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SHORT REPORT

Visceral endoderm induces specification of cardiomyocytes in mice

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Abstract The endoderm plays an inductive role in the formation of cardiomyocytes in many vertebrates. Here, we provide further evidence for this in the mouse and demonstrate enhanced cardiomyogenesis in mouse embryonic stem cells cultured in the presence of native visceral endoderm. Isolated mesoderm from late-primitive streak stage mouse embryos that still have an open proamniotic canal had a reduced capacity to form cardiomyocytes after 4 days in culture compared with mesoderm isolated from later stages but prior to cardiomyogenesis. Moreover, removal of the visceral endoderm but not the primitive streak reduced the formation of beating areas in embryo explants in culture. Coculture with the END2 cell line, which has visceral endoderm-like properties, restored the formation of beating areas. Immunohistochemical analysis showed that the expected candidate signaling pathways downstream of Wnts and bone morphogenetic proteins (BMPs) were active in the embryo at the appropriate time and place to be involved. Overall, the results show that, as in other vertebrates, the (visceral) endoderm plays an important role in the early events of mouse cardiomyogenesis.
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Introduction

For stem cell-based replacement to become part of clinical therapy, it is essential for directed differentiation to be properly and optimally controlled. In-depth understanding of analogous processes during development can provide vital clues on how best to recapitulate stepwise differentiation choices *in vitro* so that appropriate cell products become available for transplantation.

In vertebrates, the heart is the first organ to become functional, contracting rhythmically to pump blood through the developing embryo soon after gastrulation, when the three germ layers, endoderm, mesoderm and ectoderm, form. In lower vertebrates (*Xenopus*, zebrafish, and chicken), it has clearly been shown that heart development requires the adjacent endoderm (Lough and Sugi, 2000; Zaffran and Frasch, 2002). Nascone and Mercola showed in transplantation studies that both the endoderm and the Spemann organizer are necessary for the induction of *Xenopus* heart tissue (Nascone and Mercola, 1995). Similarly, Schultheiss and colleagues could induce cardiomyogenesis in noncardiogenic tissue in chicken by coexplanting this with

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anterior lateral definitive endoderm (Schultheiss et al., 1995). In *Xenopus* and in chicken, cardiac induction occurs during gastrulation (Nascone and Mercola, 1995; Ladd et al., 1998). In mammals, however, the importance of endoderm has never been formally demonstrated, although endoderm overlays the cardiogenic mesoderm throughout gastrulation, at least in mice.

Clonal analysis and fate-mapping studies have shown that the majority of the cells that form the heart are present in the posterior region of the lateral epiblast at embryonic day (E)6.5 and that at the mid-primitive streak (MS) stage around E7.0, these cells ingress though the primitive streak as it elongates to form the lateral plate mesoderm at each side of the embryo (Lawson et al., 1991; Parameswaran and Tam, 1995). Between MS and late-primitive streak (LS) (Fig. 1A), the presumptive cardiac mesoderm is displaced from distal-posterior to a proximal-anterior position in the embryo to form the paired heart-forming fields or cardiac crescent. Interestingly, there is a concerted movement of presumptive cardiac mesoderm with the overlying foregut endoderm (Lawson and Pedersen, 1987; Tam et al., 1997), suggesting that there may be interactions between endoderm and presumptive cardiac mesoderm.

The END2 cell line is derived from mouse P19 embryonal carcinoma (EC) cells and has properties similar to visceral endoderm. Previously it has been demonstrated that co-culture of a variety of mouse and human pluripotent stem cell lines with END2 cells resulted in the formation of beating cardiomyocytes (Mummery et al., 2002; Mummery et al., 2003, 1991). Here we examined the role of visceral endoderm in inducing cardiac commitment during mouse embryonic development, extending on previous work by others (Arai et al., 1997; Auda-Boucher et al., 2000) and compared native visceral endoderm with END2 cells for functional interchangeability in both mouse embryos stripped of visceral endoderm and in mouse embryonic stem cells (mESCs).

Results and discussion

After amnion closure, mesoderm is committed to form beating cardiomyocytes

Extending the findings of Arai et al. (Arai et al., 1997) and Auda-Bucher et al. (Auda-Boucher et al., 2000), we cultured mesoderm isolated from mouse embryos from E6.5 to E7.5, staged as early primitive streak (ES), early primitive streak plus (ES+), mid-primitive streak (MS), late-primitive streak (LS), early allantoic bud (EB), and late allantoic bud (LB) and scored the emergence of beating areas during the nine subsequent days. To avoid misinterpretation due to the slightly different developmental stages, the embryos were staged and pooled into three categories. ES/ES+ embryos showed mesoderm formation until 2/3 of the length of the epiblast, MS/LS embryos showed mesoderm covering more than 2/3 of the length of the epiblast and had a proamniotic canal clearly open, and EB/LB embryos had a closed amnion and a visible allantoic bud (Fig. 1A).

