

Redefining the role of ectoderm in somitogenesis: a player in the formation of the fibronectin matrix of presomitic mesoderm

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The absence of ectoderm impairs somite formation in cultured presomitic mesoderm (PSM) explants, suggesting that an ectoderm-derived signal is essential for somitogenesis. Here we show in chick that the standard enzymatic treatments used for explant isolation destroy the fibronectin matrix surrounding the anterior PSM, which fails to form somites when cultured for 6 hours. By contrast, explants isolated with collagenase retain their fibronectin matrix and form somites under identical culture conditions. The additional presence of ectoderm enhances somite formation, whereas endoderm has no effect. Furthermore, we show that pancreatin-isolated PSM explants cultured in fibronectin-supplemented medium, form significantly more somites than control explants. Interestingly, ectoderm is the major producer of fibronectin (*Fn1*) transcripts, whereas all but the anterior-most region of the PSM expresses the fibronectin assembly receptor, integrin $\alpha 5$ (*Itga5*). We thus propose that the ectoderm-derived fibronectin is assembled by mesodermal $\alpha 5 \beta 1$ integrin on the surface of the PSM. Finally, we demonstrate that inhibition of fibronectin fibrillogenesis in explants with ectoderm abrogates somitogenesis. We conclude that a fibronectin matrix is essential for morphological somite formation and that a major, previously unrecognised role of ectoderm in somitogenesis is the synthesis of fibronectin.

KEY WORDS: Somitogenesis, Fibronectin, Ectoderm, Presomitic mesoderm, Integrins, Extracellular matrix, Chick

INTRODUCTION

Somites are transient segments of the paraxial mesoderm that are formed in a rostral-to-caudal progression during vertebrate embryogenesis. Each pair of somites, epithelial structures located symmetrically on either side of the neural tube, periodically detaches from the rostral end of the presomitic mesoderm (PSM), while new immature PSM tissue is added in the posterior part of the embryo. The strict temporal and spatial regulation of somitogenesis is of crucial developmental importance because segmental structures, such as the vertebrae, trunk skeletal muscles, peripheral spinal nerves and early blood vessels, develop according to the somite segmental pattern (Christ and Ordahl, 1995; Gossler and Hrabě de Angelis, 1998).

The PSM can be divided in two regions that differ not only in terms of gene expression patterns, but also in the morphology of the PSM cells. In the caudal two-thirds of the PSM, high Fgf8 activity is believed to keep cells in a mesenchymal, undifferentiated state and oscillatory expression of segmentation clock genes occurs (Dubrulle et al., 2001; Freitas et al., 2005; Palmeirim et al., 1997). As PSM cells leave this caudal immature region, crossing what has been termed the determination front and enter the anterior third of the PSM, several changes take place. The cyclic expression of clock genes comes to a halt, retinoic acid signalling progressively replaces

Fgf signalling (Diez del Corral et al., 2003) and somite anterior-posterior polarity is established (Saga and Takeda, 2001). Furthermore, the first signs of morphological somite formation occur as peripheral PSM cells undergo a mesenchymal-to-epithelial transition (Duband et al., 1987; Kulesa and Fraser, 2002). Finally, in the anterior-most region of the PSM, somite boundaries are specified and formed through the activation of transcription factors of the Mesp family (Sawada et al., 2000), most likely also involving Ephrin (Barrios et al., 2003; Durbin et al., 2000) and Notch signalling (Sato et al., 2002).

It has been clearly demonstrated that the molecular segmentation of the anterior PSM is an intrinsic property of the PSM and does not require signalling from neighbouring tissues (Palmeirim et al., 1997). However, morphological somite formation does not occur in isolated cultured PSMs. For somites to form, ectoderm must be in contact with the PSM (Borycki et al., 2000; Borycki et al., 1998; Correia and Conlon, 2000; Palmeirim et al., 1998), suggesting that ectodermal signals, most likely Wnts (Borycki et al., 2000; Schmidt et al., 2004), are essential for morphological somite formation.

Interactions between PSM cells and the extracellular matrix molecule fibronectin have been implicated in somitogenesis (Duband et al., 1987; George et al., 1993; Lash et al., 1984; Lash and Ostrovsky, 1986; Ostrovsky et al., 1983). Fibronectin exists in plasma and cellular forms which, when assembled into a matrix, mediate a wide variety of cellular processes such as cell spreading, migration, proliferation, survival and differentiation (Pankov and Yamada, 2002). Fibronectin matrix assembly is a complex cell-dependent process that requires the engagement of fibronectin by cell surface integrins, usually the $\alpha 5 \beta 1$ integrin, and fibrillogenesis involving fibronectin-fibronectin binding (Mao and Schwarzbauer, 2005; Wierzbicka-Patynowski and Schwarzbauer, 2003). Fibronectin 1 (*Fn1*)-null mouse embryos

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initiate gastrulation normally, but, although they have paraxial mesoderm, no morphologically distinguishable somites form (George et al., 1993; Georges-Labouesse et al., 1996). Furthermore, embryos null for both $\alpha 5$ and αV integrin subunits (*Itga5* and *Itgav*) do not assemble a fibronectin matrix and fail to form somites (Yang et al., 1999). Together, these studies suggest that interactions between the fibronectin matrix and the PSM cells are crucial for normal somitogenesis.

The main objective of the present work was to address the contribution of the ectoderm and the fibronectin matrix in somitogenesis. We show that enzymatic treatments generally used to isolate PSM explants from the surrounding tissues destroy the fibronectin matrix surrounding the PSM. When these explants are cultured for 6 hours, no somites form. By contrast, we show for the first time that when collagenase is used, the endogenous fibronectin matrix remains intact and somites form even in the absence of all surrounding tissues. Moreover, addition of exogenous fibronectin to cultured pancreatin-isolated PSMs significantly improves their ability to form somites. Interestingly, *Fnl* is primarily expressed in the ectoderm, whereas the PSM strongly expresses the fibronectin receptor *Itga5*, suggesting that ectoderm and PSM collaborate in constructing the fibronectin matrix present around the PSM. Finally, we demonstrate that inhibition of fibronectin matrix assembly in the presence of ectoderm abrogates somitogenesis. Based on these findings, we conclude that a fibronectin matrix is essential for somitogenesis and that a major role of ectoderm in this process is to provide the fibronectin protein for this matrix.

MATERIALS AND METHODS

Eggs and embryos

Fertilised chicken (*Gallus gallus*) eggs were incubated in a humidified atmosphere at 37.5°C until stage HH12-14 (Hamburger and Hamilton, 1992). Somite nomenclature follows Christ and Ordahl (Christ and Ordahl, 1995).

Cryosectioning

Embryos were fixed in 4% paraformaldehyde in phosphate buffer (77 mM Na₂HPO₄, 23 mM NaH₂PO₄, 0.12 mM CaCl₂) with 4% sucrose overnight at 4°C and processed for cryoembedding (Bajanca et al., 2004). Cryostat (Bright Clinicut 3020) sections (12 µm) were processed for immunohistochemistry or, if embryos had already been subjected to whole-mount in situ hybridisation, mounted in 80% glycerol in PBS and photographed.

Embryo manipulation

Embryos were pinned down on resin-coated Petri dishes with PBS containing calcium and magnesium (PBS w/Ca²⁺Mg²⁺). PSM isolation, including removal of ectoderm and/or endoderm was achieved using tungsten needles, with or without the application of enzymes: pancreatin (4×, Gibco), an enzyme extract containing trypsin; dispase (2.4 U/ml, Roche), which cleaves fibronectin and collagen type IV (Stenn et al., 1989); and collagenase type II (125 U/ml, Sigma), which digests various collagens. None of these enzymes degraded laminin (data not shown). PSM isolation with pancreatin and dispase took 3-4 minutes, whereas collagenase isolation took about 8 minutes. Pancreatin was inactivated with fetal bovine serum (FBS, Gibco), whereas dispase and collagenase were washed away with PBS w/Ca²⁺Mg²⁺.

