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The timing of emergence of muscle progenitors is controlled by an FGF/ERK/SNAIL1 pathway

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ABSTRACT

In amniotes, the dermomyotome is the source of all skeletal muscles of the trunk and the limbs. Trunk skeletal muscles form in two sequential stages: in the first stage, cells located at the four borders of the epithelial dermomyotome delaminate to generate the primary myotome, composed of post-mitotic, mononucleated myocytes. The epithelio-mesenchymal transition (EMT) of the central dermomyotome initiates the second stage of muscle formation, characterised by a massive entry of mitotic muscle progenitors from the central region of the dermomyotome into the primary myotome. The signals that regulate the timing of the dermomyotome EMT are unknown. Here, we propose that this process is regulated by an FGF signal emanating from the primary myotome, a known source of FGF. The over-expression of FGF results in a precocious EMT of the dermomyotome, while on the contrary, the inhibition of FGF signalling by the electoporation of a dominant-negative form of FGFR4 delays this process. Within the dermomyotome, FGF signalling triggers a MAPK/ERK pathway that leads to the activation of the transcription factor Snail1, a known regulator of EMT in a number of cellular contexts. The activation or the inhibition of the MAPK/ERK pathway and of Snail1 mimics that of FGF signalling and leads to an early or delayed EMT of the dermomyotome, respectively. Altogether, our results indicate that in amniotes, the primary myotome is an organizing center that regulates the timely entry of embryonic muscle progenitors within the muscle masses, thus initiating the growth phase of the trunk skeletal muscles.

Introduction

In vertebrates, all skeletal muscles of the body derive from the dorsal epithelial part of the somites, named the dermomyotome. The dermomyotome is a transient structure formed by a central epithelial sheet delimited by four contiguous lips (Christ and Ordahl, 1995). The trunk skeletal muscle morphogenesis occurs in two sequential stages. In a first stage, cells arising from the four epithelial borders of the dermomyotome generate differentiated, post-mitotic myocytes that organize into the primitive skeletal muscle (named the primary myotome) located beneath the dermomyotome (Cinnamon et al., 1999; Gros et al., 2004; Kahane et al., 1998, 2002). Myocytes utilize the evolutionary conserved Planar Cell Polarity pathway to elongate parallel to the embryonic axis (Gros et al., 2009). During the second stage of muscle morphogenesis, the central portion of the dermomyotome undergoes an epithelial-to-mesenchymal transition (EMT), initiated in the trunk region 36 h after somites have formed. This triggers the invasion of the primary myotome by a population of muscle progenitors (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Manceau et al., 2008). Long-term lineage analyses show that satellite cells, the major muscle stem cells of the adult, derive from the same dermomyotomal population (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). Recent evidence suggests that myocytes that initially form impose their organization on those that appear later (Gros et al., 2009; Kahane et al., 2007). This indicates that the primary myotome may serve as a scaffold on which muscle progenitor-derived myocytes organize. It is therefore crucial that progenitors enter the myotomal compartment in a timely manner, once the primary myotome formation is well under way. How is the timing of the EMT regulated is unknown.

Embryonic manipulations have shown that both the ectoderm and the dorsal neural tube can promote the induction or the maintenance of the epithelial structure of the dermomyotome (Christ et al., 1992; Dietrich et al., 1997; Fan et al., 1997; Fan and Tessier-Lavigne, 1994; Kenny-Mobbs and Thorogood, 1987; Spence et al., 1996). Wnt6 in the ectoderm was shown to maintain the epithelial structure of the

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dermomyotome, through Frizzled7 and a βcatenin-dependent signalling pathway that controls the transcription of Paraxis, a well known player in somite epithelialization (Linker et al., 2005; Schmidt et al., 2004). The expression of Wnt6 coincides with the epithelial phase of the dermomyotome and the subsequent downregulation of its transcription corresponds to the time of central dermomyotome dissociation (Geetha-Loganathan et al., 2006; Linker et al., 2005). Although it is now well established that signals from the ectoderm maintain the epithelial structure of the dermomyotome, it is unclear whether the EMT of the central dermomyotome is only due to the lack of these maintenance signals or if it is also triggered by positive signals.

A variety of signalling molecules have been shown to regulate the EMT of epithelial tissues, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPS), WNTs and Notch (Barrallo-Gimeno and Nieto, 2005; De Craene et al., 2005). The EMT of the lateral dermomyotome, that allows the migration of muscle progenitors into the forming limb, is initiated by FGF signalling from the limb mesenchyme that regulates SF/HGF in this tissue. SF/HGF activates its cognate tyrosine kinase receptor Met tyrosine in the lateral dermomyotome (Bladt et al. 1995; Heymann et al., 1996; Brand-Saberi et al., 1996; Dietrich et al., 1999). The central dermomyotome does not express Met (Yang et al., 1996) and it is therefore unlikely that this mechanism is responsible for its EMT. However, FGF signalling components are expressed in the chick embryo at time and places compatible with a role in the EMT of the central dermomyotome. Several FGF family members are expressed by the myotome and two FGF receptors (FGFR1 and FGFR4) are expressed by the dermomyotome (Brent and Tabin, 2004; Kahane et al., 2001). In addition, the phosphorylated form of MAPK/ERK, one of the downstream effector of the FGF signalling pathway, is present in the dermomyotome (Brent and Tabin, 2004).

The SNAIL zinc finger transcriptional repressors, SNAIL1 (formerly SNAIL) and SNAIL2 (formerly SLUG), have been shown to control the EMT of epithelia in vertebrate and invertebrate development and during metastatic progression of cancers (Barrallo-Gimeno and Nieto, 2005). SNAIL proteins are able to repress genes coding for proteins associated with an epithelial phenotype such as E-cadherin or desmosomal proteins and can activate the expression of mesenchymal markers (Batlle et al., 2000; Cano et al., 2000; Savagner et al., 1997). In the developing vertebrate embryo, Snail family genes are required in several morphogenetic processes such as gastrulation (Ciruna and Rossant, 2001; Nieto et al., 1994), the emigration of the neural crest from the neural tube (Nieto et al., 1994) or somitogenesis (Dale et al., 2006). There is a striking interchange in both expression patterns and functions of Snail1 and Snail2 between chick and mice embryos in several tissues (Locascio et al., 2002; Sefton et al., 1998). However, in somites, the distribution of Snail1 and Snail2 transcripts are conserved in chick and mouse embryos: Snail1 is expressed in the myotome and sclerotome, while Snail2 transcripts are only observed in migratory trunk neural crest cells (Sefton et al., 1998). The expression of Snail1 at the developmental stages when the EMT of the dermomyotome and the emergence of the muscle progenitor population take place has not been examined.

