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Functional dissection of Pax3 in paraxial mesoderm development and myogenesis

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Abstract

The paired box transcription factor Pax3 is well-known as a major regulator of embryonic myogenesis. Before Pax3 expression becomes restricted to the dermomyotome, this transcription factor is also expressed in the developing somites. The role of Pax3 at this early stage is unclear, in particular because of the scarce frequency of Pax3-positive cells in the early mouse embryo. Inducible gene expression in embryonic stem (ES) cells represents an excellent tool to overcome this limitation, since it can provide large quantities of otherwise rare embryonic populations expressing a factor of interest. Here we used engineered mouse ES cells to perform a functional analysis of Pax3 with the aim to identify the molecular determinants involved in the early functions of this transcription factor. We find that Pax3 induction during embryoid body (EB) differentiation results in the up-regulation of genes expressed in the presomitic and somitic mesoderm. Moreover, we show that paraxial mesoderm induced by transient expression of Pax3 is not irreversibly committed to myogenesis, rather requires sustained Pax3 expression. Using a series of deletion mutants of Pax3, which differentially affect its transcriptional activity, we map protein domains necessary for induction of paraxial mesoderm and induction of the myogenic program. The paired, homeo- and transcriptional activation domains were each required for both processes, however the paired-c-terminal RED domain showed a paraxial mesoderm-specific activity that was dispensable for myogenesis. These findings demonstrate and provide mechanistic insight into an early role for Pax3 in the generation of paraxial mesoderm.

Keywords

Mouse embryonic stem cells; Pax3; paraxial mesoderm; skeletal muscle progenitors; deletion mutants

INTRODUCTION

During development, mesoderm arises from cells that ingress through the primitive streak, a process that is regulated by gradients of morphogens that establish the body plan axes. Mesoderm is initially patterned into four general compartments: the axial mesoderm (notochord), the paraxial mesoderm (somites), the lateral plate mesoderm (cardiac and hemato-endothelial), and the extraembryonic mesoderm (reviewed by [1]). Commitment towards one of these compartments depends on both the time of ingression in the primitive streak and position of the cells, which defines the morphogens cells will be exposed to,

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AUTHORSHIP CONTRIBUTIONS

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ultimately resulting in the activation of specific transcription factors. After specification, paraxial mesoderm undergoes re-epithelialization as presomitic mesoderm (PSM) undergoes the process of segmentation into somites. Somitogenesis is regulated by morphogen gradients of retinoic acid and FGF8 together with the synchronous oscillation of cells in the PSM that results in the cyclic expression of Notch pathway genes, i.e. *Lunatic Fringe (Lnfg)* and *Hairy/Enhancer of split (Hes)*. Notably, disruption of Notch signaling results in defective somitogenesis (reviewed by [2]). This process proceeds in an anterior to posterior direction and establishes alternating bands of cells that ultimately form an epithelial structure. After their formation, epithelial somites are patterned dorsally and ventrally to originate respectively, the dermomyotome, and sclerotome [3].

Pax3, a member of the paired-domain family of transcription factors, is an important regulator of myogenesis (reviewed in [4]). During embryogenesis, Pax3 is expressed in the dorsal neural tube, in the posterior PSM, and in the developing somites, where expression becomes restricted to the epaxial and hypaxial regions of the dermomyotome. Ablation or mutation of Pax3, as in the case of the *splotch* mice [5], is characterized by the absence of limb muscles. In these embryos, muscle progenitors do not delaminate from the hypaxial dermomyotome, and additional developmental abnormalities arise in neural crest cell derivatives. Similarly, mutation of the human *PAX3* gene is observed in patients affected by Waardenburg syndrome, which are characterized by defects in pigmentation, hearing and limb musculature. Importantly, Pax3 regulates the expression of the HGF receptor *c-Met*, which is necessary for the delamination/migration of the muscle progenitors toward the limb bud [6]. Another well-known Pax3-target gene is the myogenic factor *Myf5*, which is induced directly by Pax3 in the hypaxial somite and limb [7, 8]. Direct targets of Pax3 include also the Fibroblast Growth Factor Receptor 4 (*Fgfr4*), the Forkhead family transcription factor *FoxC2* and the Double-sex homolog *Dmrt2* [9–11].

The transcriptional activity of Pax3 depends on the presence of two independent DNA binding domains, the Paired domain and the Homeodomain, which are conserved across evolution [12, 13]. Pax3, its homologous Pax7, and other Pax-family members also share a short sequence named the Octapeptide, which has been proposed to bind the co-repressor Groucho [14]. The carboxy-terminal transactivation domain is less characterized and less conserved. Several studies have indicated that Pax3 is not a strong transactivator, however these used immortalized cell lines, which may not express relevant co-factors, and clearly do not reproduce the early events that occur in the mouse embryo [8, 15–18]. However expression of Pax3 in the P19 embryonal carcinoma cell line [19] or in mouse embryonic stem cells [20] is able to induce the myogenic program [19, 20], supporting the importance of an appropriate cellular model to investigate embryonic myogenesis. Mouse embryonic stem cells represent a powerful tool to study early developmental mechanisms particularly due to the possibility to manipulate their genome and to adapt their culture conditions [21–23]. Taking advantage of this system, we investigated the effect of Pax3 expression in differentiating mouse ES cells, focusing on the characterization of domains and how they affect Pax3 activity on paraxial mesoderm and myogenesis. Through the generation and characterization of a mutant panel of ES cell lines, we show that i) Induction of Pax3 in EB cultures recapitulates the formation of the embryonic myotome, through a process involving upregulation of somite markers; ii) Continual induction of Pax3 is required to induce the final commitment of these cells to the myogenic lineage, and iii) The paired-c-terminal domain of Pax3 plays an important role specific to paraxial mesoderm.