Explant culture experiments

Embryo explants were positioned on 0.8 µm polycarbonate filters (Millipore) floating on M199 medium with 5% FBS and 10% chick serum (Palmeirim et al., 1997). In one set of experiments, the culture medium of one PSM was supplemented with 50-100 µg/ml of rat (Calbiochem) or bovine (Sigma) plasma fibronectin, whereas the contralateral PSM was incubated in control medium (medium only, or with BSA). Both plasma fibronectins produced the same results. In another set of experiments,

posterior embryo explants were cultured with 50 or 100 µg/ml of a 70 kDa fibronectin fragment (Sigma). Control embryos were incubated in medium with BSA.

Statistical analysis

A factorial analysis of variance (ANOVA) was used to test for the effect of the isolation method (pancreatin versus collagenase) and the culture method (PSM with or without endoderm or ectoderm) on the capacity of PSMs to form somites. The statistical significance of predicted specific differences was then analysed using contrast analysis. A paired Student's *t*-test was used to test for differences between explants grown with or without the fibronectin supplement. Differences between control embryo explants and explants cultured with the 70 kDa fibronectin fragment were tested with *t*-tests following a square-root transformation of the data. Statistical tests were computed with the STATISTICA 6 (StatSoft) programme.

Antibodies and immunohistochemistry

Fibronectin immunohistochemistry was performed using a polyclonal antibody (Sigma, 1:400), or a monoclonal anti-cellular fibronectin antibody (clone FN-3e2; Sigma, 1:400) that recognises the EIIIA domain unique to cellular fibronectin (Barnes et al., 1995). Monoclonal anti-N-cadherin (cadherin 2) antibody (clone32; BD Transduction Laboratories, 1:100) was also used. Secondary antibodies were Alexa Fluor 488-, 546- or 568-conjugated anti-rabbit and anti-mouse IgG F(ab')₂ fragments (Molecular Probes, 1:1000). For F-actin staining, Alexa Fluor 568-conjugated phalloidin was used (Molecular Probes, 1:40) and TO-PRO3 (Molecular Probes, 1:500) incubated with secondary antibody and ribonuclease A (Calbiochem, 10 µg/ml) was used for nuclear staining.

Explants were fixed overnight at 4°C in 4% paraformaldehyde in PBS and washed in PBS. Both primary and secondary antibodies were incubated overnight in PBS containing 1% BSA and 0.1% Triton X-100 at room temperature, and explants were mounted in Vectashield (Vector Laboratories).

Cryosections were treated with 0.2% Triton X-100 in PBS for 20 minutes, washed in PBS and blocked for 30 minutes in 5% BSA in PBS. Incubation in primary antibody was overnight at 4°C, and incubation in secondary antibody was for 1 hour at room temperature. Sections were counterstained with 4',6-diamidino-2-phenylidole-dihydrochloride (DAPI, 5 µg/ml in PBS, 0.1% Triton X-100).

Image acquisition and analysis

Images were acquired with an Olympus DP50 digital camera attached to an Olympus BX60 microscope equipped with epifluorescence and Nomarski optics, or with a Zeiss LSM 510 Meta confocal microscope. During confocal image acquisition, the detection parameters were adjusted to avoid under- or overexposed pixels. For fibronectin quantification, the average fluorescence intensity of six non-overlapping 75 µm-diameter regions of interest along each PSM was taken from the maximum *z*-projection of the seven most-superficial slices. The average fluorescence intensity of each PSM was normalised against its background fluorescence. ImageJ and Adobe Photoshop were used for image analysis and processing.

In situ hybridisation probes

In situ hybridisation probes for *Fnl*, *Itga5* and *Itgav* were generated. The *Fnl* probe was designed against a region present in all splice variants of the chick *Fnl* mRNA (French-Constant and Hynes, 1988; Norton and Hynes, 1987). Reverse transcription (RT) PCRs were used to isolate portions of chick *Fnl*, *Itga5* and *Itgav* using the sense oligos 5'-CGTTCGTCT-CACTGGCTACA-3', 5'-AGGTGCTGAGGGGGCAA-3', 5'-TTCTCCACAGCAAACAGCC-3' and the antisense oligos 5'-GGTCCTCTGG-ATGGGATTCT-3', 5'-CACGACGGTGAGCGAAG-3', 5'-ATCCTCA-CCACAATCCAGCA-3', respectively. The DNA fragments generated were cloned into the pCRII-TOPO vector (Invitrogen) and plasmid DNA was isolated. The constructs were confirmed by sequencing.

A plasmid carrying an insert of *Paraxis* (*Tcf15*) was kindly provided by C. Jouve (Developmental Biology Institute of Marseille, Marseille, France). The digoxigenin-labelled RNA probes were obtained from linearised plasmids, according to standard procedures adapted from Sambrook et al. (Sambrook et al., 1989).

Whole-mount in situ hybridisation

Embryos and explants were fixed overnight at 4°C in 4% formaldehyde with 2 mM ethylene glycol-bis (β -amino-ethyl ether) tetra acetic acid (EGTA) in PBS, rinsed in PBT (PBS, 0.1% Tween 20), dehydrated in methanol and stored at -20°C. Whole-mount in situ hybridisation was performed according to Henrique et al. (Henrique et al., 1995), but BM Purple Substrate (Roche) was used as staining solution. Some embryos were cryosectioned as described above.

RT-PCR

PSM and epithelial somites were isolated using pancreatin. Twenty samples of each of the following were collected for RT-PCR: (1) the four posterior-most somites; (2) the anterior third of the PSM; and (3) the posterior two-thirds of the PSM. Whole embryos were collected as positive control. Samples were processed for RNA extraction using the RNeasy Mini Kit with column DNA digestion (Qiagen). Total RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). PCR was performed using the primers described above. Amplification using β -actin primers was used as positive control. Reactions from which reverse transcription (RNA control) or cDNA (water control) were omitted were used as negative controls.

RESULTS

Enzymatic PSM isolation using pancreatin or dispase destroys the surrounding fibronectin matrix

Immunoreactivity for fibronectin was observed in embryo sections around the PSM and epithelial somites, as previously reported (Duband et al., 1987; Ostrovsky et al., 1983), and short strands of fibronectin immunoreactivity were distinguishable among PSM cells (Fig. 1A,B). Non-enzymatic removal of ectoderm from the anterior PSM in whole embryos revealed a network of fibrillar fibronectin-positive matrix surrounding the PSM (Fig. 1C). However, when PSMs were isolated with pancreatin or dispase, which are commonly used for this purpose, no immunoreactivity for fibronectin was detected (Fig. 1D,E). These data show that both pancreatin and dispase destroy the PSM fibronectin matrix within the time period necessary for PSM isolation. By contrast, we found that PSM isolation using collagenase preserved a fibrillar fibronectin matrix (Fig. 1F), suggesting that this is a better method for PSM isolation than pancreatin and dispase.

Collagenase-isolated PSMs retain their fibronectin matrix and are able to form somites even in the absence of ectoderm

If fibronectin is important for somitogenesis, it is not surprising that PSMs isolated with pancreatin or dispase fail to form somites. However, ectoderm is able to support morphological somitogenesis even when explants are treated with these enzymes (Borycki et al., 1998; Correia and Conlon, 2000; Palmeirim et al., 1998), whereas endoderm does not (Palmeirim et al., 1998). To evaluate the role of ectoderm, endoderm and fibronectin matrix in somite formation, pancreatin or collagenase were used to isolate three types of PSM explants: PSM without any adjacent tissues (PSM), PSM with the underlying endoderm (PSM+endoderm), or PSM with the overlying ectoderm (PSM+ectoderm). Two identical explants were isolated from each embryo. One was immediately fixed and immunolabelled for fibronectin, whereas the other was cultured for 6 hours (time to form 4 somites) and processed for fibronectin immunohistochemistry and F-actin staining (Fig. 2). The number of somites formed in each case was recorded (Fig. 3).

