesenchymal stem cells by laminin-5 (laminin-332). Stem cells (Dayton, Ohio) 24, 2346-54.
Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature 364, 501-6.
Hayashi, Y. *et al.* (2007). Integrins regulate mouse embryonic stem cell self-renewal. Stem cells (Dayton, Ohio) 25, 3005-15.
Hinterberger, T. J., Sassoon, D. A., Rhodes, S. J., and Konieczny, S. F. (1991). Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. Developmental biology 147, 144-56.
Hirsinger, E., Malapert, P., Dubrulle, J., Delfini, M. C., Duprez, D., Henrique, D., Ish-Horowicz, D., and Pourquié, O. (2001). Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. Development (Cambridge, England) 128, 107-16.
Holowacz, T., Zeng, L., and Lassar, A. B. (2006). Asymmetric localization of numb in the chick somite and the influence of myogenic signals. Developmental dynamics : an official publication of the American Association of Anatomists 235, 633-45.
Huttenlocher, A., Lakonishok, M., Kinder, M., Wu, S., Truong, T., Knudsen, K. A., and Horwitz, A. F. (1998). Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. The Journal of cell biology 141, 515-26.
Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11-25.
Hynes, R. O. (1996). Targeted mutations in cell adhesion genes: what have we learned from them? Developmental biology 180, 402-12.
Jaffredo, T., Horwitz, a. F., Buck, C. a., Rong, P. M., and Dieterlen-Lievre, F. (1988). Myoblast migration specifically inhibited in the chick embryo by grafted CSAT hybridoma cells secreting an anti-integrin antibody. Development (Cambridge, England) 103, 431-46.
Jones, J. L., and Walker, R. a. (1999). Integrins: a role as cell signalling molecules. Molecular pathology : MP 52, 208-13.
Jostes, B., Walther, C., and Gruss, P. (1990). The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. Mechanisms of development 33, 27-37.
Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S. J., and Rudnicki, M. A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development (Cambridge, England) 124, 4729-38.
Kassar-Duchossoy, L., Giacone, E., Gayraud-Morel, B., Jory, A., Gomès, D., and Tajbakhsh, S. (2005). Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes & development 19, 1426-31.
Kaufman, S. J., and Foster, R. F. (1988). Replicating myoblasts express a muscle-specific phenotype. Proceedings of the National Academy of Sciences of the United States of America 85, 9606-10.
Kopan, R., Nye, J. S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development (Cambridge, England) 120, 2385-96.
Kopan, R., Schroeter, E. H., Weintraub, H., and Nye, J. S. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. Proceedings of the National Academy of Sciences of the United States of America 93, 1683-8.
Kuang, S., and Rudnicki, M. A. (2008). The emerging biology of satellite cells and their therapeutic potential. Trends in molecular medicine 14, 82-91.
Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. The Journal of biological chemistry 274, 7238-44.
Lathia, J. D., Rao, M. S., Mattson, M. P., and Ffrench-Constant, C. (2007). The microenvironment of the embryonic neural stem cell: lessons from adult niches? Developmental dynamics : an official publication of the American Association of Anatomists 236, 3267-82.
Linask, K. K., Ludwig, C., Han, M. D., Liu, X., Radice, G. L., and Knudsen, K. A. (1998). N-cadherin/catenin-mediated morphoregulation of somite formation. Developmental biology 202, 85-102.
Linask, K. K., Ludwig, C., Han, M. D., Liu, X., Radice, G. L., and Knudsen, K. a. (1998). N-cadherin/catenin-mediated morphoregulation of somite formation. Developmental biology 202, 85-102.
Lyons, G. E., Ontell, M., Cox, R., Sassoon, D., and Buckingham, M. (1990). The expression of myosin genes in developing skeletal muscle in the mouse embryo. The Journal of cell biology 111, 1465-76.
Mao, Y., and Schwarzbauer, J. E. (2005). Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. Matrix biology : journal of the International Society for Matrix Biology 24, 389-99.
Mayer, U., Saher, G., Fässler, R., Bornemann, A., Echtermeyer, F., von Der Mark, H., Miosge, N., Pöschl, E., and von Der Mark, K. (1997). Absence of integrin alpha 7 causes a novel form of muscular dystrophy. Nature genetics 17, 318-23.
McKeown-Longo, P. J., and Mosher, D. F. (1985). Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. The Journal of cell biology 100, 364-74.
Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., and Nonaka, I. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature 364, 532-5.
Nofziger, D., Miyamoto, A., Lyons, K. M., and Weinmaster, G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development (Cambridge, England) 126, 1689-702.
Ordahl, C. P., and Le Douarin, N. M. (1992). Two myogenic lineages within the developing somite. Development (Cambridge, England) 114, 339-53.
Ott, M. O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development (Cambridge, England) 111, 1097-107.
Parker, M. H., Seale, P., and Rudnicki, M. A. (2003). Looking back to the embryo: defining transcriptional networks in adult myogenesis. Nature reviews. Genetics 4, 497-507.
Patton, B. L. (2000). Laminins of the neuromuscular system. Microscopy research and technique 51, 247-61.
Paulin, D., and Li, Z. (2004). Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. Experimental cell research 301, 1-7.
Petersen, P. H., Zou, K., Krauss, S., and Zhong, W. (2004). Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. Nature neuroscience 7, 803-11.
Pourquié, O. (2001). Vertebrate somitogenesis. Annual review of cell and developmental biology 17, 311-50.