After 4 days in culture, the vast majority of mesodermal explants from EB/LB embryos exhibited areas of beating muscle, whereas the majority of the mesoderm explants isolated from either ES/ES+ or MS/LS embryos did not (Fig. 1B). The contractile cardiomyocytes phenotype was confirmed by the detection of alpha-actinin, which links actin filaments to each other in both skeletal and cardiac muscle cells. In the MS/LS mesodermal explants that exhibited areas of contraction alpha-actinin were detected (Figs. 1C and D) while it was absent in the explants that showed no contraction (Fig. 1E). The spacing between the sarcomeres and total length and width were similar to sarcomeres in cardiomyocytes isolated from E9.5 mouse hearts (Fig. 1F), suggesting that the formation and organization of the sarcomeres in the cardiomyocytes derived from the MS/LS mesodermal explants *in vitro* after 4 days in culture were relatively normal. After 9 days in culture, the number of MS/LS mesodermal explants exhibiting areas of beating muscle remained similar ($<20\%$). We conclude that mesoderm isolated from ES-LS embryos has a reduced capacity to form cardiomyocytes in culture. This is in contrast to mesoderm isolated from embryos with a closed amnion, which seem to form areas of beating muscle autonomously.

Our results are in agreement with those reported previously (Arai et al., 1997; Auda-Boucher et al., 2000). Although in those studies different nomenclature was used to stage embryos, it is clear that the major step in cardiac determination occurs at the time when the proamniotic canal closes. Moreover, prolonged culture (9 days) does not enhance the formation of beating areas in culture and therefore there seems to be a true impediment to the formation of cardiomyocytes, rather than a delay due to culture conditions.

In the absence of visceral endoderm, ES-LS embryo explants show reduced capacity to form areas of beating muscle

To investigate whether the visceral endoderm was necessary to induce beating areas in ES-LS embryos after 4 days of culture, we isolated E6.0/pre-primitive streak, ES/ES+ and MS/LS embryos and cultured the embryonic part of the embryo with or without the visceral endoderm, the primitive streak, or both. The culture of the whole embryonic part with or without the primitive streak resulted in the formation of beating areas in most explants from ES/ES+ and MS/LS embryos (Fig. 2A). The absence of the visceral endoderm, however, caused a striking decrease in the number of explants with beating areas. Explants from embryos cultured without visceral endoderm containing no areas of beating muscle also showed no immunostaining for alpha-actinin (Figs. 2B and C). In contrast, explants cultured with visceral endoderm showed alpha-actinin immunostaining in well-organized sarcomeres (Figs. 2D–G). This suggests that mesoderm from ES-LS embryos is highly dependent on the presence of the visceral endoderm and not on the presence of ectoderm or the primitive streak to form beating areas.

Signaling pathways active during cardiac induction in the embryo

Multiple studies largely in lower vertebrates have suggested that cardiogenic signals from both the ectoderm