MATERIAL AND METHODS

Plasmids and cell line generation

The recombination plasmids p2lox Pax3 was obtained by sub-cloning the blunted EcoRI/XhoI DNA fragment encoding Pax3 (from pSPORT Pax3 – Open Biosystems) into p2lox vector (described in [24]) digested with EcoRV and SmaI. The p2lox Pax3 3xFlag was generated in a two step procedure. First, Pax3 was PCR-amplified to remove the stop codon with the following primers FW: ACAGAATTCATGACCACGCTGGCCG RV: CAGGCGGCCGCTGCAATATCTGGCTTGAG and sub-cloned in the p2lox vector using EcoRI/NotI. Second, the sequence encoding the 3xFlag was inserted in the NotI site of the p2lox Pax3 (without Stop Codon) by ligating two annealed oligos (FLAG-FW and RV) encoding the tag sequence. FLAG-FW: GGCCCTCGAGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCG ACTACAAGGATGACGATGACAAGTAGGC FLAG-RV: GGCCGCCTACTTGTTCATCGTCATCCTTGTAAGTCGATGTCATGATCTTTATAATCA CCGTCATGGTCTTTGTAGTCCTCGAG

Deletion mutants for both Pax3 and Pax3-3xFlag were generated using the Quickchange Mutagenesis kit (Stratagene) following the manufacturer's instructions. The list of oligos used is reported in Table S1. The p2lox-Ires-GFP plasmid was generated by cloning of the blunted EcoRI-BsrGI DNA fragment encoding Ires-GFP (from pSAM2-ires-GFP – described in [25]) into p2lox vector digested with EcoRV and SmaI. The p2lox Pax3 Ires-GFP was generated by insertion of the blunted EcoRI-XhoI fragment (described above) into the p2lox-Ires-GFP vector digested with XhoI and blunted. Stable cell lines were generated by inducible cassette exchange by transfecting 20 µg of p2lox vector into the A2lox-CRE cell line [21] using the Mouse ES cell Nucleofector kit (Lonza). Cells were selected with 300 µg/ml of Geneticin (Gibco) as previously described [26]. Prior to the studies described here, all these mutants were sequenced to avoid PCR-mediated mutation (data not shown).

Cell cultures

Mouse embryonic stem cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) in Knockout DMEM (Gibco) supplemented with 15% ES qualified FBS (Gibco), penicillin/streptomycin (Gibco), 2 mM Glutamax (Gibco), 0.1 mM β-mercaptoethanol, and 1,000 U/mL LIF (Millipore), at 37°C in 5% CO₂. For differentiation, ES cells were trypsinized, resuspended in EB differentiation media (EBM) and pre-plated for 30 min on gelatin-coated dishes to remove MEFs. After counting, cells were diluted in EBM at 10,000 cells/ml, and plated as drops of 10µl in non-adherent 15 cm Petri dishes to induce EB formation. EBs were harvested after 48h and plated on non-adherent 10 cm dishes on a swirling rotator in EBM at 37°C in 5% CO₂. EBM consisted of Iscove-modified DMEM (Gibco) supplemented with 15% ES qualified FBS (Gibco), penicillin/streptomycin, 2 mM Glutamax, 200 µg/mL iron-saturated transferrin (Sigma), 4.5 mM monothioglycerol (Sigma) and 50 µg/mL ascorbic acid (Sigma). Doxycycline (Sigma – D9891) was dissolved in sterile PBS at a concentration of 1 mg/ml and stored at 4°C. Induction of the transgene was achieved by adding the doxycycline stock to the culture media at a final concentration of 0.8 µg/ml. Sorted or unsorted cells obtained from day 5 EBs were replated on gelatinized flasks cultured at 37°C in 5% CO₂ in EBM for 5 days, followed by one day in DMEM (Gibco) supplemented with 2% Horse Serum (Hyclone), penicillin/streptomycin and 2 mM glutamax to assess their differentiation into myotubes.

FACS analysis

Day 5 EBs were harvested, washed twice with PBS and then trypsinized for 1.5 min in a 37°C water-bath with continuous shaking. Trypsin was inactivated by adding 4 volumes of

PBS supplemented with 10% FBS (PBSF) and cells were resuspended and filtered through a 70 μ m strain to remove cell clumps. Cells were washed once with PBS and then incubated 5 minutes in PBSF in the presence of Fc Block (1 μ l/4 million cells – E-bioscience). Staining was performed by adding 0.5 μ l of each antibody per 1 million of cells and incubating for 25 min on ice. We used a PE-conjugated anti-mouse CD140a (PDGFR α) and an APC-conjugated anti-mouse CD309 (FLK-1) (both from E-bioscience). Cells were washed twice with PBS and then resuspended in PBSF containing propidium iodide to exclude dead cells. Samples were analyzed and sorted using a FACS Aria II (BD Biosciences).