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435, 948-53.

Rifes, P., Carvalho, L., Lopes, C., Andrade, R. P., Rodrigues, G., Palmeirim, I., and Thorsteinsdóttir, S. (2007). Redefining the role of ectoderm in somitogenesis: a player in the formation of the fibronectin matrix of presomitic mesoderm. Development (Cambridge, England) 134, 3155-65.

Roegiers, F., and Jan, Y. N. (2004). Asymmetric cell division. Current opinion in cell biology 16, 195-205.

Rosen, G. D., Sanes, J. R., LaChance, R., Cunningham, J. M., Roman, J., and Dean, D. C. (1992). Roles for the integrin VLA-4 and its counter receptor VCAM-1 in myogenesis. Cell 69, 1107-19.

Ruiz Gómez, M., and Bate, M. (1997). Segregation of myogenic lineages in Drosophila requires numb. Development (Cambridge, England) 124, 4857-66.

Santolini, E., Puri, C., Salcini, A. E., Gagliani, M. C., Pelicci, P. G., Tacchetti, C., and Di Fiore, P. P. (2000). Numb is an endocytic protein. The Journal of cell biology 151, 1345-52.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. Genes & Development 6, 2620-2634.

Sastry, S. K., Lakonishok, M., Thomas, D. a., Muschler, J., and Horwitz, a. F. (1996). Integrin alpha subunit ratios, cytoplasmic domains, and growth factor synergy regulate muscle proliferation and differentiation. The Journal of cell biology 133, 169-84.

Schaart, G., Viebahn, C., Langmann, W., and Ramaekers, F. (1989). Desmin and titin expression in early postimplantation mouse embryos. Development (Cambridge, England) 107, 585-96.

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. Developmental biology 175, 84-94.

Schultz, E., Gibson, M. C., and Champion, T. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. The Journal of experimental zoology 206, 451-6.

Schultz, E., and McCormick, K. M. (1994). Skeletal muscle satellite cells. Reviews of physiology, biochemistry and pharmacology 123, 213-57.

Schwarzbauer, J. (1999). Basement membranes: Putting up the barriers. Current biology : CB 9, R242-4.

Schwarzbauer, J. E., and Sechler, J. L. (1999). Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. Current opinion in cell biology 11, 622-7.

Seale, P., Asakura, A., and Rudnicki, M. A. (2001). The potential of muscle stem cells. Developmental cell 1, 333-42.

Sechler, J. L., Cumiskey, a. M., Gazzola, D. M., and Schwarzbauer, J. E. (2000). A novel RGD-independent fibronectin assembly pathway initiated by alpha4beta1 integrin binding to the alternatively spliced V region. Journal of cell science 113 (Pt 8, 1491-8.

Shawber, C., Nofziger, D., Hsieh, J. J., Lindsell, C., Bögler, O., Hayward, D., and Weinmaster, G. (1996). Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. Development (Cambridge, England) 122, 3765-73.

Smith, T. H., Kachinsky, A. M., and Miller, J. B. (1994). Somite subdomains, muscle cell origins, and the four muscle regulatory factor proteins. The Journal of cell biology 127, 95-105.

Sonnenberg, A., Janssen, H., Hogervorst, F., Calafat, J., and Hilgers, J. (1987). A complex of platelet glycoproteins Ic and IIa identified by a rat monoclonal antibody. The Journal of biological chemistry 262, 10376-83.

Sunada, Y., and Yamadas, Y. (1994). Deficiency of Merosin in Dystrophic. Control.