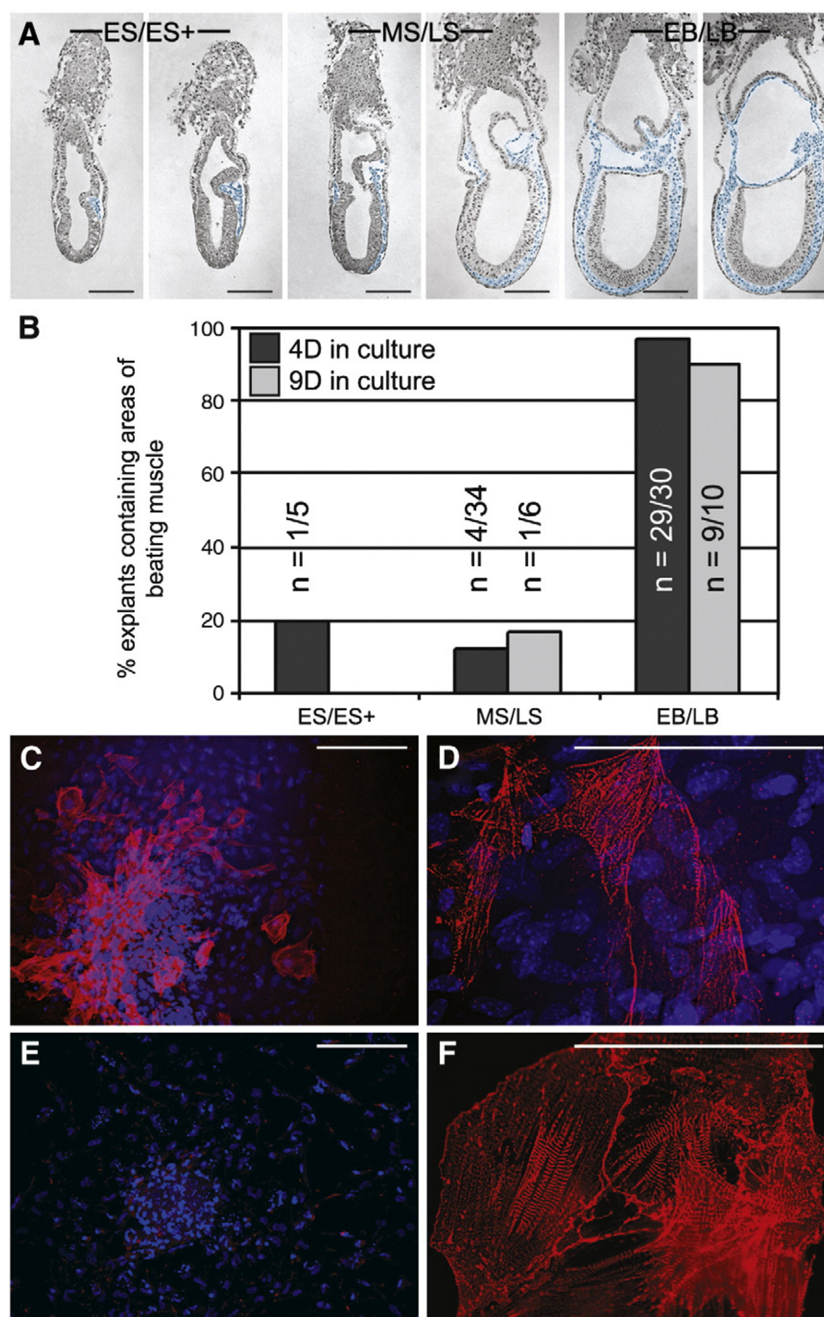

Figure 1 The potential of isolated mesoderm to generate cardiomyocytes in culture. (A) Sagittal sections of mouse embryos at different stages of development. Mouse embryos were staged and pooled into the categories ES/ES+ (early primitive streak, early primitive streak plus), MS/LS (mid-primitive streak/late-primitive streak), and EB/LB (early allantoic bud/late allantoic bud). Note the differences in mesoderm development that characterize each developmental stage (mesoderm is depicted in blue using pseudo colors). Figure adapted from (Chuva de Sousa Lopes and Mummery, 2004). (B) Graph indicating the percentage of explants of isolated mesoderm from ES/ES+, MS/LS, and EB/LB embryos that exhibited areas of beating muscle after 4 and 9 days in culture. n is the number of beating explants per total number of explants analyzed. (C, D) Immunostaining for alpha-actinin (red) of a MS/LS mesoderm explant that contained areas of beating muscle after 4 days in culture. D shows sarcomeres (alpha-actinin positive) at a higher magnification. Blue staining is DAPI. (E) Immunostaining for alpha-actinin (red) of a MS/LS mesoderm explant that did not contain areas of beating muscle after 4 days in culture, showing absence of alpha-actinin. Blue staining is DAPI. (F) Cardiomyocytes isolated from an E9.5 mouse heart stained for alpha-actinin (red), showing the characteristic shape of the sarcomeres. Scale bars are 100 μm.

and the endoderm surrounding the presumptive cardiac mesoderm could include the canonical Wnt signaling, non-canonical Wnt signaling, and BMP signaling (reviewed in (Filipczyk et al., 2007; Solloway and Harvey, 2003)). Therefore, we examined the localization of beta-catenin, phosphorylated JNK (P-JNK) and phosphorylated Smad1,5,8