In this study, we have investigated the molecular mechanisms that trigger the EMT of the central dermomyotome by gain and loss of function experiments in the chick embryo, using the technique of somite electroporation. Our data indicate that the FGFs secreted by the primary myotome might be key signals that trigger the EMT of the dermomyotome by controlling the activation of MAPK/ERK with no significant influence on the proliferation of dermomyotome cells. This in turn increases the transcription of Snail1 in the dermomyotome, resulting in the initiation of its EMT. We thus propose that the primary myotome might act as an organizing center that controls the timing of the entry of the muscle progenitors within the primary myotome, thus allowing the growth phase of trunk skeletal muscles to be initiated.

Materials and methods

Chick embryos, phenotype analyses

Fertilized chick eggs were obtained from a commercial source (EARL Morizeau, Dangers, France) and incubated at 38 °C in a humidified incubator. Embryos were staged according to the developmental table of Hamburger and Hamilton (1992) or according to days of incubation. The timing of the central dermomyotome EMT is highly reproducibly taking place 36 h after somite formation (Gros et al., 2005). Its epithelial structure can be easily observed with adherens junction markers, such as N-cadherin or F-Actin, that were used here. To demonstrate a delayed EMT, we examined manipulated embryos at developmental stages at which the EMT should have already started (after 36 h). On the contrary, to demonstrate a precocious EMT, we examined developmental stages at which the EMT should not have yet taken place (before 36 h). In each section, the non-electroporated side serves as control.

In situ hybridization and tissue sections

Whole-mount *in situ* hybridizations on chick embryos were performed as described (Henrique et al., 1995). The probes that were used are: a chicken Snail1 probe (Sefton et al., 1998, a generous gift from A. Nieto); EST probes (from BBSRC ChickEST database; Boardman et al., 2002) for chicken FGF3 (745 bp ChEST 812G6); a cSprouty 1 probe (1500 bp fragment, a generous gift from Gail Martin; Minowada et al., 1999); and a cErm probe (Brent and Tabin, 2004). Embryos were embedded in a gelatine/sucrose solution and sectioned at 20 μm using a Cryostat (Leica CM 3050S).

Plasmids and in ovo electroporation

Newly formed somites were electroporated as previously described (Gros et al., 2005; Scaal et al., 2004). The electrodes were positioned so that only the dorsal portion of interlimb somites, which gives rise to the central region of the dermomyotome, was electroporated. The electroporated plasmids co-express the gene of interest together with a GFP or RFP fluorescent reporter. Thus, the specificity of the targeting can be determined under UV examination a few hours after manipulation. Only those embryos where the dorsal portion of the dermomyotome was targeted were kept for further analyses. Chicken Snail1 cDNA, FGF8 cDNA, and the DNA for the fusion protein FGFR4-Fc "FGFR4-TM" (the extracellular and the transmembranal domains of FGFR4 (nt 1 to 930) introduced in-frame, upstream of the Fc region of human IgG1) were sub-cloned into an electroporation vector, which drives a cytoplasmic form of the GFP (pCAGGS-IRES-EGFP). Constitutively active MAPK kinase 1 (MKK1) (MKK1ca) and dominant-negative MKK1 (MKK1dn) DNA were cloned into an electroporation vector (PCIG), which drives a nuclear form of GFP (Delfini et al., 2005). A dominant-negative form of Snail1 was constructed by fusion of the Snail1 DNA binding domain to the transcription activator domain of VP16 (VP16-Snail1). We utilized the RNA interference technology to inhibit Snail1, using the pRFPRNAiC vector, which contains an RFP reporter gene (Das et al., 2006). This vector contained two siRNAs in tandem. This somewhat minimizes the off-target effects that would occur if only one siRNA was used. The two 22 nucleotide-long target sequences (GGAAATCCTTCAGCTGCAAGTA and GGCCTTTGCTGACC-GTTCTAAT) were chosen using the design tool "siRNA Target Finder" at www.genscript.com/ssl-bin/app/rnai. We verified that the electroporation of the siRNA resulted in an efficient downregulation of the Snail1 transcript. An unrelated siRNA (directed luciferase) had no effect on Snail1 transcription or on the EMT of the dermomyotome. Such procedure has been recently followed to address the roles of other molecules in somites (Gros et al., 2009). Embryos were analyzed 10 to 48 h after electroporation. All DNA constructs were injected at a

final concentration of around 1 μg/μl. At this concentration, electroporations with control vectors containing only GFP or RFP do not change the dermomyotome cell fate, and the EMT occurs at the same time in control, non-electroporated, contralateral somites (Supplementary Figs. 2B–D).

Cell transfection and injection

Chicken embryonic fibroblast cell line UMNSAH/DF-1 (Himly et al., 1998) were transfected with the FGF8 DNA expressing vector, using the FuGene Transfection Reagent (Roche, Meylan, France) according to manufacturer's instructions. One day after transfection, control or FGF8 transfected cells were injected into the somitocoele of newly formed epithelial somites at interlimb level. Injections were performed using a Picospritzer pressure injector (General Valve Corporation) and glass needles. These embryos were analyzed after overnight incubation.

Trunk section cultures

Trunks (including thoracic and limb levels) of HH stage 20 chick embryos were sectioned transversally (450 μm) using a tissue chopper and were cultured in a medium (L-15, 10% FBS, 1% pen/strep) with 50 μM SU5402 (Calbiochem, dissolved in DMSO) or an equivalent amount of DMSO. Following 6 h of incubation, trunk slices were fixed in 4% formaldehyde and processed for whole-mount *in situ* hybridization.