Takahashi, S. *et al.* (2007). The RGD motif in fibronectin is essential for development but dispensable for fibril assembly. The Journal of cell biology 178, 167-78.

Taverna, D., Disatnik, M. H., Rayburn, H., Bronson, R. T., Yang, J., Rando, T. a., and Hynes, R. O. (1998). Dystrophic muscle in mice chimeric for expression of alpha5 integrin. The Journal of cell biology 143, 849-59.

Truong, H., and Danen, E. H. (2009). Integrin switching modulates adhesion dynamics and cell migration. Cell adhesion & migration 3, 179-81.

Venters, S. J., and Ordahl, C. P. (2005). Asymmetric cell divisions are concentrated in the dermomyotome dorsomedial lip during epaxial primary myotome morphogenesis. Anatomy and embryology 209, 449-60.

Wakamatsu, Y., Maynard, T. M., Jones, S. U., and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. Neuron 23, 71-81.

Wallin, J., Wilting, J., Koseki, H., Fritsch, R., Christ, B., and Balling, R. (1994). The role of Pax-1 in axial skeleton development. Development (Cambridge, England) 120, 1109-21.

Weber, J. M., and Calvi, L. M. (2009). Notch signaling and the bone marrow hematopoietic stem cell niche. Bone.

Williams, B. A., and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development (Cambridge, England) 120, 785-96.

Yurchenco, P. D., Amenta, P. S., and Patton, B. L. (2004). Basement membrane assembly, stability and activities observed through a developmental lens. Matrix biology : journal of the International Society for Matrix Biology 22, 521-38.

Yurchenco, P. D., and Wadsworth, W. G. (2004). Assembly and tissue functions of early embryonic laminins and netrins. Current opinion in cell biology 16, 572-9.

Yusuf, F., and Brand-Saberi, B. (2006). The eventful somite: patterning, fate determination and cell division in the somite. Anatomy and embryology 211 Suppl, 21-30.

Zagris, N. (2001). Extracellular matrix in development of the early embryo. Micron (Oxford, England : 1993) 32, 427-38.

Zagris, N., Chung, a. E., and Stavridis, V. (2000). Differential expression of laminin genes in early chick embryo. The International journal of developmental biology 44, 815-8.

Zammit, P. S., Partridge, T. a., and Yablonka-Reuveni, Z. (2006). The skeletal muscle satellite cell: the stem cell that came in from the cold. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 54, 1177-91.

van der Flier, A., and Sonnenberg, A. (2001). Function and interactions of integrins. Cell and Tissue Research 305, 285-298.

von Der Mark, K., and Ocalan, M. (1989). Antagonistic effects of laminin and fibronectin on the expression of the myogenic phenotype. Differentiation; research in biological diversity 40, 150-7.