RNA isolation and gene expression

Cells or total EBs were resuspended in Tryzol (Invitrogen) and processed following the manufacturer's instructions. RNAs from total EBs were retro-transcribed using Superscript (Invitrogen). RNAs from sorted cells were retro-transcribed using Superscript Vilo (Invitrogen). Gene expression analyses were performed using an amount of cDNA solution corresponding to 12.5 ng of starting RNA for each reaction. qRT-PCR were performed using TaqMan Universal PCR Master Mix and TaqMan probes (Applied Biosystems).

Western blot and immunofluorescence analysis

Proteins were extracted from Total EBs or single cells using RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, 1% Triton, 1% Sodium Deoxycholate, 0.1% SDS) supplemented of Protease inhibitors (Complete – Roche) and quantified with Bradford reagent (Sigma). Protein samples were prepared in Laemmli buffer and loaded on gels for SDS-PAGE. Proteins were transferred on PVDF membranes (Millipore) for the detection with the indicated antibodies. Immunofluorescence staining was performed by fixing cells with 4% Paraformaldehyde/PBS for 10 minutes at 4°C, then permeabilized with 0.1% Triton/PBS and blocked with 5% BSA/PBS before incubating with the primary antibodies. Samples were rinsed with PBS, blocked with 5% BSA/PBS and then incubated with the secondary antibody. After washing, samples were mounted on the slides using Prolong Gold with DAPI (Molecular Probes). Pictures were acquired with Axioimager M1 fluorescence microscope or LSM 510 Meta confocal microscope (Zeiss).

Antibodies used in this study included anti-MYOD (BD Biosciences – 554130), anti-MYOG (BD Biosciences – 556358), anti-PAX3 (C-term directed - Developmental Studies Hybridoma Bank), anti-PAX3 (N-term directed – R&D system), anti-GAPDH (Abcam – ab8245), anti-FLAG (Clone M2 - Sigma), anti-cTNI (Abcam – ab47003), HRP-conjugated anti-mouse and anti-rabbit IgG (Amersham), Alexa488-conjugated anti-rabbit IgG, and Alexa555-conjugated anti-mouse IgG (Molecular Probes).

Statistical analysis—Student's two-tailed *t* test was used to analyze differences between non-induced and dox-induced samples whereas differences between multiple samples were assessed by ANOVA.

RESULTS

Function of Pax3 in paraxial mesoderm and myogenic specification

Analyses of the molecular mechanisms involving Pax3 during embryogenesis have been limited by the availability of a cellular model able to mimic these early developmental stages. Because *in vitro* differentiation of mouse embryonic stem cells (mESC) mimics the formation of most mesoderm derivatives, their scalable growth properties open a window into mechanistic studies to understand the activity of transcription factors during the development of rare embryonic precursors. An important aspect to be considered is that

skeletal myogenesis is not properly recapitulated during the *in vitro* differentiation of ES cells into embryoid bodies (EBs), as evidenced by the very low levels of Pax3, Pax7 and MRFs (Darabi et al, 2008) (Figure S1A). We have previously shown that inefficient myogenesis can be bypassed by induction of Pax3 from day 2 of EB differentiation, when mesoderm first arises, resulting in the efficient activation of the myogenic program [20]. Interestingly, Pax3 induction also led to an increase in the frequency of PDGFR α ⁺FLK-1⁻ cells, a surface profile consistent with paraxial mesoderm [23]. To specifically elucidate whether this population induced by Pax3 is actually paraxial mesoderm, we made use of this Pax3 doxycycline-inducible ES cell line and analyzed by quantitative reverse transcription PCR (qRT-PCR) the expression levels of paraxial mesoderm and myogenic markers in total day 5 EBs, which were induced with doxycycline from day 2. As shown in Figures 1A and S1B, Pax3 induction indeed results in an overall increase of all paraxial mesoderm markers tested, including Mesogenin1 (Msn1), Delta-like1 (Dll1), FoxC1, Paraxis (Tcf15), Meox1, Tbx18, and Uncx4.1. Msn1 and Dll1 are mainly expressed in the posterior PSM [27, 28], where they regulate somitogenesis [29]. Similarly, other mesodermal markers are up-regulated after Pax3 induction. FoxC1 is necessary for the segmentation of the presomitic mesoderm and its differentiation [30], Paraxis (Tcf15) and Meox1 are both involved in the somite epithelialization [31, 32], and Tbx18 and Uncx4.1 represent respectively anterior and posterior somite markers [33, 34]. As expected, Pax3 induction resulted in up-regulation of c-Met and Myf5 (well-known target genes of Pax3) as well as M-cadherin (Cdh15), MHox (Prrx1) and Nr2f2 (COUP-TFII) (Figure 1A and Figure S1B). Myf5 and M-cad are both expressed in the myotome [7, 35]. MHox is detected in the dorsal dermomyotome and in the limb buds [36], whereas Nr2f2 is required for limb development [37]. At this time point, expression of MyoD and Myf6 is not yet detectable (data not shown).