Antibodies, immunohistochemistry and confocal analysis

Phalloidin Alexa fluor 546 or Oregon green 514 (Molecular Probes) were used to detect F-Actin. For immunohistochemistry on sections, the following antibodies were used: chicken polyclonal antibody against GFP (Abcam); mouse monoclonal antibodies against Pax7 (1:10) (Hybridoma Bank), Light Meromyosin MF20 (1:10) (Hybridoma Bank), β-catenin (1/100) (Transduction Laboratories), N-cadherin (1:100) (C2542 sigma); rabbit polyclonal antibody against dpERK (1:10) (phospho p44–p42) (9101, Cell Signalling Technology). Cell nuclei were stained with Dapi (Calbiochem). Detection of proliferating cells in vivo was done using BrdU (Sigma) incorporation, applied for 1 h to embryos, 10 h after electroporation. Stained sections were examined using a Zeiss LSM 510 Meta confocal microscope.

Results

FGF signal triggers central dermomyotome EMT

Members of the FGF signalling family are expressed during mouse and chick somite differentiation. The expression of FGF4, 8, 13 and 19 has been reported in the myotome (Niswander and Martin, 1992; Karabagli et al., 2002; Mahmood et al., 1995; Gimeno and Martinez, 2007), and FGFR1 and FGFR4 transcripts have been observed in the dermomyotome (Marcelle et al., 1994; Orr-Urtreger et al., 1991; Stark et al., 1991; Brent and Tabin, 2004; Kahane et al., 2001). We have re-examined their expression patterns in the chick embryo specifically at the time when the dermomyotome undergoes its EMT. In the interlimb region, the EMT of the dermomyotome starts around stage HH20-21 (Gros et al., 2005), i.e. 36 h after somites have formed. The expression of FGF4, 8 and 19 in the primary myotome was confirmed (not shown). We have observed that an additional member of the FGF family, FGF3 is also expressed in this structure (Supplementary Figs. 1A and B). We also confirmed that FGFR1 and FGFR4 transcripts are expressed in the central dermomyotome at the time of its EMT (not shown). Interestingly, the expression of Sprouty1, that serves as an indicator of FGF signalling activity (Hacohen et al., 1998; Hanafusa et al., 2002), was activated in the central dermomyotome concomitantly with the initiation of its EMT (Supplementary Figs. 1C). Because the primary myotome is the main known source of FGFs in the vicinity of the central dermomyotome at this stage of development, the activation of Sprouty1 in this structure suggests that the dermomyotome responds to FGFs secreted by myocytes (Supplementary Fig. 1D).

We then tested what the action of FGF signalling may be on dermomyotome cells through a gain-of-function approach. This was done by electroporating a plasmid coding for cFGF8 in the central dermomyotome or by injection of FGF8-transfected cells into the nascent myotome. The epithelial structure of dermomyotome cells was analyzed using epithelial markers such as N-cadherin, β-catenin or F-actin (Cinnamon et al., 2006; Gros et al., 2005). One day after electroporation, the dermomyotome EMT has not yet started on the control side, where all dermomyotomal cells are epithelial with a strong apical β-catenin localization (Figs. 1A, B). In contrast, the over-expression of FGF resulted in an extensive EMT of the dermomyotome where cells displayed a typical mesenchymal-like morphology with no apical β-catenin localization (Figs. 1A, C). A precocious EMT was observed as early as 12 h after electroporation, i.e. 24 h before the dermomyotome normally undergoes an EMT. However, we did not systematically analyze whether an EMT could be observed earlier than 12 h after electroporation. The over-expression of FGF8 in somites at the interlimb level led to the induction of an ectopic limb bud in the flank (arrowhead in Fig. 1A), a result that has been already described after FGF bead implantation experiments (Isaac et al., 2000). The injection of Fgf8-transfected cells in somites also induced a precocious EMT (data not shown), showing that dermomyotome cells can respond to an external source of FGF. The electroporation with control vectors or injection of cells expressing GFP only did not affect the timing of the dermomyotome EMT (Supplementary Figs. 2B–D). These experiments indicate that FGF signalling promotes the EMT of the dermomyotome.

To test whether the effect of FGF signalling on dermomyotome (i.e. triggering of EMT) is not due to a modulation of cell proliferation, we analyzed the incorporation of BrdU in FGF8-electroporated embryos. This analysis was done 10 h after electroporation of cFGF8 in the dermomyotome. At this stage, the dermomyotome is a highly proliferative structure. We did not observe any significant difference in BrdU labeling (Suppl. Figs. 3 A–F), indicating that FGF signalling does not modify dermomyotome proliferation.

To determine whether FGF signalling is not only sufficient, but also necessary for the EMT, we used a vector expressing a dominant-negative transmembranal FGFR4 (FGFR4-TM) lacking its intracellular tyrosine kinase domain (Marcelle et al., 1994). By heterodimerizing with heterologous FGF receptors expressed in dermomyotome cells, such construct should block the signalling of all FGF receptors in this tissue (Bellot et al., 1991; Ueno et al., 1992). Embryos were analyzed 1.5 days after electroporation, when the dermomyotome EMT has already started on the control side (Figs. 1D, F). The electroporation of FGFR4-TM resulted in a delay in the induction of the EMT of the dermomyotome (Figs. 1E, G). While most of the electroporated cells remained within the dermomyotome, GFP-positive cells were sometimes observed within the myotome. We believe this is due to a limitation of the electroporation technique. In the best cases, 30–50% of dermomyotome cells are electroporated (Gros et al., 2005). Thus, transfected cells are typically interspersed by wild type cells that normally undergo an EMT, occasionally carrying along electroporated cells within the myotome.

Altogether, the gain and loss of function experiments described above allow us to propose that FGFs secreted by the primary myotome controls the timing of the central dermomyotome EMT.

MAPK/ERK1/2 signalling pathway mediates the effect of FGF on the dermomyotome EMT

FGF signalling activates several intracellular effectors, including MAPK/ERK (Bottcher and Niehrs, 2005). In chick somites, phosphorylated MAPK/ERK is observed in the dorsal sclerotome, the myotome