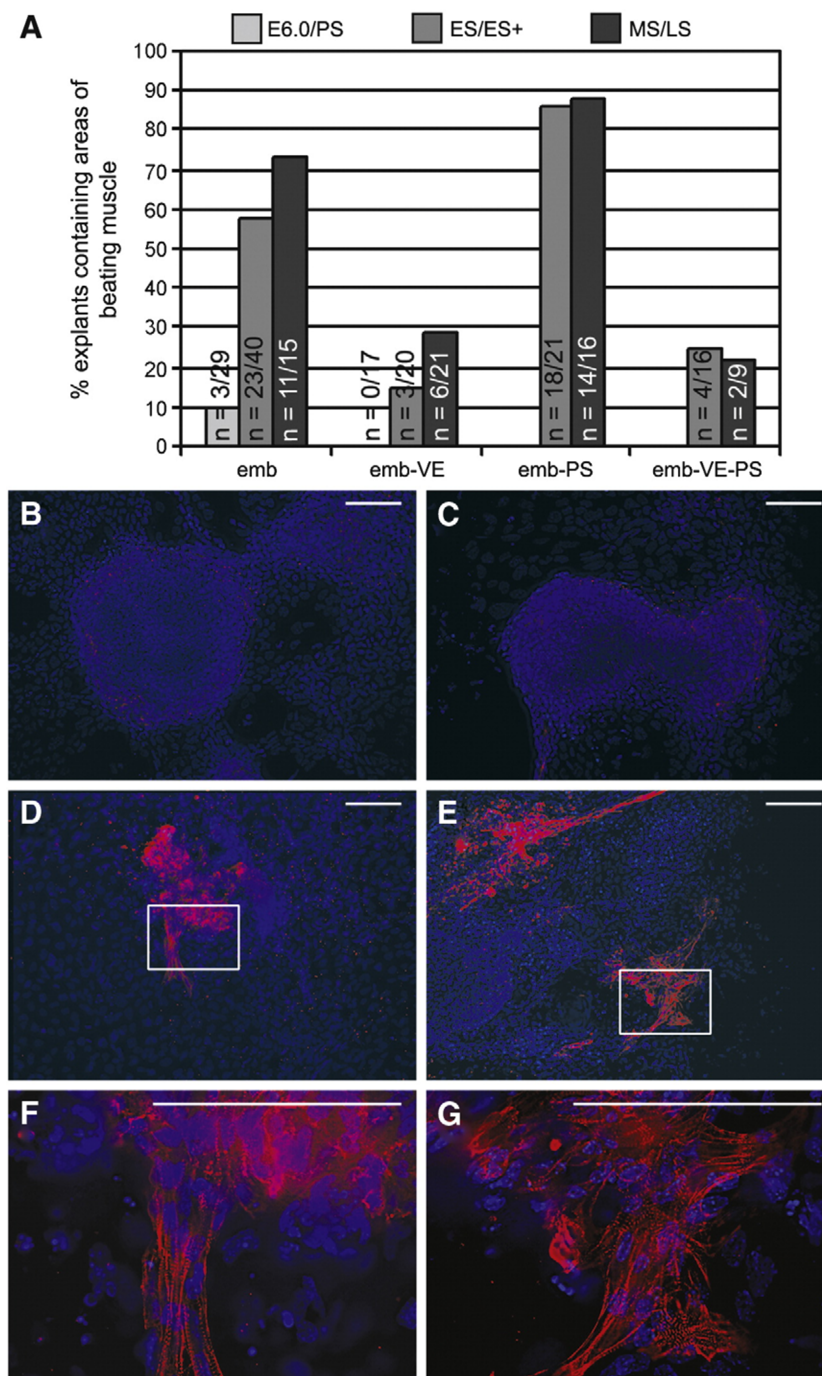

Figure 2 The visceral endoderm is important for the generation of beating areas in the mouse embryo during gastrulation. (A) Graph indicating the percentage of embryonic explants that exhibited areas of beating muscle after 4 days in culture. The developmental stages used were embryonic day (E)6.0/pre-primitive streak (PS), ES/ES+ (early primitive streak, early primitive streak plus), and MS/LS (mid-primitive streak/late-primitive streak). Embryos were cultured whole (emb), without the visceral endoderm (emb-VE), without the primitive streak (emb-PS), or without both (emb-VE-PS). n is the number of beating explants per total number of explants analyzed. (B, C) MS/LS embryonic explants cultured 4 days without the visceral endoderm (B) and without both the visceral endoderm and primitive streak (C), immunostained for alpha-actinin (red), showing no sarcomeric structures. Blue staining is DAPI. (D–G) MS/LS embryonic explants cultured 4 days whole (D, F) and without the primitive streak (E, G), immunostained for alpha-actinin (red), showing sarcomeric structures. Blue staining is DAPI. F and G are magnified views of the white box in D and E, respectively. Blue staining is DAPI. Scale bars are 100 μm.

(P-Smad1/5/8), as a reflection of active canonical WNT signaling, noncanonical WNT signaling, and BMP signaling, respectively.