The up-regulation of genes that are important for paraxial mesoderm development in day 5 total EBs suggests that Pax3 also plays a role in the stages that precede myotome formation. To identify which of these genes might be directly regulated by Pax3, we generated a bicistronic inducible cell line expressing Pax3 and GFP (iPax3-Ires-GFP). After dox treatment, this cell line allows for the purification of cells that express Pax3 within 6h of induction. As shown in Figure 1B, GFP positive cells can be identified and sorted by FACS and used for gene expression analysis. When compared to respective controls (Pax3-Ires-GFP no dox and ires-GFP no/+dox), we were able to identify three genes up-regulated in this short time window of Pax3 induction. As expected, Myf5, a well characterized Pax3 target [7], was one of them (Figure 1C). Among the paraxial mesoderm genes, we identified Meox1 and Dll1 as candidate targets of Pax3 (Figure 1C). Given the importance of these genes in somite formation, it is possible that Pax3 positively regulates their expression. The role of Pax3 in the regulation of these genes is supported by previous observations: i) a Pax3 binding site has been identified in rhabdomyosarcoma cells expressing PAX3-FKHR [16], and ii) expression of Dll1 is impaired in the somites of *spotch* homozygous mouse embryos at E9.5 [38].

These initial results prompted us to further investigate how markers of paraxial mesoderm and myogenesis were induced by Pax3 within different sub-populations of embryoid bodies. Taking advantage of the sub-division into lateral plate and paraxial mesoderm provided by the differential expression of PDGFR α and FLK-1 in the early stages of EB differentiation [23], we sorted these four populations (named P+F+, P+, F+ and NEG) from both Pax3-induced and non-induced day 5 EBs, as shown in Figure 2A. Since the purity of sorted cell populations is critical for the subsequent analysis, we re-analyzed each cell sorted fraction by FACS, which confirmed their purity above 95% (Figure S1C). Gene expression analysis on the sorted cell populations revealed that the majority of the genes (Meox1, Myf5, c-Met, Prrx1 and Nr2f2) up-regulated in the total EBs had a similar pattern of expression in the PDGFR α ⁺FLK-1⁻ (P+) fraction (Figure 2B). Interestingly this was not the case for genes

known to play a role in regulating somitogenesis, such as *Dll1*, *Mgn1* and *FoxC1*. These genes were instead up-regulated in the $\text{PDGFR}\alpha^+\text{FLK-1}^+$ (P+F+) and $\text{PDGFR}\alpha^-\text{FLK-1}^+$ (F+) fractions, suggesting that Pax3 may induce other mesodermal cells to gradually acquire a paraxial fate. As mentioned above, genes involved in the subsequent steps of differentiation (*Meox1*, *Paraxis*, *Myf5*, *c-Met*, *Prrx1* and *Nr2f2*) were mainly up-regulated in the $\text{PDGFR}\alpha^+\text{FLK-1}^-$ (P+) as well as the $\text{PDGFR}\alpha^+\text{FLK-1}^+$ (P+F+) cell fractions. In the case of *Paraxis*, up-regulation was also observed in the $\text{PDGFR}\alpha^-\text{FLK-1}^+$ (F+) fraction. As expected the expression of most of the paraxial/myogenic markers was significantly lower in the $\text{PDGFR}\alpha^-\text{FLK-1}^-$ (NEG) fraction. All together, these data suggest that Pax3 can induce the paraxial/myogenic program mainly within cells already restricted to the mesodermal lineage. The $\text{PDGFR}\alpha^+\text{FLK-1}^-$ paraxial mesoderm cell population that emerges from EBs recapitulates somitogenic gene expression, and is followed by differentiation towards the dermomyotome/myotome.

Maintained expression of Pax3 is necessary to induce the myogenic program

Based on the above results, we then aimed to determine whether the $\text{PDGFR}\alpha^+\text{FLK-1}^-$ cell fraction is fully committed to the myogenic lineage or represents a transitional plastic intermediate population. Our previous findings show that persistent induction of Pax3 drives the generation of a proliferating myogenic population that abundantly expresses Pax3 and *Myf5*, with up-regulation of *MyoD* under differentiation conditions [20]. Therefore, we performed a time course analysis by inducing Pax3 at two time windows, either during EB differentiation (d2–d5), or during the monolayer (d5–d10) stage, or both as outlined in Figure 3A. Continuous expression of Pax3 (dox/dox) resulted in *MyoD* and *MyoG* expression, and as expected, this was not observed in the non-induced group (cont/cont), as shown by immunofluorescence staining and RT-qPCR for *MyoD*, as well as western blot for *MyoG* (Figure 3B–D). Induction of Pax3 from day 5 (cont/dox), only at the monolayer stage, resulted in the expression of *MyoD* at levels comparable to the group that had been induced from day 2 (Figure 3B–D). On the other hand, when Pax3 induction was applied solely at the EB stage (D2–D5) (dox/cont), the Pax3 effect on *MyoD* expression was much reduced (Figure 3B–D). Doxycycline withdrawal after day 10 does not affect expression of *MyoD* at day 15 (Figure S2), suggesting that at this point, cells are fully committed to the skeletal myogenic lineage. Along these lines, *Myogenin*, which is associated with terminal differentiation, is slightly upregulated upon Pax3 removal, both at protein and mRNA level (Figure S2). These data suggest that the $\text{PDGFR}\alpha^+\text{FLK-1}^-$ cell fraction is not fully committed to the myogenic fate after only transient Pax3 exposure (D2–D5), while later withdrawal of Pax3 is associated with terminal differentiation, in agreement with our previous observations [20].