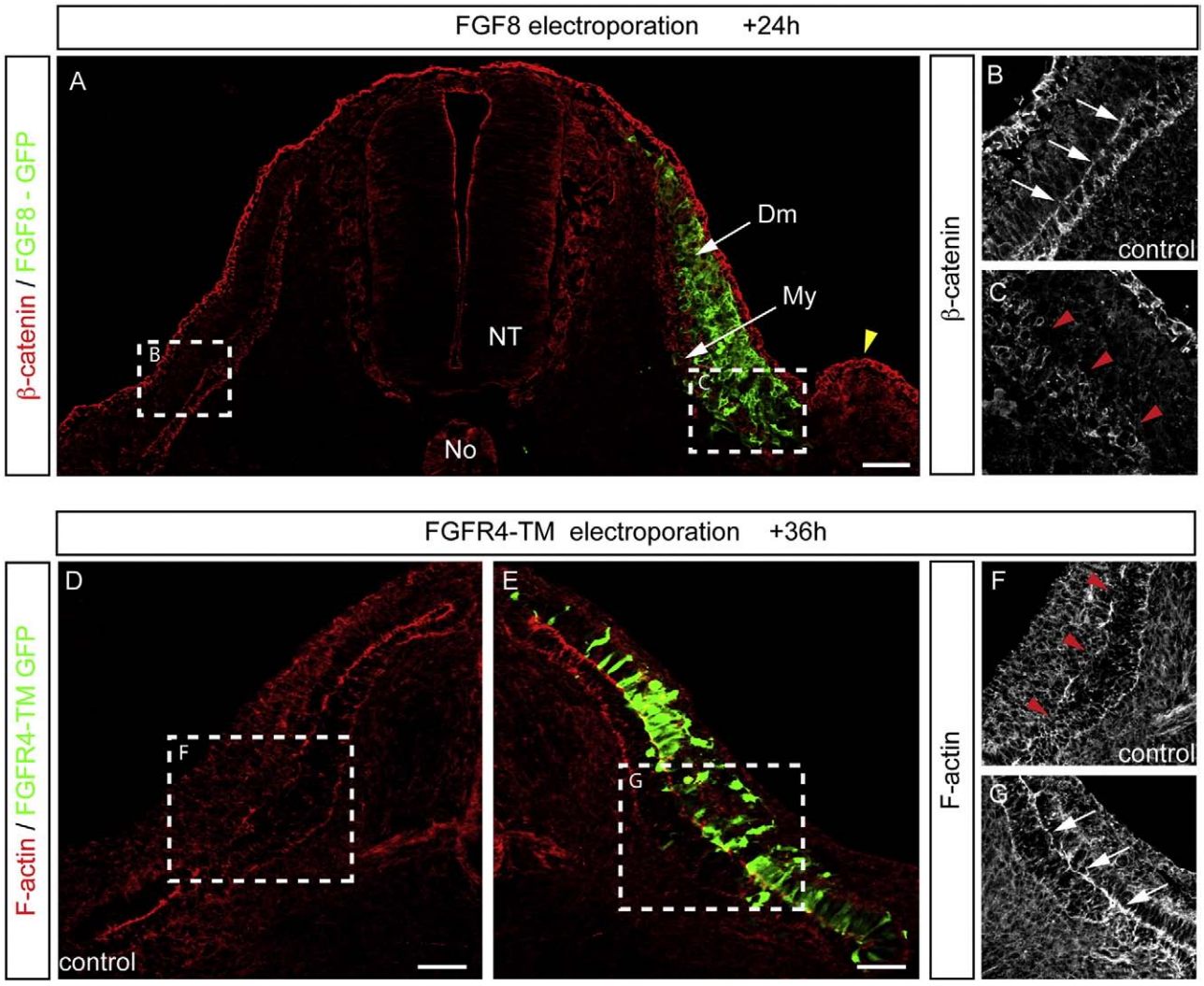
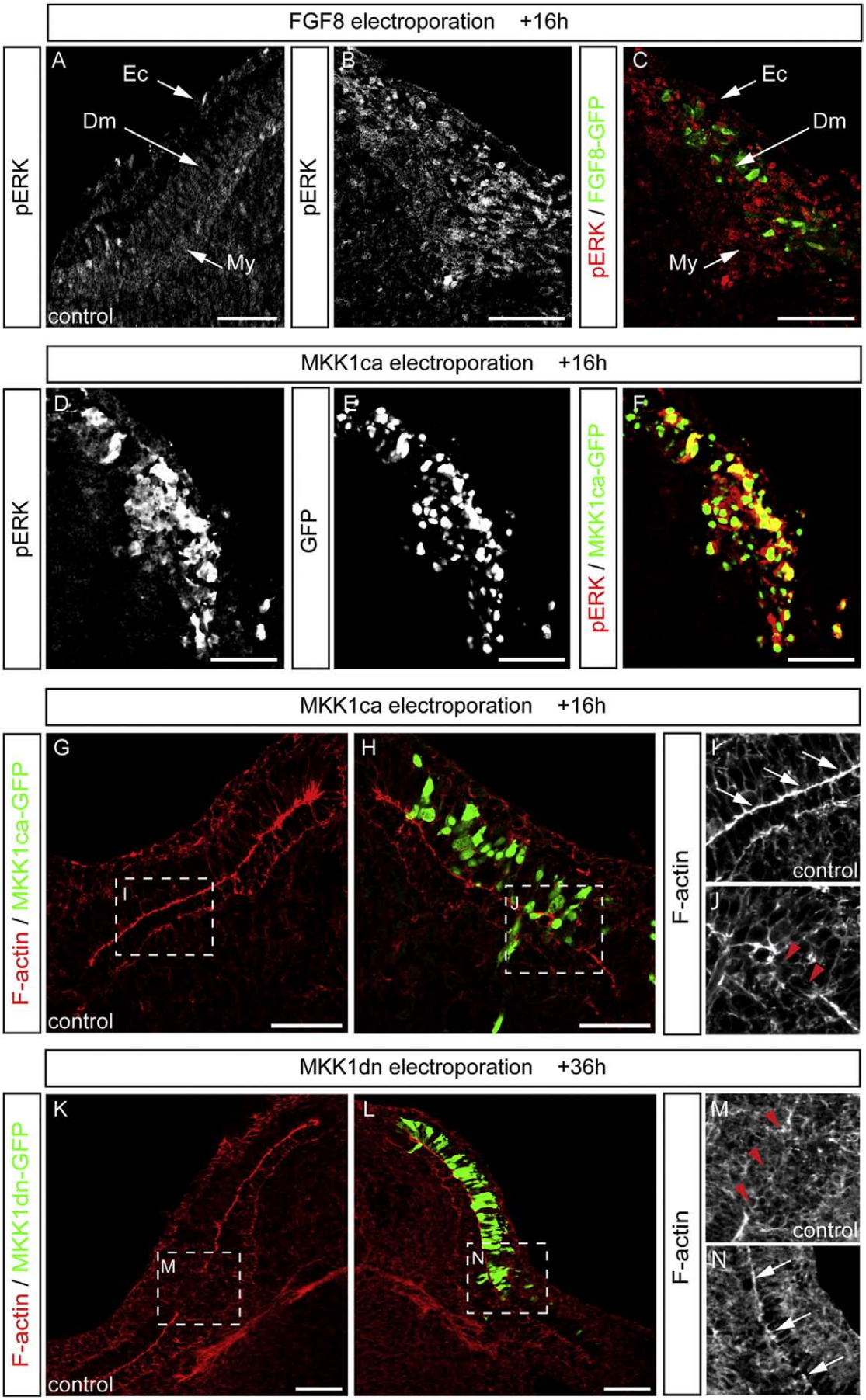