Active BMP signaling (P-Smad1/5/8) was observed as expected in the developing mesoderm from E6.5 to E8.5 (Fig. 3), suggesting a direct role of the BMP pathway in early

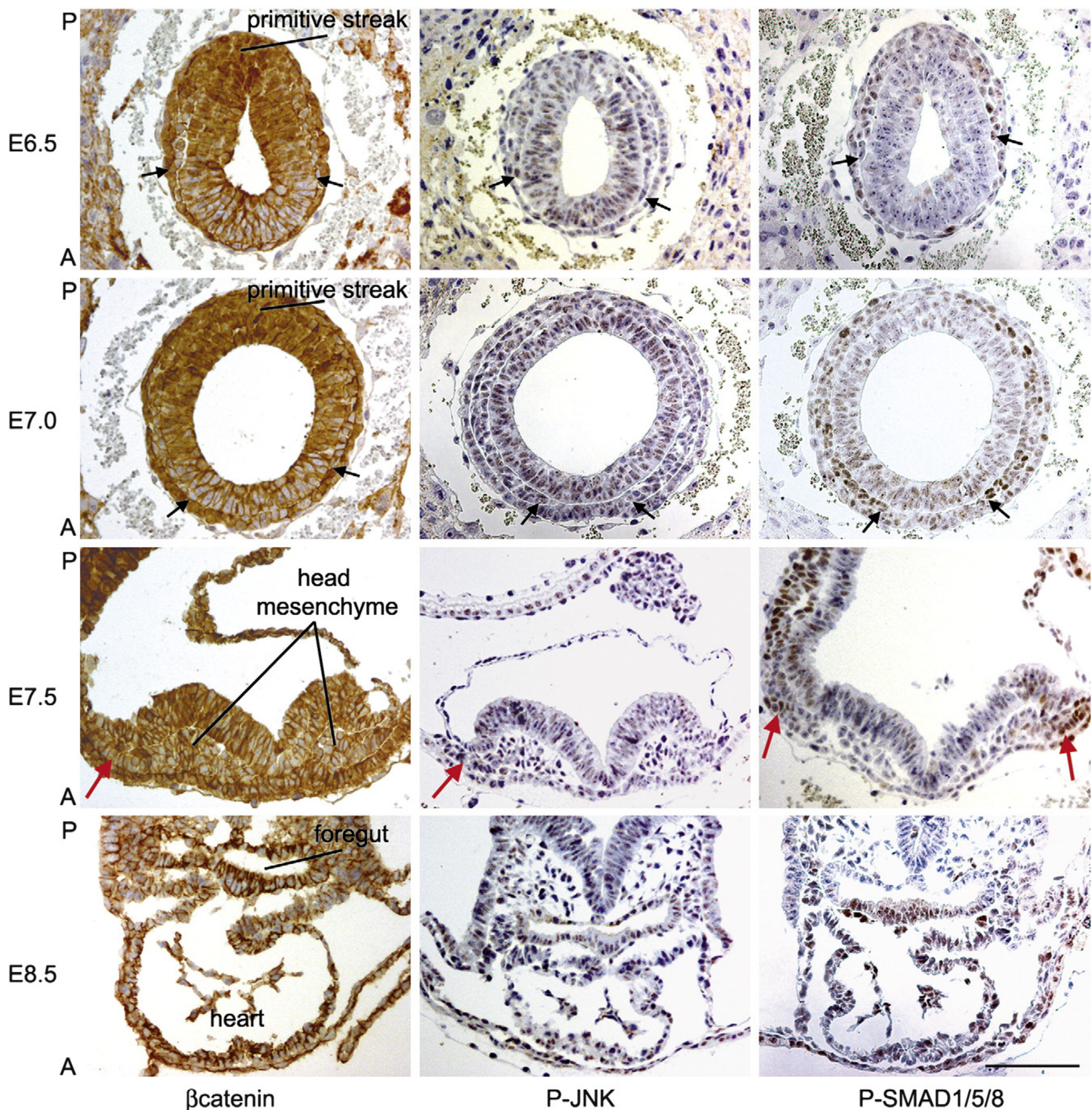

Figure 3 Signaling pathway activation in mice between E6.5 and E8.5. Transverse sections of E6.5, E7.0, E7.5, and E8.5 mouse embryos showing the circumnavegating mesoderm (see black arrows), cardiac crescent (red arrows), and forming heart (at E8.5) immunostained for beta-catenin, P-JNK, and P-Smad1/5/8. P is the posterior part and A the anterior part of the embryo. Scale bar is 100 μm.

cardiac induction in the mouse embryo. In agreement, the conditional ablation of BMP signaling, via the deletion of the BMP receptor I, in MesP1-positive cardiac progenitors resulted in the absence of a cardiac crescent (Klaus et al., 2007). Moreover, net BMP signaling was indeed observed in the cardiac crescent of transgenic mice that report in real time the transcriptional response to BMP-Smads activation (Monteiro et al., 2008).