Deletional analysis of Pax3 domains

To dissect how the different domains of Pax3 play a role in muscle determination during EB differentiation, we generated different mouse ESC lines which allow the doxycycline-dependent induction of deletion mutants of Pax3 (as shown in Figure 4A). Besides mutants deleted for the whole paired domain (ΔPD), octapeptide ($\Delta 8$), homeodomain (ΔHD) and transactivation domain (ΔTAD), we generated a deletion mutant of the carboxy-terminal part of the paired domain ($\Delta\text{PD-C}$), also known as RED domain, and one missing a small conserved region of 40 aminoacids inside the TAD ($\Delta 352-391$) (Figure S3). Following the generation of each respective ESC line, Pax3 induction was confirmed by western blot using two Pax3 specific antibodies (Figure 4B). The intracellular localization of the deletion mutants was also assessed by confocal microscopy. To perform this analysis, we generated a further series of cell lines expressing Pax3 and its deletion mutant fused with the 3XFLAG tag. To avoid any interference due to the 3XFLAG sequence, we compared the myogenic differentiation of Pax3-FLAG to the unmodified Pax3, which demonstrated no differences

between these 2 cell lines (data not shown). All the Pax3 deletion mutants localized to the nucleus, in a similar fashion to the full-length (Figure 4C). Indeed, using nuclear localization signal (NLS) prediction softwares (NucPred and pSortII [39, 40]), the putative NLS represents a short region of 6–7 aminoacids N-terminal to homeodomain, which was not altered by any deletion mutant.

The RED domain is important for Pax3-mediated paraxial mesoderm induction

Since induction of Pax3 in this context increases the frequency of the PDGFR α ⁺FLK-1[−] cell fraction (Figures 2A, S1C and [20]), we therefore investigated the necessity of the different domains of Pax3 during mesoderm differentiation. FACS analysis (Figure 5A–B) shows that the frequency of PDGFR α ⁺FLK-1[−] cells is unaffected by deletion of the octapeptide ($\Delta 8$), suggesting that this sequence, although conserved in some Pax genes, is dispensable for Pax3 paraxial mesoderm functions. On the other hand, deletion of the paired domain (ΔPD) and the homeodomain (ΔHD) completely abolished Pax3 activity (Figure 5A–B), confirming the importance of both domains in Pax3-dependent transcriptional activity [41], and the inability of the paired domain only to bind the Pax3 target sequences. Similarly, deletion of the transactivation domain (ΔTAD) resulted in the complete loss of function of Pax3. Among the other mutants tested, deletion of the carboxy-terminal part of the paired domain ($\Delta PD-C$) decreased, but did not abolish, the induction of PDGFR α ⁺FLK-1[−] cells, indicating that this domain affects the function of Pax3 on paraxial mesoderm. Based on the crystal structure of the paired domain, the carboxy-terminal part is not involved in DNA binding and it has been proposed to be involved in protein-protein interaction [42]. However, the transactivation domain of Pax3 is not well characterized and therefore the structural basis of Pax3 function is not well understood.

To further investigate the effect of the deletion mutants of Pax3, we evaluated the expression levels of selected markers of paraxial mesoderm or skeletal myogenesis on total day 5 EBs (Figure 6). In agreement with the FACS data, Pax3 and Pax3 $\Delta 8$ have a comparable ability to induce Myf5 and c-Met, two well characterized targets of Pax3 [6, 7], as well as M-cadherin and Paraxis, confirming the dispensability of the octapeptide. On the other hand, Pax3 ΔPD , ΔHD and ΔTAD completely lack any ability to induce the paraxial/myogenic markers. Interestingly, deletion of the RED domain ($\Delta PD-C$) drastically impairs the induction of Myf5, M-cadherin and Paraxis and this is consistent with the reduced ability of this mutant to induce the PDGFR α ⁺FLK-1[−] cell fraction. However the $\Delta PD-C$ mutant was able to induce c-Met as efficiently as wild-type Pax3, suggesting that this mutation might not completely inactivate Pax3, and that this domain might have a distinct function.

To determine effects of Pax3 and mutants on other lineages, we analyzed the expression of additional markers to neural crest/melanocyte (Mitf), chondrogenic (Runx2), cardiac (Nkx2-5) and hematopoietic (Runx1) development (Figure S4). Mitf is a well-known target of Pax3 in the neural tube [18], and was induced by WT, $\Delta 8$, and $\Delta PD-C$ versions of Pax3 (Figure S4). Runx2 is a chondrogenic marker expressed in the sclerotome-derived population [43]. Consistent with the general increase of paraxial mesoderm caused by Pax3, Runx2 was induced by the same constructs (Figure S4). This further supports a role for Pax3 in early somite patterning, as suggested by analysis of *spotch* mouse embryos [38, 44]. Among the other mesodermal lineages, induction of WT, $\Delta 8$, and $\Delta PD-C$ versions of Pax3 during EB differentiation resulted in decreased expression of Nkx2-5 (Figure S4), an early marker of cardiac development [45]. In all cases, the HD, PD, and TAD domain deletions abolished the ability to change gene expression (Figure S4). In terms of hematopoietic development, no major differences were observed at the levels of Runx1 (Figure S4).