Fig. 1. FGF signalling regulates the dermomyotome EMT. (A–C) Confocal view of a transversal section of a chick embryo electroporated at HH17 in the dorsal portion of interlimb somites with a pCAGGS FGF8-iresGFP expression vector. One day after electroporation, the endogenous EMT has not yet started on the control side and the adherens junction marker β-catenin is localized at the apical end of dermomyotome cells (A and arrows in B). The over-expression of FGF8 (A, in green) results in an extensive EMT ($n = 16/16$), characterized by a loss of β-catenin at the apical end of dermomyotome cells (A and arrowheads in C). Arrowhead in A indicates the induction of an extra limb bud at the site of FGF8 over-expression. The section is stained for β-catenin (red (A)/white (B, C)) and GFP (green). (D–G) Confocal view of a transversal section of a chick embryo electroporated at HH17 at the interlimb level with an expression vector coding for a dominant-negative form of FGFR4 (FGFR4-TM-iresGFP), observed 1.5 day after electroporation. The EMT has started on the control side, as shown by a loss of actin staining at the apical ends of dermomyotome cells (D and arrowheads in F). On the electroporated side, all cells remain epithelial (in green, E and arrows in G; $n = 5/9$). Sections are stained for F-actin (in red (D, E) or white (F, G)) and GFP (green). NT, neural tube; Dm, dermomyotome; No, notochord; My, myotome. Scale bar: 50 μm.

and the dermomyotome (Brent and Tabin, 2004). To test whether MAPK/ERK1/2 might be an effector of the FGF pathway regulating the EMT of the dermomyotome, we first examined whether FGF8 can activate the phosphorylation of ERK in the central dermomyotome. One day after electroporation of FGF8 in the dorsal somite, immunohistochemistry on transversal sections labelled with the anti-phospho ERK antibody revealed a significant activation of ERK phosphorylation in the dermomyotome in and around the FGF8 electroporated cells (Figs. 2B, C), compared to the control side (Fig. 2A), indicating that FGF8 can activate non-cell-autonomously the ERK pathway in the dermomyotome.

We then investigated whether the modulation of the MAPK/ERK intracellular effectors of FGF signaling mimics the gain and loss of function of FGF and of its putative receptors at the membrane. We first electroporated a constitutively active form of MKK1 (MKK1ca) in the dorsal somite (Delfini et al., 2005; Mansour et al., 1994). MKK1 (also called MEK1) is a MAP kinase kinase that phosphorylates and thus activates MAPK/ERK (Johnson and Lapadat, 2002). As expected, 1 day

Fig. 2. A MAPK/ERK1/2 signalling pathway mediates the effect of FGF on the dermomyotome EMT. (A–F) Transversal sections of chick somites stained with antibodies directed against a phosphorylated form of ERK (white (A, B, D)/red (C,F)) or against GFP (white (E)/green (C, F)) 16 h after over-expression of FGF8 (leading to a non-cell-autonomous increase of ERK phosphorylation) (A–C; $n = 3/3$) or of a constitutively active form of MKK1 (leading to a cell-autonomous increase of ERK phosphorylation) (MKK1ca, D–F; $n = 3/3$) in interlimb somites. (G–J) Transversal sections of chick somite stained for F-actin (red (G, H))/white (I, J)) and GFP (green) 16 h after dorsal electroporation of MKK1ca, i.e. before the beginning of the endogenous EMT. While on the control, un-electroporated side, the dermomyotome is entirely epithelial (G and arrows in I), the over-expression of MKK1ca induces a de-epithelialisation of cells, as indicated by the loss of apical actin staining (H and arrowheads in J; $n = 8/8$). (K–N) Transversal sections of chick somite stained for F-actin (red (K, L) or white (M, N)) and GFP (green), 36 h after electroporation of the dorsal somites with a dominant-negative form of MKK1 (MKK1dn), just after the initiation of the EMT on the un-electroporated control side (K and arrowheads in M). The over-expression of MKK1dn delays the EMT of electroporated somites (L and arrows in N; $n = 6/10$). Ec, Ectoderm; Dm, dermomyotome; My, myotome. Scale bar: 50 μm.


FGF8 electroporation +16h
pERK
A
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Dm
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control
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pERK / FGF8-GFP
C
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MKK1ca electroporation +16h
pERK
D
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pERK / MKK1ca-GFP
F
MKK1ca electroporation +16h
F-actin / MKK1ca-GFP
G
control
H
F-actin
I
control
J
MKK1dn electroporation +36h
F-actin / MKK1dn-GFP
K
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control
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F-actin
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after electroporation, ERK phosphorylation was upregulated in a cell-autonomous manner in dermomyotome cells expressing MKK1ca (Figs. 2D–F). This prematurely triggered the EMT of the dermomyotome (Figs. 2G–J), while blocking the pathway by electroporating a dominant-negative form of MKK1 (MKK1dn; Delfini et al., 2005; Mansour et al., 1994) delayed the EMT of the dermomyotome (Figs. 2K–N). These results phenocopy the data obtained after FGF8 or FGFR4-TM electroporations, respectively.

Although dermomyotome cells expressing MKK1ca or MKK1dn de-epithelialize precociously or with a delay, respectively, they maintain the expression of the dermomyotome marker Pax7 (Supplementary Figs. 2E–H), indicating that their fate is not grossly altered, and they are still able to colonize both the subectodermal space and the myotome, as they would do at later stages in normal conditions (Cinnamon et al., 2006; Gros et al., 2005).

Altogether, these data suggest that the myotomal-FGF signal that might control the timing of the central dermomyotome EMT is mediated by the MAPK/ERK pathway.