PSMs isolated from all surrounding tissues using pancreatin were negative for fibronectin immunolabelling (Fig. 2A) and generally did not present fully formed somites after 6 hours of culture ($n=206$;

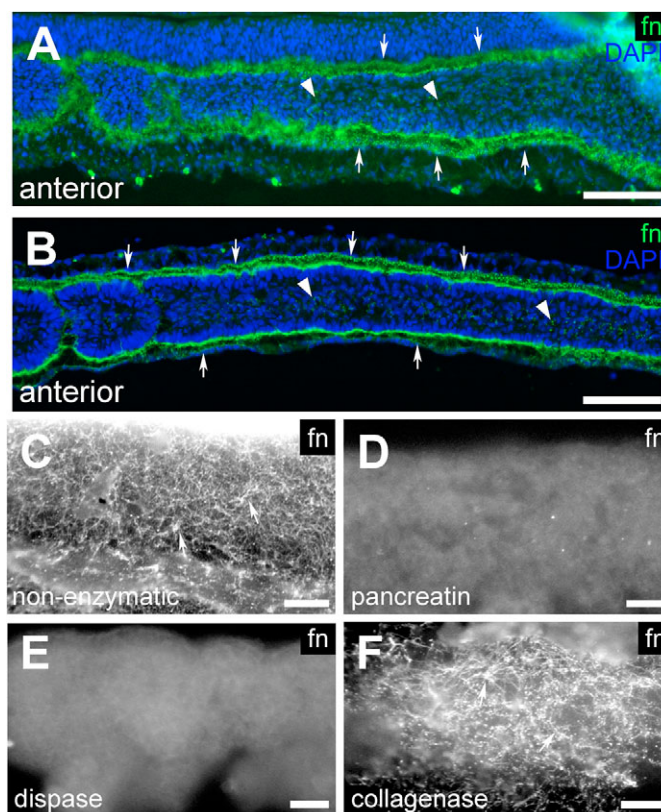


Fig. 1. Distribution of fibronectin in the chick PSM and surrounding tissues. (A,B) Longitudinal (A) and sagittal (B) sections reveal short strands of fibronectin immunoreactivity within the PSM (arrowheads in A,B), and an extensive fibronectin immunoreactivity surrounding the anterior PSM. This immunoreactivity is distinguishable from that of the neural tube, ectoderm and endoderm (arrows in A,B). (C) Non-enzymatic removal of ectoderm exposes fibrillar fibronectin immunoreactivity on the PSM. (D,E) By contrast, PSMs isolated with pancreatin (D) or dispase (E) are completely negative. (F) When collagenase is used an intact fibronectin matrix is maintained. Polyclonal anti-fibronectin (A,B) and monoclonal anti-cellular fibronectin (C-F) antibodies were used. fn, fibronectin. Scale bars: 100 μ m in A,B; 25 μ m in C-F.

Fig. 2C, Fig. 3). Curiously, in pancreatin-treated PSM+ectoderm explants, fibronectin was observed between the PSM and the ectoderm (Fig. 2G) and these formed somites after 6 hours of culture ($n=46$; Fig. 2I, Fig. 3). By contrast, although some fibronectin is preserved between the PSM and the underlying endoderm (Fig. 2D), pancreatin-treated PSM+endoderm explants rarely formed somites ($n=19$; Fig. 2F, Fig. 3).

All PSM explants treated with collagenase ($n=157$) retained a fibrillar fibronectin matrix on their surface (Fig. 2J,M,P), and exhibited fully formed somites (Fig. 2L,O,R, Fig. 3). This was true even for the PSMs cultured in complete isolation from surrounding tissues ($n=66$).

We conclude that PSMs isolated with an enzymatic treatment that retains the fibronectin matrix are able to form somites in the absence of neighbouring tissues. Pancreatin-treated PSM+ectoderm explants retain fibronectin matrix between the two tissues, which may facilitate the formation of somites. Curiously, somites rarely form in pancreatin-treated PSM+endoderm explants even though some fibronectin is retained between the two apposed tissues.

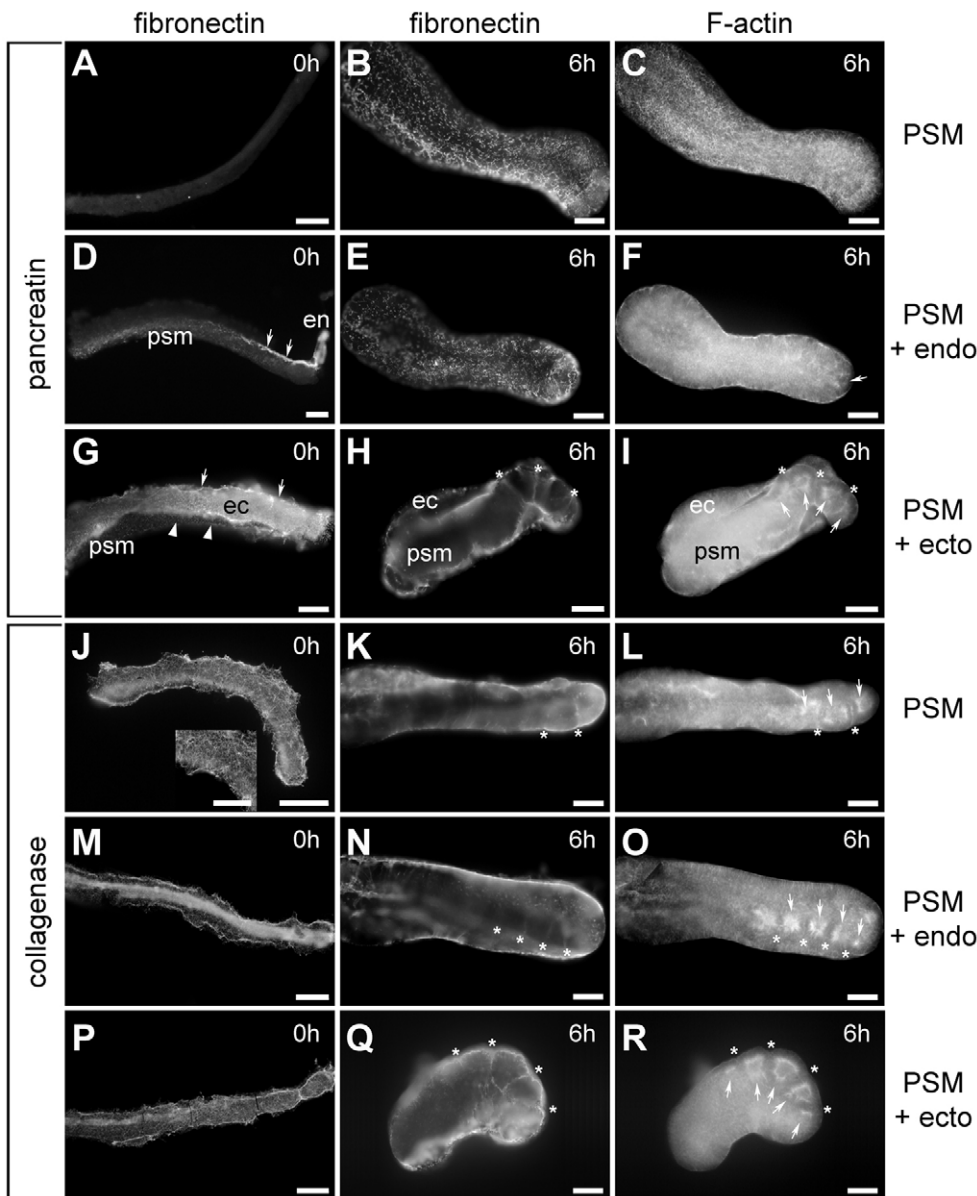


Fig. 2. Collagenase-isolated chick PSMs form somites in the absence of both ectoderm and endoderm. All explant types were either fixed immediately (0 hours, 0h) and labelled for fibronectin or cultured for 6 hours (6h) and processed for fibronectin and F-actin staining. (A-I) Pancreatin-isolated explants. Isolated PSMs are fibronectin-negative at 0 hours (A). At 6 hours, fibronectin staining is visible (B) but somites have not formed (C). In PSM+endoderm explants, some fibronectin immunoreactivity is present between endoderm and PSM at 0 hours (arrows in D). These explants show fibronectin immunoreactivity at 6 hours (E), but no somites have formed (arrow in F). PSM+ectoderm explants have fibronectin immunoreactivity between ectoderm and PSM (arrows in G), whereas the remaining PSM is negative (arrowheads in G). At 6 hours, clefts (asterisks in H,I) containing fibronectin immunoreactivity (H) and rings of F-actin staining (arrows in I) are observed. (J-R) Collagenase-isolated explants. All explants isolated with collagenase retain their endogenous, fibrillar (see insert in J) fibronectin matrix (J,M,P). At 6 hours, all explant types have clefts (asterisks in K,L,N,O,Q,R) containing fibronectin (K,N,Q) and several epithelialised somites (arrows in L,O,R). Anterior, right. psm, presomitic mesoderm; endo and en, endoderm; ecto and ec, ectoderm. Scale bars: 100 μ m.