Myogenic activity of Pax3 is not compromised by deletion of the RED domain

Although the up-regulation of Myf5 is indicative of the activation of the myogenic program, irreversible commitment is achieved only by continuous induction of Pax3, ultimately leading to MyoD and MyoG expression (Figure 3). To determine the ability of these deletion mutants to fully induce the myogenic program, day 5 EBs were plated as monolayers and cultured for 5 days, at which point cells were analyzed by immunofluorescence and western blot (Figure 7A). As shown in Figure 7B, Pax3-induced cells were positive for MyoD, while control non-induced cultures did not express this transcription factor. These results were confirmed by western blot analysis (Figure 7C). The mutations that abolished the ability to induce the PDGFR α ⁺FLK-1⁻ fraction and the paraxial/myogenic markers (as shown in Figure 5 and 6) also abolished myogenic activity. Interestingly, the Δ PD-C mutation showed a slight decrease in expression of MyoD (Figure 7B–C). As mentioned above, Pax3 induction negatively affects the expression of Nkx2-5 (Figure S4) and ultimately results in the repression of the cardiac program as shown by immunofluorescence and western blot for cTnI (Figure S5). Similar results were reported by Quattrocchi and colleagues [46]. Notably, the ability of Pax3 to repress the cardiac program inversely correlates with the skeletal myogenic activity of the Pax3 mutants.

We reasoned that mutations specifically interfering with paraxial mesoderm specification but not myogenesis would have such a weak readout in this assay. To test whether the RED domain is required for myogenic commitment, we sorted PDGFR α ⁺FLK-1⁻ cells from both Pax3 and Pax3 Δ PD-C day 5 EBs and cultured sorted presumptive paraxial mesoderm in monolayer. Interestingly, after 5 days of differentiation, we observed that Pax3 bearing the Δ PD-C mutation was able to induce the terminal differentiation at levels comparable to the full length Pax3, as shown by immunostaining for Myogenin and western blot of MyoD and Myogenin (Figure 7D and E). This indicates that the C-terminal non-DNA-binding part of the paired domain plays an essential role in the early activity on paraxial mesoderm, but is dispensable for myogenesis.

DISCUSSION

In this study we investigated the role of the transcription factor Pax3 in paraxial mesoderm formation and early myogenesis using differentiating mouse embryonic stem cells. Using this system, we show that cells derived from embryoid bodies have the potential to differentiate toward the skeletal muscle lineage in a process that depends upon maintained Pax3 expression. This is preceded by up-regulation of several somite marker genes, particularly Paraxis and Meox1, which suggests that Pax3 drives the generation of an intermediate population with features similar to the epithelializing somite population. In addition, Pax3 expression resulted in the induction of Myf5, c-Met, Nr2f2 and Prrx1 but not MyoD or Myf6, indicating that Pax3 drives cell fate changes that mimic the events happening in the early myotome. Interestingly, we observed that early markers of paraxial mesoderm (Dll1, Msn1 and FoxC1) were up-regulated in cells representing lateral and cardiac mesodermal fractions (PDGFR α ⁻FLK-1⁺ and PDGFR α ⁺FLK-1⁺, respectively), while markers associated with the epithelial somite (Meox1 and Tcf15) and myotome formation (Myf5, c-Met, Prrx1 and Nr2f2) were mainly up-regulated in the presumptive cardiac and paraxial mesodermal fractions (PDGFR α ⁺FLK-1⁺ and PDGFR α ⁺FLK-1⁻, respectively). These findings suggest that Pax3 might induce the other mesodermal populations to acquire a paraxial fate and subsequently commit them toward the myogenic lineage. This observation is also in agreement with a previous report showing the ability of Baf60c to induce the cardiac tissue from mouse mesoderm [47], suggesting these cells represent a non-committed intermediate able to generate different lineages. Although Pax3-induced EBs become primed, maintained expression of Pax3 is required to drive commitment to the myogenic lineage.

Because Pax3 null embryos show defects in both skeletal muscles and vertebrae [38, 44], it has been proposed that Pax3 might be involved in the process of epithelialization of the somite. Using the ES/EB system we show up-regulation of paraxial mesodermal genes following Pax3 induction, with a particular induction of the epithelial somite markers Meox1 and Paraxis, and the myogenic genes Myf5 and c-Met. Notably, our approach indentified Dll1 and Meox1 as potential candidate targets of Pax3, even though more analyses are needed to clarify the role of Pax3 in this process. These findings are supported by *in vivo* studies using homozygous *splotch* E10.5 embryos, which showed that the expression levels of Paraxis as well as Myf5 and c-Met in the somites were much reduced in the absence of Pax3 [44], while expression of Dll1 was perturbed [38]. Moreover, a Pax3 binding site was reported in the promoter region of the Meox1 gene [16]. Another interesting finding of the present study was the up-regulation of Nr2f2 and Prrx1. This indicates that in the presence of Pax3, differentiating cells express markers characteristic of the delaminating dermomyotome [36, 37]. Therefore, our data support the involvement of Pax3 in the process of somite differentiation prior to myogenic commitment and provide a cellular model in which to study the molecular mechanism underlying Pax3 function in both the epithelial somite and the dermomyotome.