6. Appendix

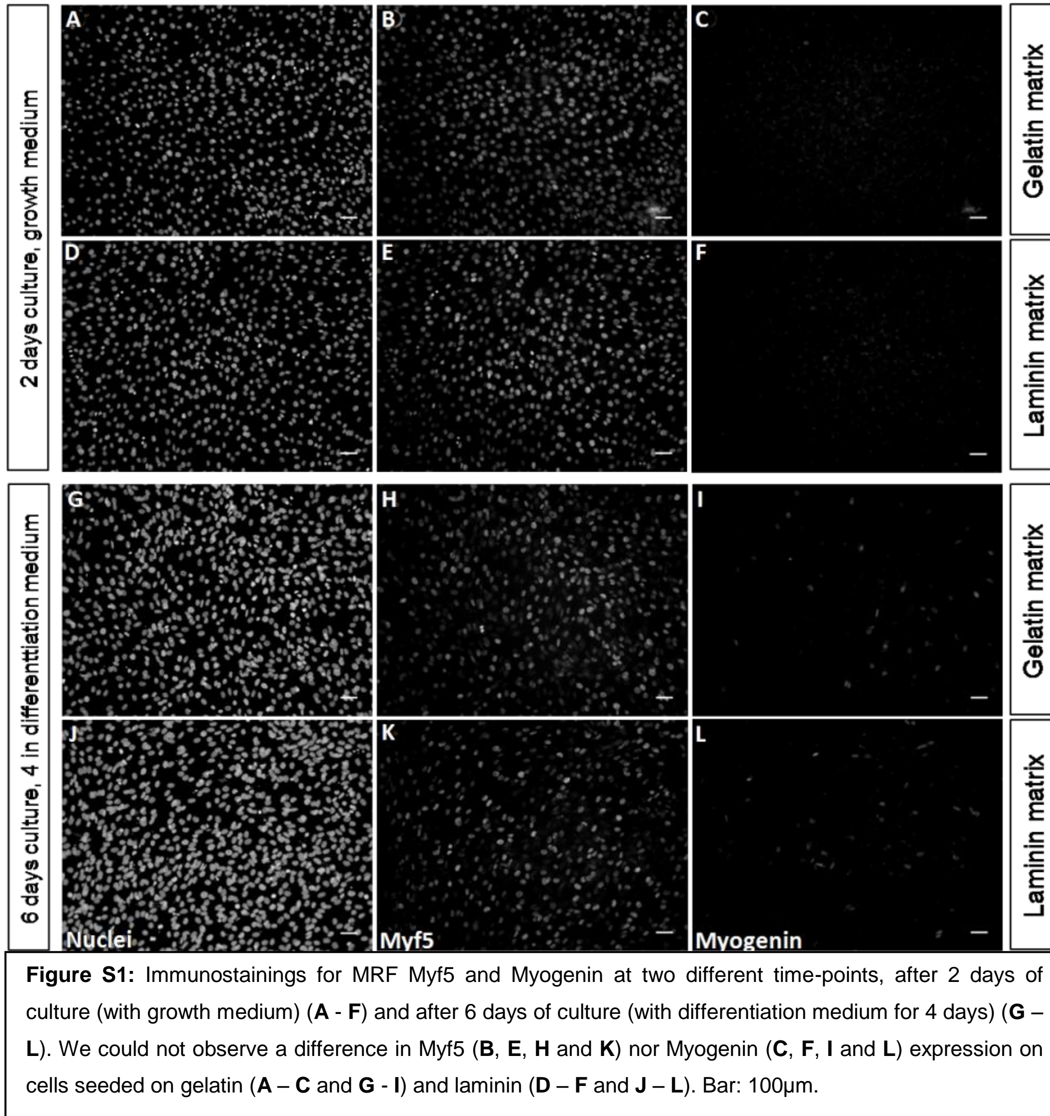


Figure S1: Immunostainings for MRF Myf5 and Myogenin at two different time-points, after 2 days of culture (with growth medium) (**A** - **F**) and after 6 days of culture (with differentiation medium for 4 days) (**G** – **L**). We could not observe a difference in Myf5 (**B**, **E**, **H** and **K**) nor Myogenin (**C**, **F**, **I** and **L**) expression on cells seeded on gelatin (**A** – **C** and **G** - **I**) and laminin (**D** – **F** and **J** – **L**). Bar: 100μm.