Snail1 mediates FGF/ERK signaling in the dermomyotome

In early chick embryos, Snail1 but not Snail2 is expressed in somites (Sefton et al., 1998). We examined whether Snail1 is expressed at developmental stages when the central dermomyotome undergoes an EMT. We found that at the time of the EMT (HH20), Snail1 transcript is detected in a salt-and-pepper expression pattern in the central dermomyotome (Figs. 3A, B), as well as in the myotome and sclerotome.

Snail1 is regulated transcriptionally and post-transcriptionally in different cellular contexts by several signalling pathways, including the FGF pathway (Barrallo-Gimeno and Nieto, 2005; De Craene et al., 2005; Dominguez et al., 2003). Coherent with this, the electroporation of FGF8 and MKK1ca, which induce a precocious EMT (see above), resulted in an upregulation of Snail1 transcription (Figs. 3C–F). On the contrary, blocking FGF pathway using the FGF inhibitor SU5402 leads to a downregulation of Snail1 in the dermomyotome (Supplementary Fig. 4). These observations are compatible with a role of Snail1 downstream of FGF/ERK signalling, during dermomyotome EMT.

To determine whether Snail1 itself is able to induce dermomyotomal EMT, we over-expressed chick Snail1 in the central dermomyotome of interlimb somites. Embryos were examined 1 day after electroporation, when the dermomyotome EMT has not yet started on the control side (Figs. 4A, C). In contrast, Snail1-electroporated dermomyotomal cells displayed a typical mesenchymal-like morphology with no apical N-cadherin localization, indicating that these cells had initiated an EMT (Figs. 4B, D). The electroporated dermomyotomal cells colonized both the subectodermal space and the myotome (Supplementary Fig. 2J), as they do in normal conditions (Cinnamon et al., 2006; Gros et al., 2005), showing that Snail1 over-expression triggers the EMT of the dermomyotome without altering cell migration.

We modulated Snail1 activity with a fusion construct of VP16 and Snail1, in which the DNA binding domain of Snail1 was fused to the transcription activator domain of VP16 (Mayor et al., 2000). This chimeric protein acts as a dominant-negative for endogenous Snail. Thirty six hours after electroporation of this construct, at a time when EMT is well under way on the control side of the embryo (Figs. 4E, G), the electroporated dermomyotome displayed an epithelial morphology (Figs. 4F, H).

We also used the RNA interference technology to inhibit Snail1 function, by electroporating a vector expressing two siRNA directed against Snail1 within the dermomyotome ((Das et al., 2006) see Materials and methods). We tested the efficiency of this strategy by *in situ* hybridization. One day after electroporation, dermomyotome cells transfected with siRNA directed against Snail1 showed a strong downregulation of Snail1 transcripts (Figs. 4I, K). We then examined whether the dermomyotome morphology is affected by siRNA-mediated reduction of Snail1 expression. Embryos were analyzed 1.5 days after electroporation, when the central dermomyotome EMT had already started on the control side (Figs. 4L, N). Analysis of epithelial markers in Snail1 siRNA-electroporated dermomyotome cells showed that those cells retained the apical epithelial marker localization compared to the control side, indicating that the EMT was delayed confirming the results obtained with VP16/Snail1 (Figs. 4M, O). The electroporation with a siRNA control, directed against the luciferase, did not affect the timing of the dermomyotome EMT (data not shown). The expression pattern of Snail1, together with the gain and loss of function experiments, suggest that Snail1, downstream of

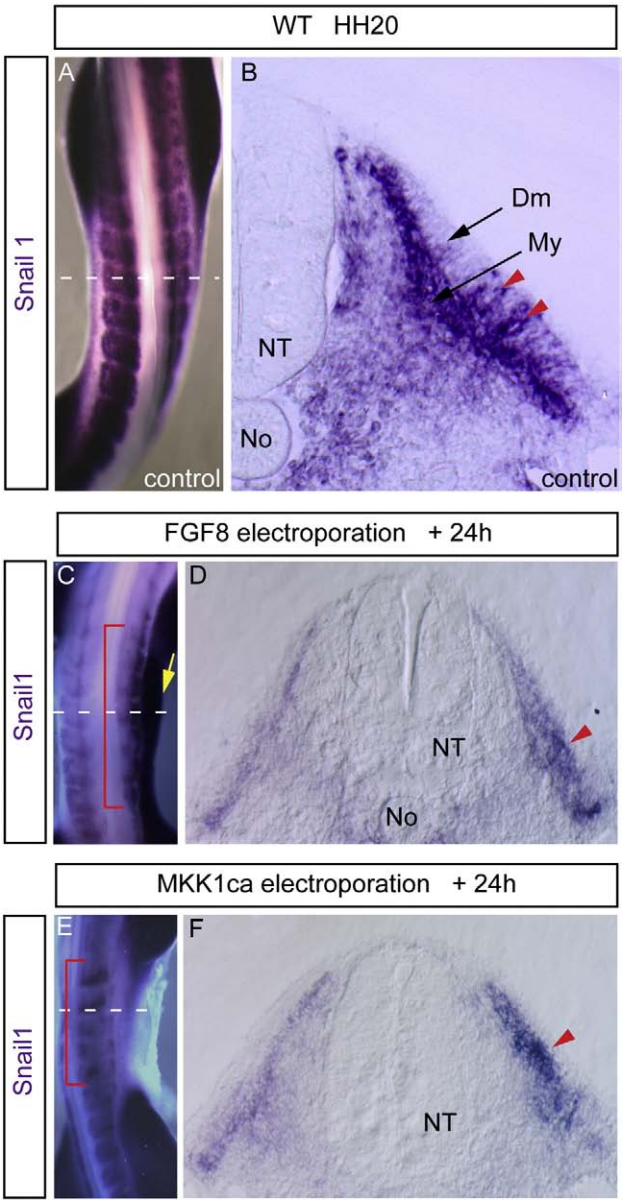

Fig. 3. Snail1 is positively regulated by FGF/ERK signaling in the dermomyotome. (A) Dorsal view at the interlimb level of a whole-mount *in situ* hybridization for Snail1 performed on a HH20 chick embryo, i.e. just before the dermomyotome EMT has been initiated. (B) Transversal section at the interlimb level of the embryo shown in (A), showing the expression of Snail1 in the dermomyotome (arrowheads in B). (C–F). Dorsal view at the interlimb level of whole-mount *in situ* hybridization for Snail1 in HH21 chick embryo electroporated with FGF8 (pCAGGS-FGF8-iresGFP; C; $n=4/4$) or MKK1ca (pCIG-MKK1ca, E; $n=4/4$), in the dorsal somites at HH17. The embryos were fixed 1 day after electroporation. D and F are transversal sections of C and E, respectively. Both FGF8 and MKK1ca over-expression lead to an activation of Snail1 transcription (brackets in C, E, arrowheads in D, F). Yellow arrow shows ectopic limb bud in the flank. NT, neural tube; Dm, dermomyotome; My, myotome; No, Notochord.