Our functional analysis using deletion mutants of Pax3 evaluated the requirement for each conserved domain of Pax3 during the induction of both paraxial mesoderm and skeletal muscle differentiation. All the mutants are able to localize to the nucleus, similarly to the full length protein. *In silico* analyses suggested the presence of a small nuclear localization signal immediately N-terminal to the homeodomain sequence. Nevertheless, since our deletion strategy excluded some regions of Pax3 (before the PD and between PD and HD), we cannot rule out the possibility that Pax3 localizes to the nucleus upon interaction with a co-factor. Mutants lacking the paired domain or homeodomain were unable to efficiently induce the myogenic program, and these results confirm previous structural studies suggesting a critical role exerted by Pax3 DNA binding domains and the importance of their interaction [29, 41]. Similarly, deletion of the transactivation domain completely abolished Pax3 activity or, as in the case of the $\Delta 352-391$ deletion, strongly impaired it. On the other hand, although the octapeptide is conserved in several members of the Pax family of transcription factors, and reported to interact with the transcriptional repressor Groucho [14], deletion of this sequence did not affect Pax3 function in any of our assays. This discrepancy could be explained by a different requirement of binding to Groucho between the Pax proteins, since this interaction was reported for the subgroup Pax2/5/8. Our results corroborate the hypothesis raised by Relaix and colleagues [48], which proposes that Pax3 functions principally as a transcriptional activator during embryogenesis.

Expression of Pax3 during EB differentiation resulted also in the repression of the cardiac program, as evidenced by down-regulation of Nkx2-5 and cardiac Troponin I (cTnI). This effect correlated with the skeletal myogenic activity of Pax3 since deletion mutants showed a different ability to repress the cardiac program. These findings are corroborated by similar observations published recently by Quattrocelli and colleagues [46].

Pax3 function in promoting paraxial mesoderm gene expression and induction of PDGFR α ⁺Flk1⁻ cells was impaired by deletion of the C-terminal part of the paired domain, the so-called RED domain. Surprisingly, the RED domain was not required for myogenesis, since PDGFR α ⁺Flk1⁻ presumptive paraxial mesoderm cells expressing Pax3 Δ PD-C were able to differentiate similarly to the full-length Pax3. As shown in the crystal structure of the paired domain [42], and subsequently confirmed by other studies, the RED domain is not in direct contact with DNA. We speculate that this domain interacts with protein co-factors that are specific to paraxial mesoderm formation and not required or absent in later myogenic

specification. Identifying the co-factors that interact with this domain may illuminate the context-specific differential effects of Pax3 expression.

CONCLUSIONS

Our study shows that using inducible mouse ES cells we can recapitulate early stages of paraxial mesoderm differentiation, allowing us to study the early function of Pax3. Interestingly, Pax3 up-regulates the epithelial somite markers Meox1 and Paraxis, supporting the involvement of this transcription factor in the process of somite epithelialization. Moreover, we conclude that the ability of Pax3 to induce myogenesis is mainly restricted to mesoderm, via the generation of PDGFR α ⁺FLK-1⁻ cells. Time course analysis demonstrated that Pax3 induction is critical to promote the commitment of PDGFR α ⁺FLK-1⁻ cells toward the myogenic lineage, suggesting a distinct function of Pax3 in the induction of paraxial mesoderm vs. the myogenic lineage. Finally, our deletion analysis identifies the RED domain (Pax3 Δ PD-C) as an important determinant for Pax3 function in paraxial mesoderm formation, and thus represents a candidate region to identify co-factors specific to early activity of Pax3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Pax3 is necessary for the commitment of ES cells toward the paraxial mesoderm cell fate and skeletal myogenic lineage. A) Paraxial mesoderm and myogenic markers are up-regulated by Pax3 induction, as shown by quantitative RT-PCR analysis of total day 5 EBs. Results were normalized to Gapdh endogenous levels. Graphs represent the mean $\pm$ SD of at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B) FACS plots show GFP expression following a short window of dox induction (6 hours) in induced and non-induced iPax3-Ires-GFP and Ires-GFP (empty vector control) day 5 EBs. C) Expression of Myf5, Meox1 and Dll1 is up-regulated within 6h of Pax3 induction, as shown by quantitative RT-PCR analysis performed on sorted cells (B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2.

A) FACS profile of total and sub-fractionated day 5 EBs from dox-induced and non-induced cultures. Cells were stained with anti-PDGFR α and anti-FLK-1 antibodies, sorted and re-analyzed to evaluate their purity. PDGFR α ⁺FLK-1⁺ (P+F+), PDGFR α ⁺FLK-1⁻ (P+), PDGFR α ⁻FLK-1⁺ (F+), PDGFR α ⁻FLK-1⁻ (NEG). The average frequency of cells in each sub-fraction is reported as percentage in the respective graph. B) Quantitative RT-PCR analysis of sorted day 5 EBs sub-fractions. Following Pax3 induction, paraxial mesoderm and myogenic genes were mainly up-regulated in P+F+ and P+ fractions. Graphs represent the mean $\pm$ SD of at least 3 independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

Figure 3.