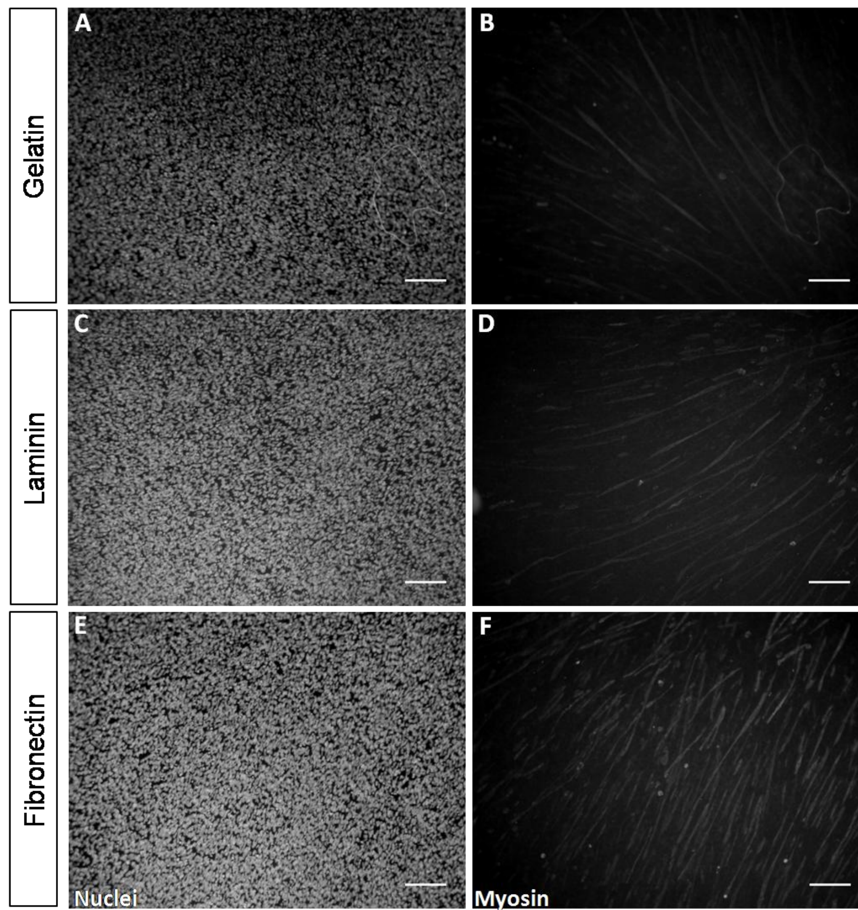


Figure S2: C2C12 myoblasts maintained for 8 days with growth medium on gelatin (**A** – **B**), laminin (**C** – **D**) or fibronectin (**E** – **F**) immunostained for Myosin (**B**, **D** and **F**). No significant difference was observed in myotubes, although cells seeded on fibronectin appear to have more myotubes. Bar: 200µm.

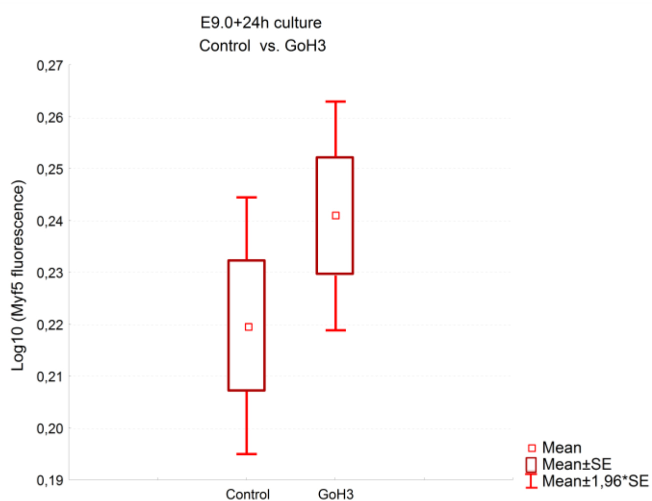


Figure S3: Graphic representation of Log_{10}-transformed dermomyotome Myf5 fluorescence of E9.0 mouse embryos cultured for 24 hours with GoH3. No statistically significant difference is observed between culture conditions.

Supplementary video 1: Time-lapse image acquisition of cells seeded on gelatin, starting at subconfluency (less than 60% confluency), coupled with the cell tracks (showed the last 20 time-points).

Supplementary video 2: Time-lapse image acquisition of cells seeded on gelatin, starting at confluency (more than 90% confluency), coupled with the cell tracks (showed the last 20 time-points).

Supplementary video 3: Time-lapse image acquisition of cells seeded on fibronectin, starting at subconfluency (less than 60% confluency), coupled with the cell tracks (showed the last 20 time-points).

Supplementary video 4: Time-lapse image acquisition of cells seeded on fibronectin, starting at confluency (more than 90% confluency), coupled with the cell tracks (showed the last 20 time-points).

Matrix	Confluency	Track	Track length (µm)	Displacement (µm)	ARMean
FN	Subconfluent	Track 1	425,78	136,77	0,50
FN	Subconfluent	Track 2	454,43	51,36	0,53
FN	Subconfluent	Track 3	401,21	105,35	0,54
FN	Subconfluent	Track 4	562,92	156,24	0,57
FN	Subconfluent	Track 5	551,27	88,91	0,57
FN	Subconfluent	Track 6	432,66	142,76	0,46
FN	Subconfluent	Track 7	444,38	37,78	0,45
FN	Subconfluent	Track 8	589,52	171,70	0,52
FN	Subconfluent	Track 9	799,68	113,18	0,55
FN	Subconfluent	Track 10	432,59	152,94	0,49
FN	Subconfluent	Track 11	440,29	182,22	0,53
FN	Subconfluent	Track 12	478,17	154,96	0,48
FN	Subconfluent	Track 13	465,58	237,13	0,49
FN	Subconfluent	Track 14	519,53	200,64	0,52
FN	Subconfluent	Track 15	547,36	46,12	0,52
FN	Confluent	Track 1	408,71	31,20	0,52
FN	Confluent	Track 2	549,70	103,83	0,53
FN	Confluent	Track 3	641,74	363,62	0,47
FN	Confluent	Track 4	672,24	78,78	0,55
FN	Confluent	Track 5	626,88	261,66	0,44
FN	Confluent	Track 6	442,92	186,37	0,36
FN	Confluent	Track 7	509,74	118,97	0,49
FN	Confluent	Track 8	742,78	279,21	0,57
FN	Confluent	Track 9	687,59	110,26	0,51
FN	Confluent	Track 10	278,83	97,46	0,50
FN	Confluent	Track 11	565,19	111,45	0,54
FN	Confluent	Track 12	733,67	265,08	0,38
FN	Confluent	Track 13	665,93	73,58	0,56
FN	Confluent	Track 14	819,43	438,62	0,53
FN	Confluent	Track 15	802,49	90,86	0,55
Gel	Subconfluent	Track 1	410,96	165,01	0,48
Gel	Subconfluent	Track 2	314,85	61,04	0,41
Gel	Subconfluent	Track 3	323,79	82,74	0,37
Gel	Subconfluent	Track 4	360,87	89,19	0,39