Ectoderm, but not endoderm, enhances the capacity of collagenase-isolated PSMs to form somites

In order to uncover the relative contribution of ectoderm, endoderm and the fibronectin matrix in morphological somite formation, we analysed our quantitative data in more detail (Fig. 3). A comparison of PSM and PSM+endoderm explants isolated with pancreatin versus collagenase, indicates that the difference in the capacity to form somites is dependent both on the enzyme ($P<0.0001$) and the presence of endoderm ($P=0.03$). To determine whether this effect is mediated by endoderm itself, or by the fibronectin retained between the two apposed tissues, we compared the somite-forming capacity of collagenase-isolated PSM and PSM+endoderm explants. No significant difference ($P=0.71$) in the capacity to form somites was found between these two explant types. We conclude that endoderm does not by itself enhance somite formation in cultured PSMs and that the slight increase in somite-forming capacity of pancreatin-isolated PSM+endoderm explants must be due to the fibronectin matrix that is retained between the two tissues.

We then compared PSM and PSM+ectoderm explants isolated with pancreatin versus collagenase. We found that the difference in the capacity to form somites is both dependent on the enzyme ($P<0.0001$) and on the presence of ectoderm ($P<0.0001$). Unlike in the case of endoderm, a significant interaction between these two variables was detected ($P<0.0001$), indicating that they mutually enhance each other's effect. To determine whether ectoderm enhances somite formation by itself, we compared collagenase-isolated PSM and PSM+ectoderm explants and found that these differ significantly in their somite-forming capacity ($P<0.0001$). We conclude that, although isolation with collagenase enables PSMs to form somites, the additional presence of ectoderm enhances this capacity.

Pancreatin-treated PSM+ectoderm explants retain fibronectin matrix only between ectoderm and PSM, whereas collagenase-treated PSM+ectoderm explants retain an extensive fibronectin matrix all around. A comparison between these two situations did not reveal a significant difference between the number of somites formed ($P=0.075$), indicating that somite formation in PSMs in

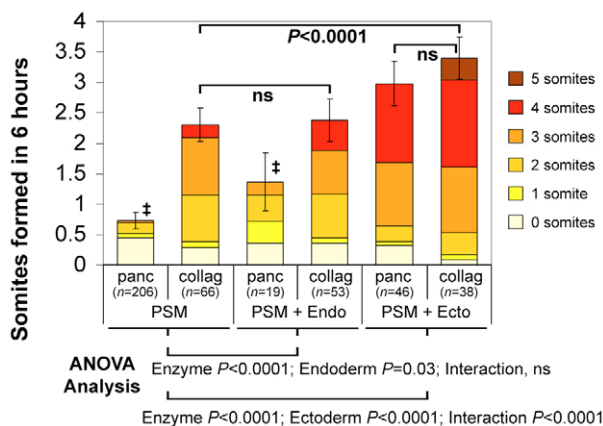


Fig. 3. Quantitative representation of the capacity of all PSM explant types treated with pancreatin or collagenase to form somites. The average number of somites formed for each explant type and enzyme treatment is depicted, and the proportion of explants forming 0, 1, 2, 3, 4 and 5 somites during the 6-hour culture period is indicated with a colour code. Note that the somites formed in PSM and PSM+endoderm explants isolated with pancreatin (marked with †) were frequently less compacted and had incomplete clefts as compared with all other experimental situations. A factorial ANOVA (results depicted at the bottom of the figure) followed by a contrast analysis of predicted differences (results shown in graph) were performed to test for differences among treatments. ns, not significant. Error bars represent 95% confidence limits.

contact with ectoderm is not improved by the presence of a fibronectin matrix all around the PSM at the beginning of the culture period.

Altogether, these results demonstrate that whereas collagenase-isolated PSMs are clearly capable of forming morphological somites, the additional presence of ectoderm enhances this capacity further, whereas endoderm has no effect.

Paraxis expression is independent of ectoderm and fibronectin matrix

Paraxis expression in the anterior PSM is necessary for epithelial somite formation (Burgess et al., 1996) and it has been suggested that ectoderm is essential to induce its expression (Correia and Conlon, 2000; Sosić et al., 1997). To determine if fibronectin and/or ectoderm influence *Paraxis* expression, pancreatin- or collagenase-isolated PSMs (Fig. 4B,D) and their contralateral control halves (Fig. 4A,C) were cultured for 6 hours and assayed for *Paraxis* expression. Both pancreatin-isolated ($n=11$) and collagenase-isolated ($n=11$) PSMs showed *Paraxis* expression (Fig. 4B,D), identical to the contralateral control halves (Fig. 4A,C), although only collagenase-isolated PSMs formed somites (Fig. 4D). We conclude that *Paraxis* expression in the anterior PSM and newly formed somites is independent of all surrounding tissues and of an intact fibronectin matrix. Furthermore, *Paraxis* expression in pancreatin-isolated PSMs cultured for 6 hours is not sufficient to drive somite formation.

Exogenous fibronectin increases the ability of pancreatin-isolated PSMs to form somites

Pancreatin-isolated PSMs show a clear fibronectin-immunoreactivity after 6 hours of culture. This fibronectin matrix was recognised by a cellular fibronectin-specific antibody (Fig. 5A),

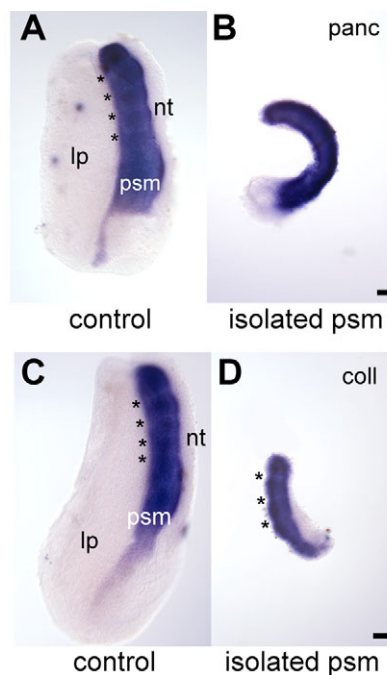


Fig. 4. *Paraxis* expression is maintained in both pancreatin- and collagenase-isolated chick PSMs cultured for 6 hours. After 6 hours of culture, control sides (intact halves, A,C) strongly express *Paraxis* in the anterior PSM in a pattern identical to that of the contralateral isolated PSMs, regardless of whether these were isolated with pancreatin (B) or collagenase (D). Only the collagenase-isolated explants formed somites (asterisks in D). Anterior, top. nt, neural tube; lp, lateral plate; psm, presomitic mesoderm; panc, pancreatin-isolated PSM; coll, collagenase-isolated PSM. Scale bars: 100 μm .

indicating that it is at least partially PSM-derived and not only from the serum supplement (McKeown-Longo and Mosher, 1983). However, practically no somites were formed in these PSM explants. Thus, the possibility arises that the amount of fibronectin (PSM- plus serum-derived) available to the fibronectin-stripped PSMs is insufficient to support somite formation. PSM isolation using pancreatin or dispase removes all the endogenous fibronectin (Fig. 1D,E, Fig. 2A) and after 2 hours of culture these PSMs showed only a faint immunoreactivity for fibronectin (Fig. 5B,E). Only after 4 hours of culture had a fibronectin matrix formed, which was similar in labelling intensity to the one observed after 6 hours (Fig. 5C-E).