We observed that beta-catenin was nuclear in the posterior region of the embryo, but cytoplasmic in the anterior region from E6.5 to E8.5 (Fig. 3). Interestingly, beta-catenin seems to be nuclear in the visceral endoderm at E6.5—E7.0 MS/LS embryos suggesting active WNT signaling in the visceral endoderm. The presence of beta-catenin in the visceral endoderm has been shown to be important to repress heart-fate (Lickert et al., 2002), whereas expression in MesP1-positive cardiac progenitors does not seem to be necessary for the formation of the cardiac crescent (Klaus et al., 2007). P-JNK was observed overall in a salt-and-pepper pattern from E6.5 to E8.5 (Fig. 3), adding to the still unclear role of the noncanonical WNT signaling during early cardiomyogenesis.

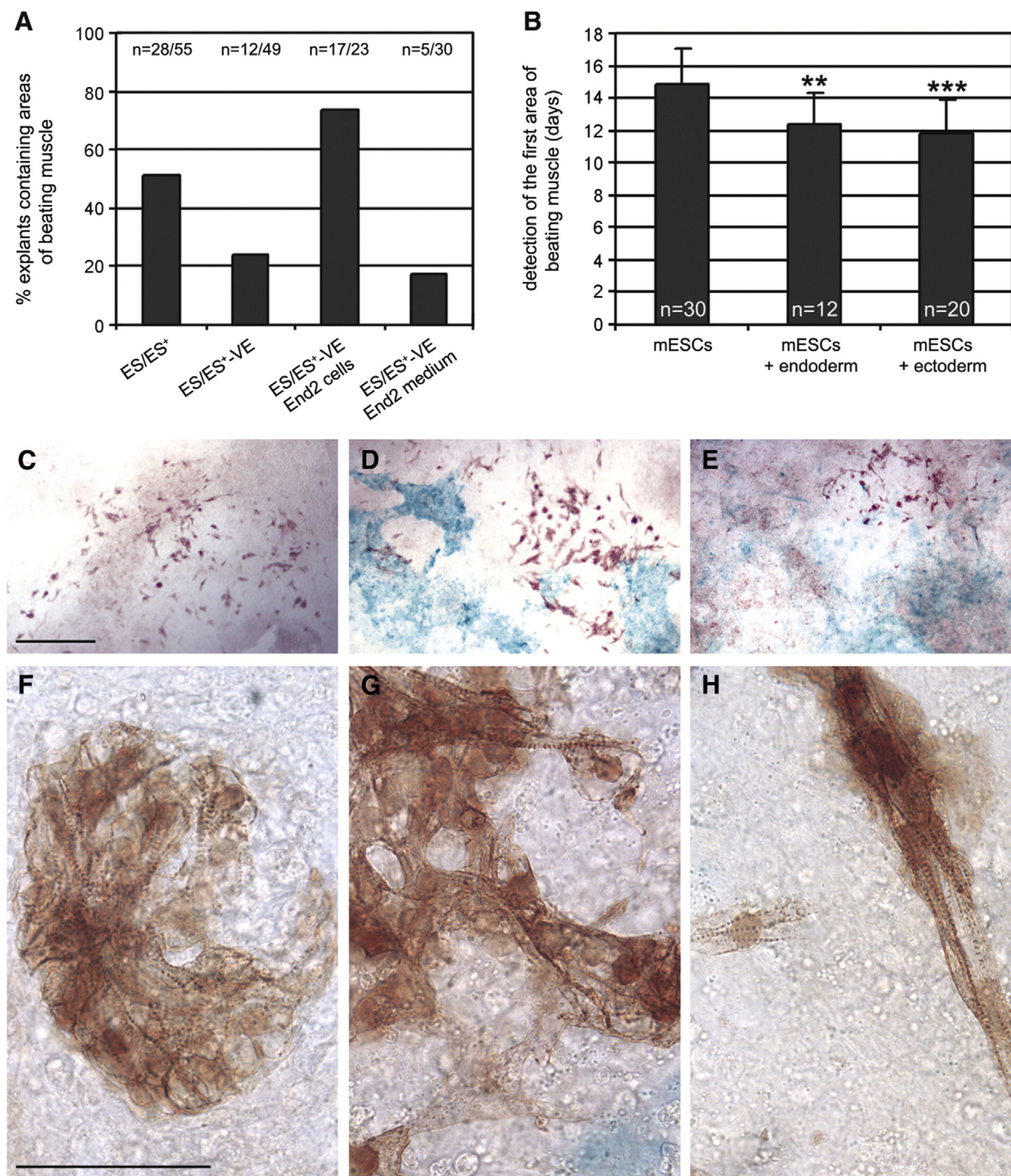

Figure 4 Endoderm induces cardiomyocyte formation in culture. (A) Graph indicating the percentage of embryonic explants from ES/ES+ (early primitive streak, early primitive streak plus) that exhibited areas of beating muscle after 4 days in culture. Embryos were cultured whole, without the visceral endoderm, or without the visceral endoderm in the presence of END2 cells or cultured in conditioned medium from END2 cells. n is the number of beating explants per total number of explants analyzed. (B) Graph showing the average number of days when the first area of beating muscle was detected in differentiating mESCs and mESCs cultured in the presence of MS/LS (mid-primitive streak/late-primitive streak) isolated endoderm or ectoderm. Student's *t* test was used for statistical analysis (** $P<0.01$; *** $P<0.001$). (C–H) Immunostaining of the mESCs after 18 days of differentiating in culture alone (C, F), with endoderm (D, G), or with ectoderm (E, H), with alpha-actinin (brown) showing sarcomeric structures and stained for LacZ (β-galactosidase activity, in blue). F–H are magnifications of C–E, respectively. Scale bars are 500 μm in C–E and 50 μm in F–H.

Visceral endoderm-like cells END2 can replace visceral endoderm inducing cardiomyocytes in ES-LS embryo explants

Our results suggest that the absence of visceral endoderm leads to a reduced capacity of the mesoderm to generate beating areas in culture. To test whether the visceral endoderm could induce formation of cardiomyocytes directly, we attempted to rescue the induction in ES/ES+ explants stripped of visceral endoderm by culturing them in the presence of the visceral endoderm-like cells END2. Interestingly, culture of ES/ES+ explants without visceral endoderm on a layer of END2 cells rescued the number of explants exhibiting beating areas completely (Fig. 4A). Culturing those explants on END2 conditioned medium alone, however, had no effect. This suggested that direct contact of the mesoderm cells with the endoderm cells was needed for the induction of beating cardiomyocytes. In