Gel	Subconfluent	Track 5	401,38	53,29	0,50
Gel	Subconfluent	Track 6	363,64	121,16	0,26
Gel	Subconfluent	Track 7	407,79	54,29	0,44
Gel	Subconfluent	Track 8	495,40	65,59	0,47
Gel	Subconfluent	Track 9	474,06	19,58	0,38
Gel	Subconfluent	Track 10	493,91	144,95	0,04
Gel	Subconfluent	Track 11	434,44	146,95	0,51
Gel	Subconfluent	Track 12	515,17	141,33	0,52
Gel	Subconfluent	Track 13	509,47	98,08	0,50
Gel	Subconfluent	Track 14	488,46	94,71	0,54
Gel	Subconfluent	Track 15	347,87	42,49	0,41
Gel	Confluent	Track 1	669,26	89,43	0,17
Gel	Confluent	Track 2	532,91	244,81	0,38
Gel	Confluent	Track 3	555,83	95,29	0,56
Gel	Confluent	Track 4	419,10	24,92	0,39
Gel	Confluent	Track 5	495,22	88,33	0,41
Gel	Confluent	Track 6	501,38	8,69	0,45
Gel	Confluent	Track 7	405,95	108,28	0,29
Gel	Confluent	Track 8	288,30	83,22	0,51
Gel	Confluent	Track 9	618,77	88,29	0,48
Gel	Confluent	Track 10	332,67	104,36	0,54
Gel	Confluent	Track 11	425,55	157,60	0,52
Gel	Confluent	Track 12	405,51	111,38	0,44
Gel	Confluent	Track 13	572,47	156,15	0,38
Gel	Confluent	Track 14	684,08	197,88	0,53
Gel	Confluent	Track 15	403,67	121,56	0,48

Supplementary table1: Description of track length, displacement and ARMean measurements for all tracks.