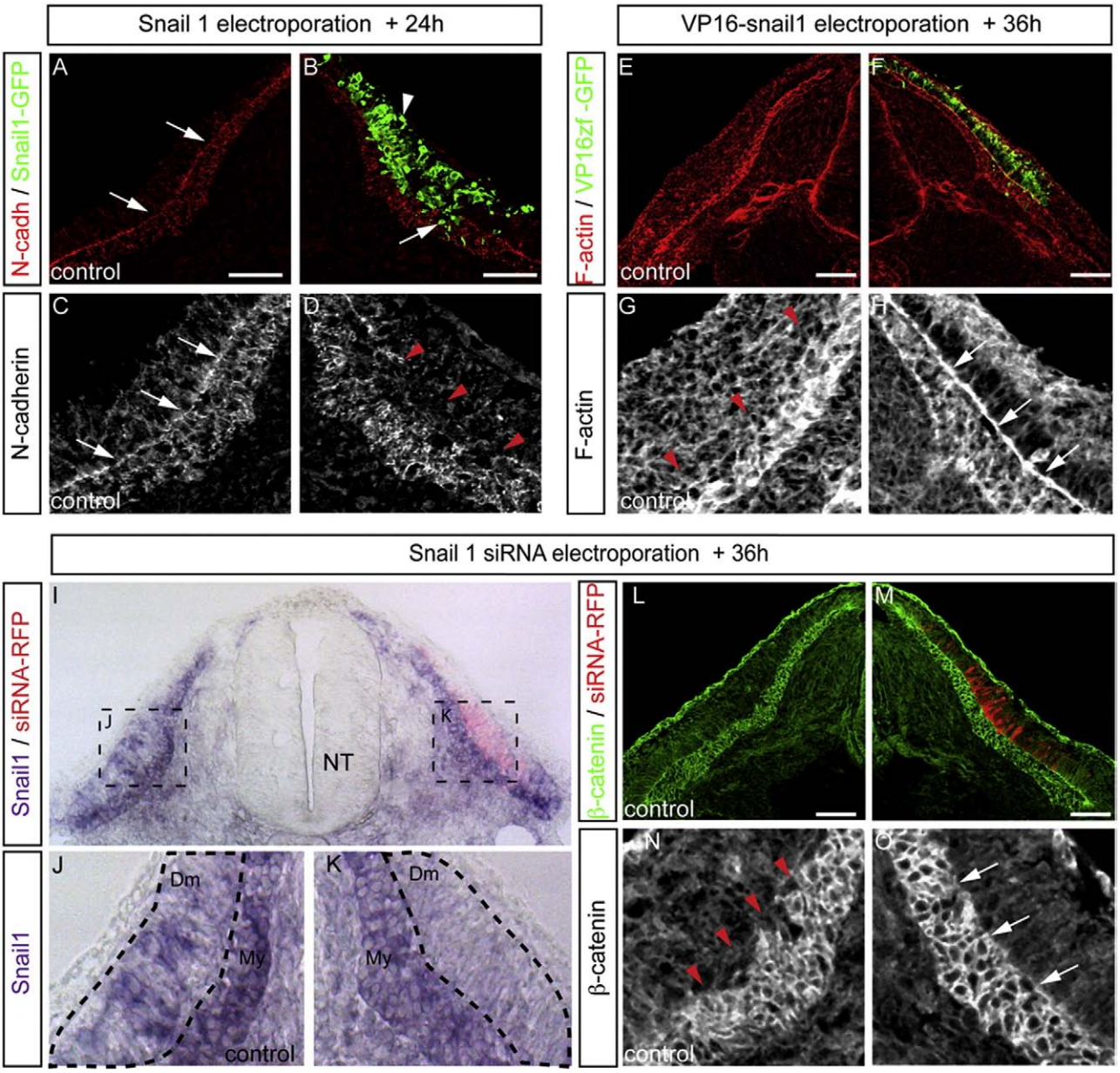

Fig. 4. Snail1 regulates the EMT of the dermomyotome. (A–D). Transversal sections at the interlimb level of a chick embryo electroporated at HH17 with cSnail1 (cSnail1-iresGFP), stained for N-cadherin (red (A, B)/white (C, D)), and GFP (in green, B) and harvested 1 day after electroporation, i.e. just before the EMT of the dermomyotome has been initiated. While on the control side, the adherens junction marker N-cadherin is observed at the apical end of dermomyotome cells (arrows in A, C) the EMT is precociously initiated on the cSnail1-over-expressing side (B and arrowheads in D; $n=5/5$); arrowhead in B indicates cells of the dermis. (E–H) Transversal sections at the interlimb level of a chick embryo electroporated at HH17 with a dominant-negative form of cSnail1 (VP16 Snail-iresGFP), stained for F-actin (red (E, F)/white (G, H)), and GFP (in green, F) and harvested 36 h after electroporation. While on the control side, the EMT of the dermomyotome has been initiated (arrowheads in G), the dominant-negative form of Snail1 on the electroporated side results in a maintenance of the epithelial structure of the dermomyotome (arrows in H; $n=6/6$). (I–K) Transversal sections at the trunk level of a chick embryo electroporated in dorsal somites at HH17 with a siRNA directed against Snail1 (siRNA Snail-CMV RFP), harvested at HH20, and hybridized with a Snail1 probe. J and K are enlargements of I that show the significant decrease of Snail1 mRNA level in the dermomyotome (delineated by dotted lines) after siRNA electroporation (K) compared to the control side (J; $n=4/4$). (L–O) Transversal sections at the trunk level of a chick embryo electroporated at HH17 with siRNA targeted against Snail1, stained for RFP (red in M) and for β-catenin (green (L, M) or white (N, O)). The embryo was harvested just after the beginning of the EMT on the control side (L, arrowheads in N). On the siRNA-electroporated side, the EMT is delayed (M, arrows in O; $n=5/8$). NT, neural tube; Dm, dermomyotome; My, myotome. Scale bar: 50 μm.