To determine whether the amount of fibronectin available to the PSMs could be the limiting factor for their capacity to form somites, pancreatin-isolated PSM pairs ($n=20$) were cultured in fibronectin-supplemented medium versus control medium. The average number of somites formed by PSMs cultured with fibronectin for 6 hours was significantly higher ($P=0.002$) than that formed by the contralateral PSMs grown in control medium (Fig. 5F-J). Cells of PSMs cultured with fibronectin had aligned, elongated nuclei and apical enrichment of F-actin and N-cadherin, which was not observed in PSMs cultured in control medium (Fig. 6, compare B,E with A,D). In fact, cell morphology in PSMs cultured with fibronectin was very similar to that of collagenase-isolated PSMs (Fig. 6, compare B,E with C,F). We conclude that the culture of fibronectin-stripped PSMs in fibronectin-supplemented medium significantly improves their capacity to form somites and that their cells exhibit a morphology typical of somitic cells.

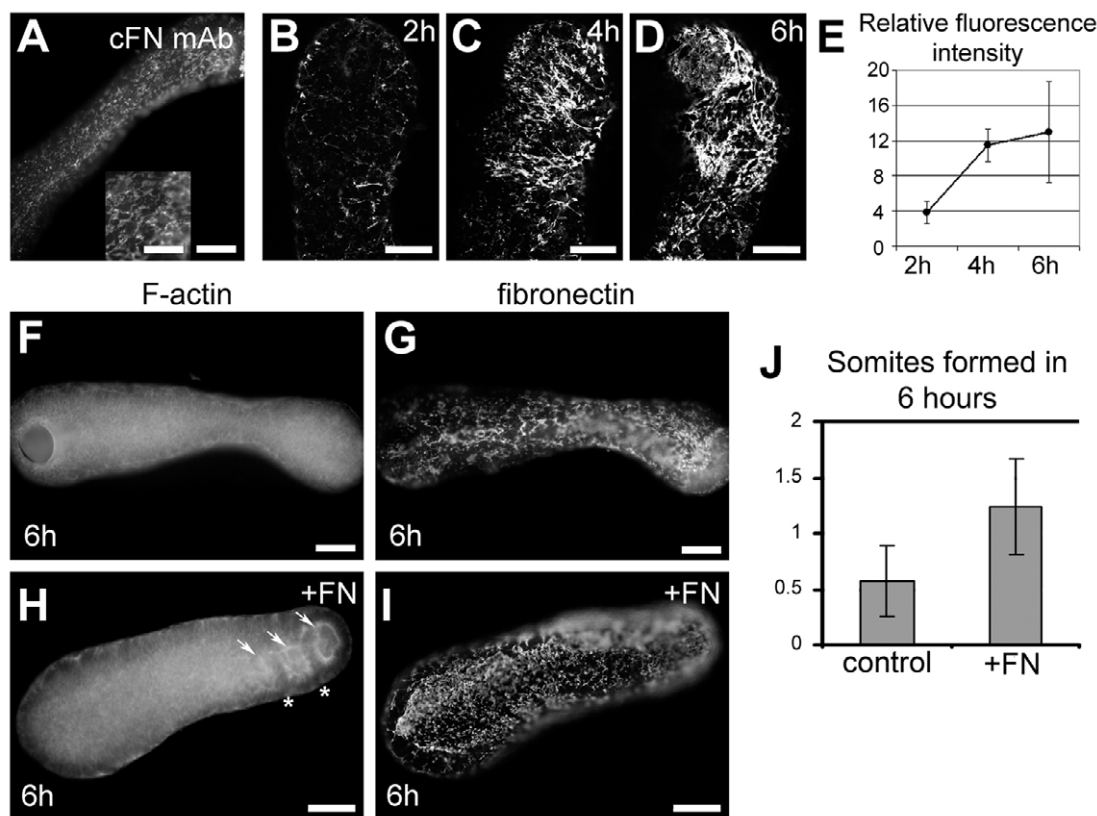


Fig. 5. Partial rescue of somite formation in pancreatin-isolated PSMs cultured with exogenous fibronectin. (A) Pancreatin-isolated chick PSMs present immunoreactivity for cellular fibronectin after 6 hours in culture. (B–D) z-projection of confocal images of dispase-isolated PSMs cultured for 2 hours (B) show faint fibronectin immunoreactivity, whereas after 4 (C) and 6 (D) hours it is more extensive. (E) Average fluorescence labelling intensity relative to background in dispase-isolated PSMs cultured for 2 ($n=7$), 4 ($n=4$) and 6 hours ($n=6$). (F–I) Contralateral pancreatin-isolated PSMs, cultured in control (F,G) or fibronectin-supplemented (H,I) medium and stained for F-actin (F,H) and fibronectin (G,I). (J) Bar chart of the average number of somites formed ($n=20$ pairs) showing that PSMs cultured with fibronectin form significantly more somites ($P=0.002$). Error bars represent 95% confidence limits. cFN, cellular fibronectin; +FN, PSM cultured in fibronectin-supplemented medium. Scale bars: 100 μm in A,F–I; 50 μm in A insert, B–D.

PSM and ectoderm collaborate in the assembly of the PSM fibronectin matrix

Our previous observations indicate that although isolated PSMs are able to produce and assemble a fibronectin matrix in culture, the amount of fibronectin available to them is insufficient to support somitogenesis. This led us to investigate where fibronectin is normally produced.

In situ hybridisation for chick *Fnl* transcripts revealed strong expression in the ectoderm and in the posterior part of epithelial somites (Fig. 7A–D). Furthermore, *Fnl* was expressed in the endoderm underlying anterior PSM and somites (Fig. 7B–D). Unexpectedly, *Fnl* mRNA was not detected in the rostral PSM and only a faint signal was present in the caudal PSM (Fig. 7B,C). This was confirmed by removal of the ectoderm prior to fixation (Fig. 7E–I). Interestingly, RT-PCR analysis showed that both anterior and posterior PSM express *Fnl* mRNA (Fig. 7J), which is in accordance with the cellular fibronectin immunoreactivity present on pancreatin-isolated PSMs after 6 hours of culture (Fig. 5A). Together, these data demonstrate that the major site of *Fnl* transcription is the ectoderm overlying the PSM, whereas the PSM expresses only low levels of *Fnl* mRNA.

Our next step was to identify where the integrins known to mediate fibronectin fibrillogenesis (Yang et al., 1999) are expressed. The main integrin responsible for fibronectin fibrillogenesis, *Itga5*,

was strongly expressed in the posterior two-thirds of the PSM (Fig. 7L,N), whereas *Itgav* transcripts were not detected in the PSM by in situ hybridisation (data not shown). Little or no *Itga5* mRNA expression was detected in the anterior-most PSM (Fig. 7L,M), but was again detected in epithelial somites (Fig. 7L). These data demonstrate that ectoderm is the major source of fibronectin, whereas the PSM expresses *Itga5*, the receptor necessary for its assembly into a fibrillar matrix.

Inhibition of fibronectin matrix assembly in the presence of ectoderm inhibits somite formation

We have shown that somite formation occurs in collagenase-isolated PSMs and in pancreatin-isolated PSMs cultured with fibronectin. Our results also demonstrated that although collagenase-isolated PSMs were able to form somites, the presence of ectoderm improved this capacity further. We now show that ectoderm is the major producer of fibronectin protein in the region of the PSM. The question arises, is the beneficial effect of ectoderm due to its production of fibronectin or does it provide some other essential signal to the PSM that contributes to somitogenesis?