this regard, the effect of known mediators of cell contact between endoderm and mesoderm during heart development, such as fibronectin (Trinh and Stainier, 2004) or integrins (Liu et al., 2009; Zagris et al., 2004), could help explain our results. Alternatively, maybe the concentration of necessary factor(s), like BMPs, was not sufficiently high in the END2 conditioned medium as used to cause an effect. Another potential factor missing (or reduced) in the END2 conditioned medium could be TNFα, which has been shown to induce secreted factors favorable to cardiac differentiation (Arrell et al., 2008; Behfar et al., 2007).

Moreover, the lack of effect of the END2 conditioned medium inducing areas of beating muscle contrasted with the clear effect on hESCs embryoid bodies (Xu et al., 2008), suggesting that cell geometry and 3D organization may also play an important role in cardiomyogenesis. This was observed previously when END2 cells were cocultured with either P19EC cells or hESC: cell aggregates were induced in both cases before beating commenced (Mummery et al., 2002; Mummery et al., 1991). Although not tested here specifically, in all other studies cell lines with visceral-endoderm-like properties, as END2 cells, have proven more effective inducing cardiomyogenesis than mesoderm derivatives.

Visceral endoderm accelerates differentiation of mESCs to cardiomyocytes

The inducing influence of the visceral endoderm presumably takes place in the MS/LS developmental stage. To determine whether there was a direct role of the visceral endoderm in the induction of cardiomyocytes *in vitro*, we isolated both visceral endoderm and ectoderm of MS/LS mouse embryos and we cocultured this native visceral endoderm with differentiating E14 mESCs in monolayer. If the endoderm induces the formation of cardiomyocytes in mouse, as in other vertebrates, we expected that it should be able to promote the differentiation of mESCs to cardiomyocytes. In order to distinguish the mESCs from the primary mouse embryonic tissue, Rosa embryos expressing β-galactosidase ubiquitously, were used. We monitored the appearance of beating areas daily and detected the first beating areas in mESCs cocultured with visceral endoderm (as well as with ectoderm) significantly earlier than in mESCs differentiating alone (Fig. 4B). After 18 days of coculture, all explants were immunostained for alpha-actinin and showed well-organized sarcomeres in small clusters of cardiomyocytes scattered through the culture dish, faithfully reflecting the beating areas observed (Figs. 4C–H). The beating areas were always LacZ-negative, indicating their mESC origin. In agreement with the inducing role of the visceral endoderm, many beating clusters were observed in the vicinity of the embryonic tissue (LacZ-positive) and those may have been the first areas to be formed in culture.