FGF/ERK signalling, plays a key role in controlling the EMT of the dermomyotome.

Discussion

Our results allow us to propose that close interaction between the primary myotome and the dermomyotome is pivotal for the timing of the EMT of the dermomyotome, which triggers the emergence of muscle progenitors. This study also describes Snail1 as a key regulator during this process.

FGF/ERK signalling might orchestrate the timing of emergence of muscle progenitors

The aim of the present work was to gain insight into the tissue and the molecular interactions that regulate the timing of emergence of muscle progenitors during embryogenesis. During the first day and a half of somite differentiation, trunk muscles form solely from a contribution of post-mitotic cells originating from the epithelial borders of the dermomyotome, thereby generating a primitive muscle, the primary myotome. The EMT of the central dermomyotome,

initiated 36 h after somite formation, allows the entry of the muscle progenitors within this organized tissue. This precise sequence of events is intriguing. The primary myotome is thought to provide a scaffold on which later waves of muscle cells organize (Gros et al., 2008; Kahane et al., 2007). It is thus conceivable that the proper morphogenesis of progenitor-derived muscle cells requires the presence of a well organized primary myotome, hence the requirement for a tight regulation of the timing of this process. In support of this hypothesis, we observed that the long term effect of a precocious EMT has a striking effect on myotome organization: 72 h after electroporation of FGF8, the orientation of myocytes within the myotome was greatly perturbed (Supplementary Figs. 3G, H).

Our data underline how remarkably simply the timing of this process might be orchestrated in the developing embryo. We propose that it is the primary myotome itself that secretes the FGF signals that trigger the EMT, thus allowing the entry of progenitors in the muscle compartment. The ectoderm, which is in direct contact with the dermomyotome, may be an alternative source of FGF signal. However, it was shown that this tissue is rather a source of signals that maintain the epithelial structure of the dermomyotome (through a Wnt6/β-catenin pathway; Schmidt et al., 2004; Linker et al., 2005) than the opposite. Thus, although the experiments presented here do not formally demonstrate that the myotome is the source of the FGF signals that triggers the dermomyotome EMT, they provide evidence that this is the case. Interestingly, the observation that the over-expression of FGF results in a premature EMT, suggests that the exact timing of the emergence of progenitors is regulated by the amount of FGF signal emanating from the myotome, which is likely proportional to the number of myocytes present in the primary myotome. In this way, muscle progenitors enter the primary myotome only when it is sufficiently developed. Through this mechanism, a process (the EMT) and the time at which it occurs are regulated with one signal. Since the downregulation of the expression of the epithelializing factor Wnt6 in the ectoderm precedes the dermomyotome EMT (Linker et al., 2005), it is possible that dermomyotome cells can determine the balance between the amounts of Wnt and FGF signaling. While our experiments show that FGF8 may be this signal, they did not address whether the FGFs may cooperate in this process. Solving this question will require the specific knock-down of each of the FGFs expressed by the myotome.

The gain and loss of function experiments we have performed support the hypothesis that FGF mediates its activity through a MAPK/ERK pathway. However, we cannot exclude that MAPK/ERK phosphorylation is also controlled by other tyrosine kinase receptors, since it is not a specific mediator of the FGF pathway. Additional factors may also cooperate or allow the initiation of this process. It was previously shown that exogenous Neurotrophin-3 (expressed by the neural tube) is able to induce the EMT of the dermomyotome (Brill et al., 1995). However, it is unclear whether this molecule plays this role in vivo.

FGF4 bead implantation experiments had suggested that FGF from the myotome promotes the migration of (FGFR4-positive) muscle progenitors emanating from the rostral and caudal borders of the epithelial dermomyotome and their proliferation in the myotomal compartment (Kahane et al., 2001). However, other work (Gros et al., 2004) argues against the generation of muscle progenitors from the rostral and caudal borders of the epithelial dermomyotome, which were shown to emanate from the central domain of the dermomyotome (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). Since FGFs are known mitogens, a possibility was that the EMT of the dermomyotome might be due to an overproliferation of dermomyotome cells. However, the observation that BrdU uptake was not significantly altered upon FGF8 electroporation indicates that this is not the cause of the FGF-induced EMT.

While the data presented here suggest that FGFs from the myotome might act on the dermomyotome to regulate the timing of emergence of muscle progenitors, previous work had identified a role of FGFs on tendon formation. Both in the mouse and the chick embryo, myotomal-FGF is acting on sclerotomal cells located at the somite borders, leading to the establishment of a tendon progenitor population located at the junction between the segmental muscles and cartilages (Brent et al., 2003, 2005; Brent and Tabin, 2004; Smith et al., 2005). Together with the data presented here, this suggests that the primary myotome may be an organizing center, which orchestrates the pace of muscle growth in concordance with tendon and cartilage formation. This organizing activity is likely mediated by FGF signals that induce in a timely fashion the emergence of muscle progenitors from the dermomyotome and the specification of tendon progenitors from the sclerotome lineage.

A Snail1 pathway regulates the EMT of the dermomyotome

The experiments described here indicate that the EMT of the dermomyotome is mediated by a Snail1 pathway. Snail transcription factors are known to directly repress E-cadherin transcription, which is thought to be the key event in triggering tissue de-epithelialization during development or tumor metastasis during cancer (Nieto, 2002). Since E-cadherins are not expressed in the DM (Marcelle et al., 2002), Snail1 protein may regulate other cadherins. N-cadherin is a potential candidate, since it is strongly expressed in dermomyotome and it has been implicated in promoting the derivation of dermomyotome progenitors towards the muscle lineage (Cinnamon et al., 2006). However, in our study, we do not observe a downregulation of N-cadherin proteins, but rather a loss of its apical distribution. It is therefore unclear whether this class of cadherins is a direct target of Snail repressor or whether Snail acts on a yet unidentified cadherin whose downregulation directly or indirectly disrupt the subcellular distribution of N-cadherin. Further studies will determine the molecular targets regulated by Snail1 during dermomyotome EMT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.05.544.

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