To test this hypothesis, we cultured posterior embryo explants for 12 hours in medium containing a 70 kDa fragment of fibronectin known to block fibronectin fibrillogenesis (McKeown-Longo and Mosher, 1985). Thus, fibronectin matrix assembly is blocked in the

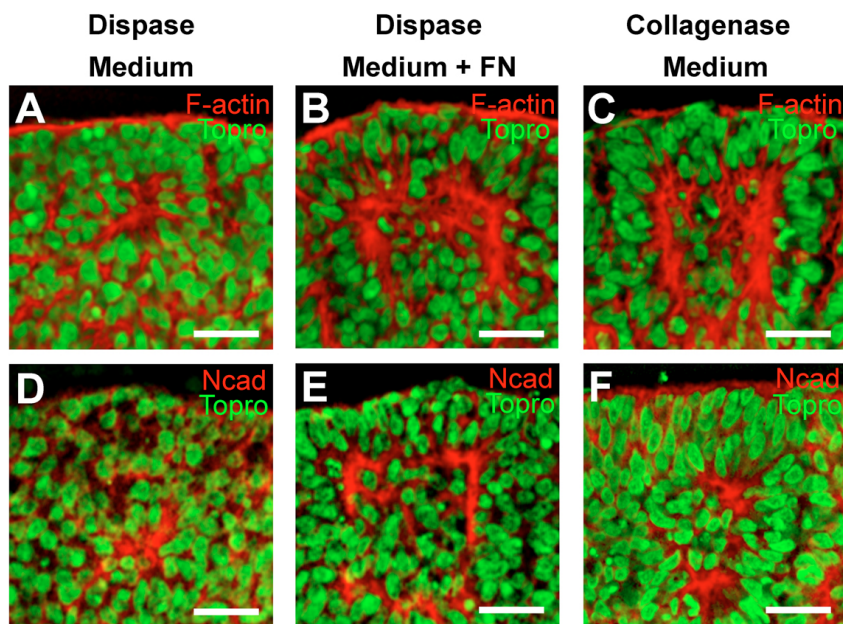


Fig. 6. F-actin and N-cadherin distribution in dispase-isolated PSMs cultured with and without fibronectin. Somite III regions (i.e. somite -II at 0 hours) of dispase-isolated chick PSMs after 6 hours of culture in control medium show only small foci of F-actin (A) and N-cadherin (D) clustering. By contrast, the same region of dispase-isolated PSMs cultured with fibronectin shows a prominent apical enrichment of F-actin (B) and N-cadherin (E) and a nuclear alignment and elongation typical in epithelial somites (B,E). Interestingly, this cellular arrangement is very similar to that observed in collagenase-treated PSMs (C,F). Anterior, left. Scale bars: 25 μ m.

presence of ectoderm. Endoderm was removed from one side of the embryo to improve the accessibility of the peptide, while the other side was kept intact. Control embryos formed the expected 7-8 somites (Fig. 8A,C,E). However, when explants were cultured in the presence of 50 μ g/ml of the fibronectin peptide (Fig. 8B,E), somitogenesis was less efficient (this was only significant in the absence of endoderm; $P=0.005$). Strikingly, when embryos were cultured in the presence of 100 μ g/ml of the peptide, somitogenesis was practically abolished, both in the presence ($P=0.002$) and absence ($P=0.001$) of endoderm (Fig. 8D,E). We conclude that fibronectin matrix assembly is absolutely necessary for morphological somite formation, even when ectoderm is left intact over the PSM.

DISCUSSION

PSMs isolated from all surrounding tissues form somites if their fibronectin matrix is left intact

The notion that fibronectin might be an important player in somitogenesis is not new. A fibronectin matrix outlines epithelial somites in both chick and mouse and its distribution pattern in the PSM shows a more complex matrix anteriorly than posteriorly, such that it has been suggested that this matrix is progressively laid down as the PSM matures (Lash et al., 1984; Lash and Ostrovsky, 1986; Ostrovsky et al., 1983; Ostrovsky et al., 1988). Experiments involving the addition of fibronectin to dissociated chick PSM cells caused their aggregation, suggesting that fibronectin might normally play a role in compacting and/or increasing the cell-cell adhesiveness of anterior PSM cells (Lash et al., 1984; Lash et al., 1987). In agreement with this, we show that adding exogenous fibronectin to pancreatin-isolated PSMs significantly improved their capacity to form somites.

In *Fnl*-null mouse embryos, paraxial mesoderm initially forms normally but does not generate any morphologically distinguishable somites (Georges-Labouesse et al., 1996). The *Fnl*-null phenotype thus represents one of the most severe somitogenesis phenotypes in a single mouse gene (reviewed by Pourquié, 2001). Moreover, a fibronectin matrix is required for normal somitogenesis and axial

extension in *Xenopus* (Marsden and DeSimone, 2003) and both *fn1* and *itga5* knock-down interfere with somite epithelialisation and boundary maintenance in zebrafish (Julich et al., 2005; Koshida et al., 2005).

Many authors have reported that PSMs cultured in the absence of all surrounding tissues do not form somites (Borycki et al., 1998; Borycki et al., 2000; Correia and Conlon, 2000; Lash et al., 1984; Lash and Ostrovsky, 1986; Lash and Yamada, 1986; Linker et al., 2003; Packard, 1976; Packard, 1980; Palmeirim et al., 1998). In all these studies, PSMs were isolated with pancreatin (which contains trypsin), trypsin or dispase. Here we demonstrate that 3-4 minutes of exposure to pancreatin or dispase is sufficient to remove all fibronectin immunoreactivity from isolated PSM, whereas collagenase-isolated PSMs retain their fibronectin matrix and form somites. We thus show for the first time that isolated PSMs can form somites in the absence of all surrounding tissues as long as the fibronectin matrix is preserved. We propose that the major cause for the reported failure in morphological somite formation in isolated PSMs is that the enzymes commonly used for their isolation degrade the fibronectin matrix.

Ectoderm and PSM collaborate to produce PSM fibronectin matrix

Our results demonstrate that ectoderm strongly expresses *Fnl*, whereas the PSM expresses very little (see proposed model in Fig. 9). Cultured ectoderm assembles an extensive, basally located fibronectin matrix (Newgreen and Thiery, 1980), showing that ectodermal *Fnl* expression results in the production of fibronectin, which is predominantly secreted basally towards the PSM. Furthermore, fibronectin-positive 'dense bodies' appear to come off the basal surface of the ectoderm as fibronectin-positive extracellular material accumulates on the mesoderm (Sanders, 1986). In fact, extracellular matrix material has been described as travelling freely between germ layers in the early chick embryo (Harrison et al., 1985). Thus, we suggest that the bulk of the PSM fibronectin matrix originates from fibronectin protein produced and secreted by the ectoderm (Fig. 9).