Temporal windows of Pax3-responsiveness during EB differentiation. A) Outline of time windows of Pax3 induction during EB differentiation (D2–D5) and monolayer stage (D5–D10). The following 4 experimental groups were assessed: i) no dox added in any differentiation stage (cont/cont), ii) dox added in both stages, EB and monolayer, from day 2 to day 10 (dox/dox), iii) dox added only from day 5 at the monolayer stage (cont/dox), and iv) dox added only at the EB stage, from day 2 to day 5 (dox/cont). B–D) Pax3 is necessary to drive the myogenic commitment of mesodermal precursors. In all experimental arms, day 5 EBs were sorted for PDGFR α ⁺FLK-1[−] cells, and then cultured as monolayer, on gelatin-coated glass coverslips for additional 5 days (D5–D10): the first 4 days in the presence of

proliferation medium, and in the last 24 hours in differentiation myogenic medium to induce myotubes formation, at which point samples were analyzed by immunofluorescence staining (B), real time PCR analyses (C) or western blot (D). Pax3 was induced as outlined in (A). (B) Immunofluorescence staining for MYOD. Cells were fixed and stained with anti-MYOD (red) antibody and Dapi (nuclei – blue). Pax3 induction at the monolayer stage results in expression levels of MYOD that are comparable to continuous induction. Bar: 50µm. C) MyoD expression by RT-qPCR analysis. Results were normalized to Gapdh. Graphs represent the mean $\pm$ SD of at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$. D) Western blot analyses for MYOG and PAX3. Samples were lysed with RIPA buffer and analyzed with the indicated antibodies. Histone H3 antibody was used for loading control. Cont/Cont (lane 1), Dox/Dox (lane 2), Cont/Dox (lane 3) and Dox/Cont (lane 4).

Figure 4.

Generation of Pax3 deletion mutants. A) Schematic representation of Pax3 mutant constructs used to generate inducible ES cell lines (PD: paired domain, 8: octapeptide, HD: homeodomain, TAD: transactivation domain). B) Expression of the Pax3 mutant genes upon Dox induction during EB differentiation of inducible ES cell lines. Day 5 EBs were lysed with RIPA buffer and protein extracts were analyzed by western blot using anti-GAPDH and anti-PAX3 antibodies directed toward the amino- (lanes 11–16) and carboxy-terminals (lanes 1–10). Pax3 (lanes 1, 6, 11 and 14); Pax3Δ8 (lanes 2 and 7); Pax3 ΔPD-C (lanes 3 and 8); Pax3 ΔHD-C (lanes 4 and 9); Pax3 ΔPD (lanes 5 and 10); Pax3 ΔTAD (lanes 12 and 15); Pax3 Δ352-391 (lanes 13 and 16). C) Day 5 EBs were trypsinized, plated on glass

coverslips in the presence of dox, and after 2 days, cells were fixed and subjected to immunofluorescence staining with anti-PAX3 antibody. Pictures were acquired with a confocal microscope. Bar: 10 μ m.

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Figure 5.

Effects of Pax3 mutants on mesoderm patterning. A) A representative FACS profile for PDGFRα and FLK-1 in induced and non-induced ES cells encoding wild-type Pax3 or Pax3 mutated on the selected domains at EB day 5. Transgene expression was induced by adding dox to the culture medium from day 2. Fluorescence intensity for FLK-1 is indicated on the y axis and PDGFRα on the x axis. B) Frequency of paraxial mesoderm progenitors, measured by the percentage of PDGFRα⁺FLK-1⁻ cells. Data represent mean ±SD of 3 independent experiments. * p<0.05.

Figure 6.

Gene expression analysis of Pax3 mutants. Relative levels of Myf5, M-cadherin, c-Met, and Paraxis in day 5 EBs. Transcripts are normalized to Gapdh, and reported as relative expression to the non-induced sample. The graphs report the mean $\pm$ SD from at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 7.

Identification of critical domains for Pax3 activity on the myogenic program. A) Schematic representation of the experiment layout. To investigate the ability of Pax3 to induce the paraxial mesoderm and the myogenic lineage, unsorted (panel B and C) or sorted PDGFR α ⁺FLK-1⁻ cells (panel D and E) were plated as monolayer on gelatin-coated glass coverslips and cultured for 4 days in proliferation medium and 1 day in differentiation medium. B) Cells were fixed and stained with anti-MYOD (red) antibody and Dapi (nuclei – blue). Samples were analyzed by epifluorescence microscopy. Bar: 100 μ m. C) Similarly, cells from differentiated cultures were analyzed by western blot for the expression of MYOD. Cells were lysed with RIPA buffer and protein extracts were analyzed using anti-

MYOD, anti-GAPDH antibodies. Pax3 (lanes 1, 6, 11 and 14); Pax3 Δ 8 (lanes 2 and 7); Pax3 Δ PD-C (lanes 3 and 8); Pax3 Δ HD-C (lanes 4 and 9); Pax3 Δ PD (lanes 5 and 10); Pax3 Δ TAD (lanes 12 and 15); Pax3 Δ 352-391 (lanes 13 and 16). D) Immunostaining with anti-MYOG antibody (red) and Dapi (nuclei – blue) of differentiated sorted PDGFR α ⁺FLK-1⁻ cells from dox-treated Pax3 and Pax3 Δ PD-C cell lines. Bar: 100 μ m. E) Similarly to panel D, differentiated cultures were analyzed by western blot with anti-MYOD, anti-MYOG and anti-PAX3 antibodies. Pax3 (lane 1) and Pax3 Δ PD-C (lane 2).

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