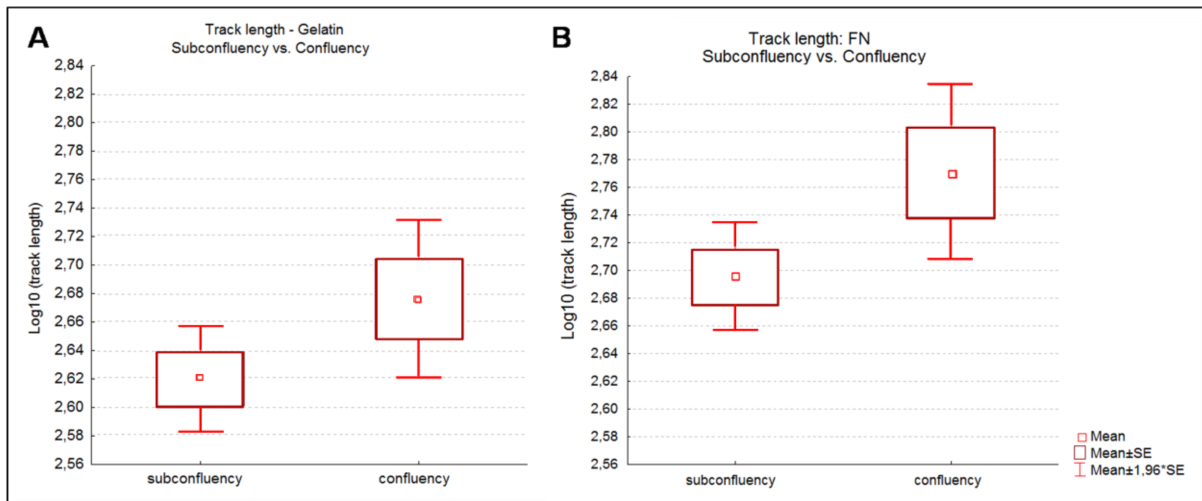


Figure S4: Graphic representation of subconfluency-confluency comparisons of Log_{10}-transformed track lengths of cells seeded on gelatin (**A**) on fibronectin (**B**). No statistically significant difference was observed between them ($t=1.65$, $n_{subconfluency}=15$, $n_{confluency}=15$, $p=0.111$ and $t=1.996$, $n_{subconfluency}=15$, $n_{confluency}=15$, $p=0.056$, respectively).

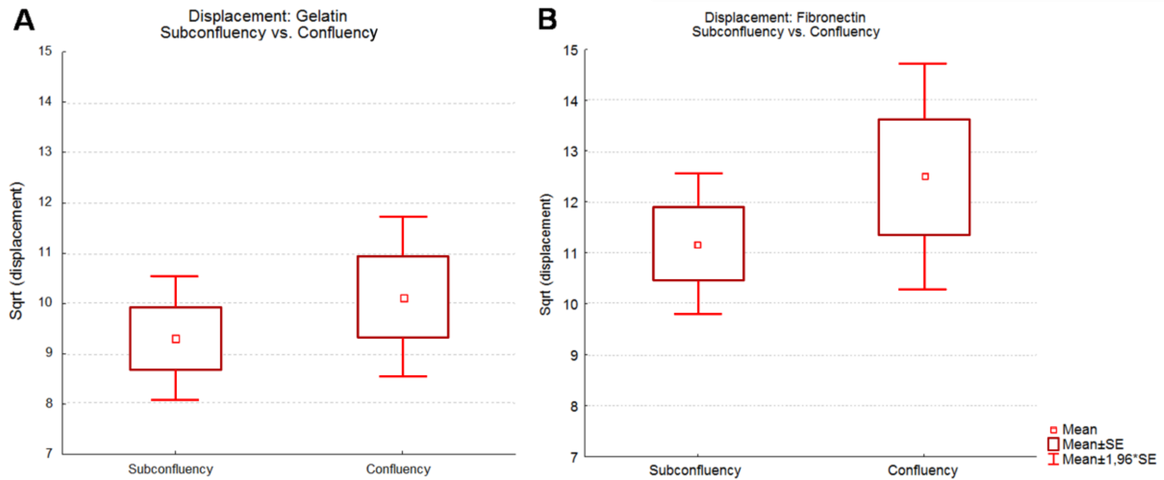


Figure S5: Graphic representation of subconfluency-confluency comparisons of cell displacements of cells seeded on gelatin (**A**) on fibronectin (**B**). No significant difference was observed between square root-transformed displacements (t=0.811, $n_{subconfluency}$=15, $n_{confluency}$=15, p=0.424 and t=0.992, $n_{subconfluency}$=15, $n_{confluency}$=15, p=0.33, respectively).