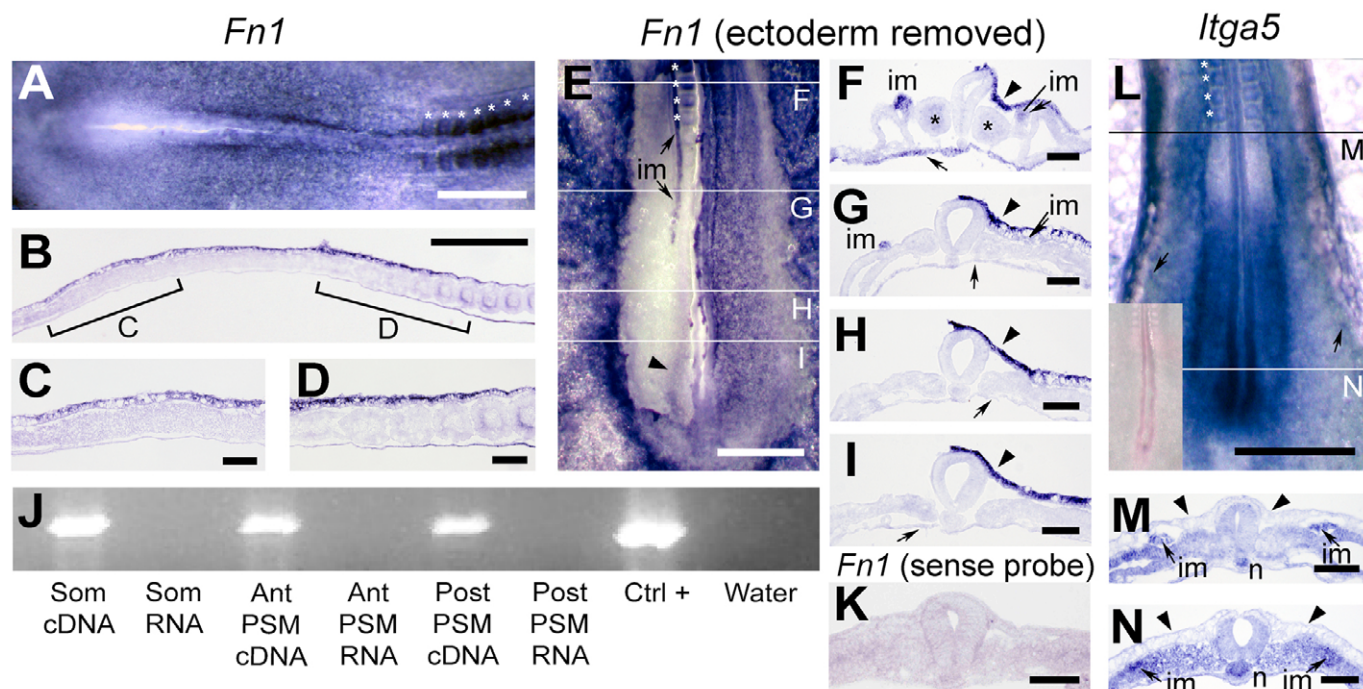


Fig. 7. mRNA expression pattern of fibronectin (*Fn1*) and its integrin receptor (*Itga5*) in the PSM and surrounding tissues. (A-D) In situ hybridisation for *Fn1* on HH12-14 stage chick embryos shows that *Fn1* mRNA is strongly expressed in the ectoderm overlying the PSM (A-D) and in the posterior part of epithelial somites (A,B,D). Endoderm is negative posteriorly but expression is detected at the level of anterior PSM and epithelial somites (B-D). PSM appears negative for *Fn1* mRNA except for a faint staining posteriorly (B,C). (E-I) Removal of ectoderm before fixation confirms this pattern and shows that *Fn1* expression is very strong in the intermediate mesoderm (E,F,G). (J) However, *Fn1* expression is detectable in both the anterior third and posterior two-thirds of the PSM by RT-PCR. (K) Negative hybridisation control with *Fn1* sense probe. (L-N) In situ hybridisation for *Itga5* shows that it is strongly expressed in the posterior two-thirds of the PSM and in epithelial somites. Insert (L) shows sense probe control. Sections of *Itga5*-hybridised embryos show that the signal in PSM is faint anteriorly (M), but strong posteriorly (N). *Itga5* is also expressed in notochord, intermediate (arrows) and lateral mesoderm (M,N), and extraembryonic blood vessels (arrows in L). Faint staining is detectable in neural tube and ectoderm (arrowheads, M,N). Anterior, up. *, Epithelial somite; im, intermediate mesoderm; n, notochord; som, somites; ant PSM, anterior PSM; post PSM, posterior PSM; Ctrl+, positive control (whole embryo cDNA); RNA, reverse-transcription omitted; water, cDNA omitted. Scale bars: 500 μ m in A,B,E,L; 100 μ m in C,D,F-K,M,N.

The posterior two-thirds of the PSM strongly express *Itga5*. Immunoreactivity for the $\beta 1$ integrin subunit has been shown to be enriched on the surface of the PSM and is much lower within the PSM (Duband et al., 1986; Krotoski et al., 1986). Given that fibronectin matrix accumulates almost exclusively on the surface of the PSM, we suggest that $\alpha 5\beta 1$ protein is enriched on the outer surface of the PSM where it regulates fibronectin assembly (Fig. 9).

Our results also show that the amount of fibronectin available to the PSM is crucial for the formation of a fibronectin matrix that can support somitogenesis. As the PSM-derived fibronectin is not sufficient to support somitogenesis, the PSM appears to depend on an external source of fibronectin to construct its matrix. Our observations strongly suggest that this external source is ectoderm.

A new role for ectoderm in morphological somite formation

It is clear that *Paraxis* is necessary for somitogenesis (Burgess et al., 1996), and its long-term expression depends on the overlying ectoderm (Correia and Conlon, 2000; Schmidt et al., 2004; Sosić et al., 1997). However, *Paraxis* expression is maintained in the paraxial mesoderm for up to 10 hours without any interaction with ectoderm (Linker et al., 2005), which agrees with our observation of strong *Paraxis* expression in isolated PSMs cultured for 6 hours

(Palmeirim et al., 1998) (this study). Thus, although ectoderm is essential to maintain *Paraxis* in the dermomyotome (Linker et al., 2005), it is dispensable for initial *Paraxis* expression in PSM and early somites.

Our results instead provide evidence of a previously unrecognised role of ectoderm in somitogenesis, which is the production of the bulk of fibronectin necessary for morphological somite formation. However, we also show that if enough fibronectin has assembled into a matrix in the anterior PSM, the ectoderm is no longer required for somite formation. How can these results be reconciled with the existing literature that advocates an essential signalling role of ectoderm in morphological somite formation? We suggest that when ectoderm is removed from the full extension of the chick PSM in ovo (Sosić et al., 1997), or from the caudal PSM (Schmidt et al., 2004), somites do not form because the PSM is deprived of the major source of fibronectin necessary to construct its matrix (Fig. 9). In fact, if ectoderm is replaced over the PSM after its manipulation, there is little effect on somitogenesis (Packard et al., 1993). Conversely, we propose that when ectoderm is physically separated from the anterior PSM, epithelial somites form (Linker et al., 2005) because the fibronectin matrix in the anterior PSM remains undamaged. Furthermore, when dispase-treated PSMs are cultured within a bag of tail ectoderm, somite formation is

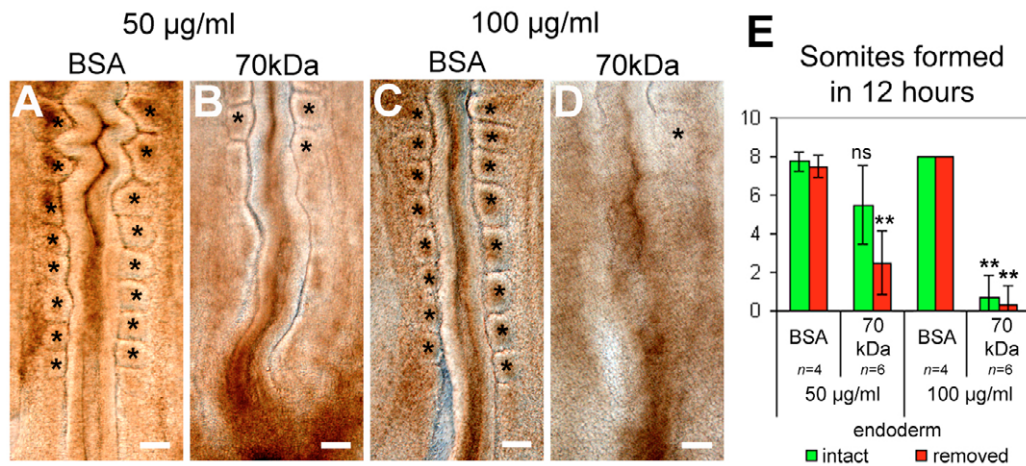


Fig. 8. A 70 kDa peptide containing the fibronectin assembly domain inhibits somitogenesis in embryo explants cultured for 12 hours. (A-D) Ventral view of chick embryo explants cultured with BSA (control) or the 70 kDa fibronectin fragment. Endoderm was removed on the left while the other side was kept intact. Embryos incubated with BSA form the expected number of somites in 12 hours (A,C), whereas embryos cultured with the 70 kDa fibronectin fragment form fewer somites (B,D). When embryos are cultured with 100 µg/ml of the peptide, somitogenesis is practically abolished (D). (E) Bar chart of the results obtained in A-D. Error bars represent 95% confidence limits. *, Somites formed in culture; BSA, bovine serum albumin; 70 kDa, 70 kDa fibronectin fragment; ns, not significant; **, $P \leq 0.005$. Scale bars: 100 µm.