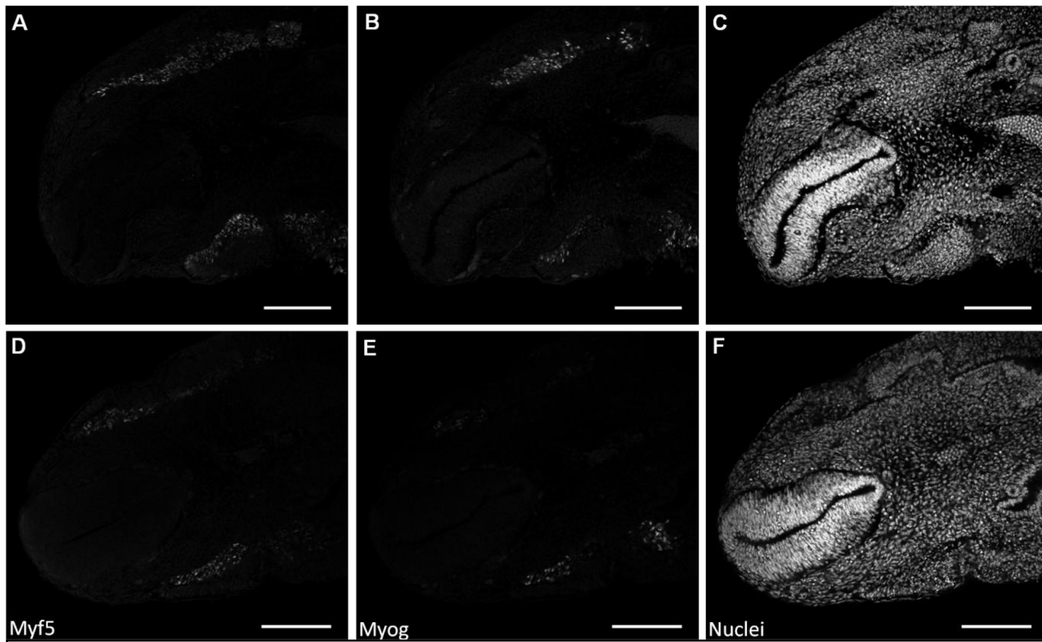


Figure S6: E10.0 embryos stained for Myf5 (**A** and **D**) and Myogenin (Myog; **B** and **E**) cultured with 70kDa fragment (**D**-**F**) show no difference in myoblast alignment when compared to the control embryos (**A**-**C**). Bar: 100μm.

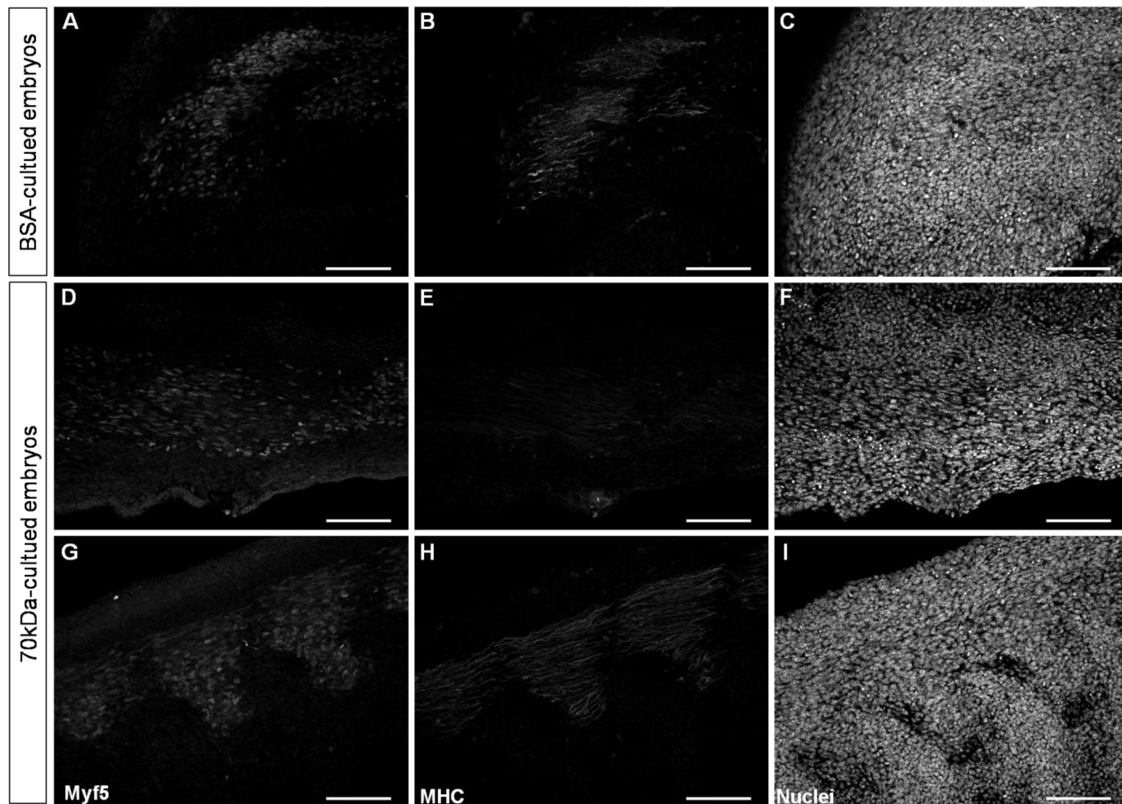


Figure S7: E12.0 embryos cultured with BSA (control; **A**-**C**) or the 70kDa fragment (**D**-**I**). When compared inter-vertebrae myocytes location and alignment by Myf5 (**A**, **D** and **G**) and MHC (**B**, **E** and **H**) immnunostaining, we did not detected any difference. Bar: 100μm.