partially restored (Correia and Conlon, 2000). We show that pancreatin-treated PSMs significantly increased their ability to form somites when cultured in fibronectin-enriched medium. It is thus tempting to suggest that the ectoderm bags used by Correia and Conlon (Correia and Conlon, 2000) provided a rich source of fibronectin protein to the dispase-isolated PSMs, allowing them to assemble a fibronectin matrix and partially restore somitogenesis. We propose that for morphological somites to form, PSMs need a major external source of fibronectin – the overlying ectoderm – in order to build a fibronectin matrix able to support somite formation.

Does ectoderm play additional roles in morphological somite formation?

Although the role of Wnts in the segmentation clock and in the differentiation of epithelial somites is well documented (reviewed by Auhehla and Pourquié, 2006; Rida et al., 2004; Yusuf and Brand-Saberi, 2006), their role in the epithelialisation of somites is much less clear. Wnt6, the only Wnt expressed in the ectoderm overlying the anterior PSM (Cauthen et al., 2001; Rodriguez-Niedenfuhr et al., 2003; Schubert et al., 2002), is the most likely candidate to play a role in somite formation. Wnt6-expressing cells placed in the caudal PSM after ectoderm removal rescue *Paraxis*, *Pax3* and *MyoD* expression after 18 hours of culture (Schmidt et al., 2004). However, expression of these genes is independent of somite epithelialisation (Linker et al., 2005; Palmeirim et al., 1998; Burgess et al., 1996; Wilson-Rawls et al., 1999) (this study). Furthermore, our results show that somites form in collagenase-isolated PSMs in the absence of ectoderm and that somitogenesis is severely affected when fibronectin matrix assembly is inhibited in its presence. These data therefore strongly argue against a direct role of ectodermal Wnts on the epithelialisation of the anterior PSM. Although ectodermal canonical Wnt signalling cannot be ruled out as a player in morphological somite formation [some somite-like condensations form in dispase-isolated quail PSM explants cultured with LiCl

(Borycki et al., 2000)], our experiments show that its role in somite epithelialisation can only be minor in comparison with that of the fibronectin matrix.

Fibronectin matrix assembly as a part of the somitogenesis machinery

We have demonstrated that the PSM fibronectin matrix is essential for morphological somite formation. Fibronectin matrix assembly is a complex process (reviewed by Wierzbicka-Patynowski and Schwarzbauer, 2003; Mao and Schwarzbauer, 2005) and its strengthening and maintenance is dependent on the amount of free fibronectin available for continuous incorporation, even after an initial fibrillar matrix has formed. This might explain why collagenase-isolated PSM+ectoderm explants form significantly more somites than collagenase-isolated PSMs. Since ectoderm produces fibronectin during the 6-hour culture period, the matrix of PSMs cultured with ectoderm increases in strength, whereas PSMs isolated without ectoderm rely on the matrix they have at the time of isolation. The 70 kDa fibronectin fragment used in our experiments prevents the fibronectin-fibronectin binding essential for the formation of new fibrils and it also binds to and displaces fibronectin within young fibrils (McKeown-Longo and Mosher, 1985), explaining its dramatic effect on somite formation. We propose that the presence of ectoderm promotes a continuous fibronectin fibrillogenesis that maintains and increases the strength of the PSM fibronectin matrix, essential to support somitogenesis.

Is the PSM fibronectin matrix solely providing a structural framework or could fibronectin also be playing an instructive role? Studies of *Xenopus* convergent extension have demonstrated that fibronectin affects cadherin-mediated cell-cell adhesion, which is important for morphogenetic movements (Marsden and DeSimone, 2003). Fibronectin signalling through $\alpha 5 \beta 1$ integrin upregulates N-cadherin expression in cultured myoblasts (Huttenlocher et al., 1998). Since N-cadherin has been implicated in somitogenesis (Horikawa et al., 1999; Linask et al., 1998; Radice et al., 1997) and the presence of fibronectin correlates with the cellular redistribution

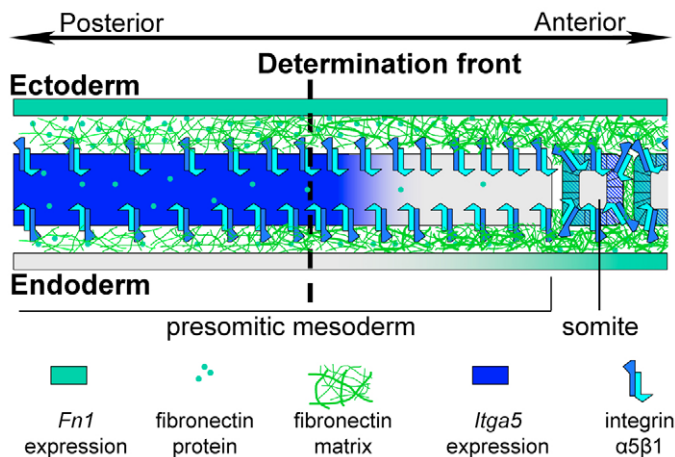


Fig. 9. Model of fibronectin assembly in the chick PSM. Sagittal view of PSM and first epithelial somite, showing ectoderm dorsally and endoderm ventrally. Based on our data, we suggest that PSM fibronectin matrix assembly occurs primarily in the posterior two-thirds of the PSM and that the fibronectin matrix in the anterior-most PSM is (if left unperturbed) sufficiently extensive to support somite formation. We propose that ectoderm is the major source of fibronectin protein for this matrix, and that only a minor contribution comes from the PSM itself. The PSM does however express $\alpha 5\beta 1$ integrin, which is essential for the assembly process. Although *Itga5* mRNA is not detected in its surface, we hypothesize that the $\alpha 5\beta 1$ protein remains on its surface. *Itga5* mRNA is again detected in epithelial somites, whereas *Fn1* is expressed in their posterior half, suggesting a potential role in the stabilisation of somite clefts (Koshida et al., 2005). According to our model, ectoderm ablation over the posterior two-thirds of the PSM compromises fibronectin matrix assembly and the formation of somites by the underlying PSM. By contrast, ectoderm ablation over the anterior-most PSM, if performed without destroying the endogenous fibronectin matrix, does not affect somite formation.

of N-cadherin and F-actin (this study), $\alpha 5\beta 1$ -mediated signalling might affect N-cadherin during somitogenesis. Integrin $\alpha 5\beta 1$ and fibronectin have also been shown to regulate cell behaviour during convergent extension in *Xenopus* (Davidson et al., 2006). This is controlled by small GTPases of the Rho family (Fukata et al., 2003), which have been implicated in the regulation of the mesenchymal-to-epithelial transition of the anterior PSM (Nakaya et al., 2004).

In summary, we conclude that fibronectin is an essential player in somitogenesis and that the PSM fibronectin matrix is the product of a collaboration between the PSM and the overlying ectoderm. We propose that the continuous build-up of the fibronectin matrix is essential for somitogenesis and that the removal of this matrix or the inhibition of its assembly compromises morphological somite formation. It is also tempting to suggest that the increasing complexity of the fibronectin matrix might transmit specific signals, so that cell behaviour in the anterior PSM changes, promoting epithelialisation. Whether and how fibronectin collaborates with other factors to achieve this morphological transition into somites will be interesting issues to explore.

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