Surprisingly, the induction of cardiomyocytes was also accelerated when mESCs were differentiated in the presence of embryonic ectoderm (Fig. 4B). We reasoned that the ectoderm tissue from the LS embryo, as the embryo is still gastrulating, may be able to give rise to mesoderm and endoderm in culture. The accelerated induction of cardiomyocytes observed after coculture of mESCs with LS ectoderm tissue could therefore be an indirect effect mediated by *de novo* endoderm formation. A new model (Kwon et al., 2008) proposing that there is an intercalation of definitive endoderm cells of epiblast origin with visceral endoderm cells instead of a massive displacement during gastrulation strengthens our interpretation.

Conclusions

We have investigated the role of the visceral endoderm in inducing the formation of cardiomyocytes in the mouse embryo during gastrulation. Our results demonstrate that both native visceral endoderm and a visceral endoderm-like cell line have the capacity to directly induce mesoderm cells to a cardiac fate *in vivo*. Also, visceral endoderm is capable *in vitro* of accelerating the differentiation of mESCs to cardiomyocytes.

Materials and methods

Embryo isolation

F2 embryos obtained from $(C57BL6 \times CBA)_{F1}$ crossings were used. For coculture of isolated germ layers with mESCs, embryos of $(C57BL6 \times CBA)_{F1} \times (C57BL6 \times CBA)_{Rosa}$ (Friedrich and Soriano, 1991) crossings were used. Mice were terminated by cervical dislocation. The uteri were dissected and kept in ice-cold phosphate-buffered saline (PBS). Embryos between E6.0 and E9.5, were isolated out of the decidua with sharpened tweezers and thin tungsten needles. Noon of the day the copulation plug was observed was regarded as E0.5, but all individual embryos were subsequently staged (Fig. 1A). Embryos were kept on a plastic platform on ice in Dulbecco's minimal essential medium (DMEM) supplemented with 10 mM Hepes and 7.5% fetal calf serum (FCS).

Germ layer separation and explant culture

The embryonic and extraembryonic parts of the E7.5 embryo were separated. The node and primitive streak were removed from the embryonic part in order to allow complete separation of the germ layers. The embryonic part was then incubated for maximally 8 min at 4 °C in 2.5% pancreatin and

0.5% trypsin in Hank's balanced salt solution (HBSS). After incubation, the embryos were transferred to warm (37 °C) DMEM containing 15% FCS. The ectoderm, mesoderm and endoderm were separated mechanically using thin tungsten needles. To increase embryo viability, embryos were processed, as described above, in groups of 2.

Embryos younger than E7.5 were incubated whole in 2.5% pancreatin and 0.5% trypsin in HBSS and the visceral endoderm was removed first. The extraembryonic and embryonic parts were separated thereafter. If present, the node and the primitive streak were removed from the embryonic part and subsequently the germ layers were separated.

E9.5 embryo hearts were isolated, trypsinized, and cultured on coverslips; the E6.5–E7.5 embryos or the isolated germ layers were cultured for maximally 9 days in 4-well plates (Nunc) on either glass or plastic coverslips coated with poly-L-lysine (50 μg/ml) and cultured in DMEM containing 15% FCS at 37 °C in 7.5% CO_2 humidified atmosphere. When necessary, the medium was refreshed.

Cell culture and coculture with mouse embryonic germ layers

END2 cells and END2 conditioned medium (containing 7.5% FCS) were prepared as described (Mummery et al., 1991; van den Eijnden-van Raaij et al., 1991). Extra FCS was added freshly to obtain medium supplemented with 15% FCS before use. END2 conditioned medium was used 1:1 with DMEM containing 15% FCS.

E14 mESCs were cultured on irradiated STO-snl feeder cells in DMEM conditioned by Buffalo rat liver cells, supplemented with 20% FCS, penicillin (10 U/ml), streptomycin (10 μg/ml), β-mercaptoethanol (0.1 mM), and 1 u/ml Leukemia Inhibitory Factor (LIF). For coculture experiments, isolated ectoderm and visceral endoderm from MS/LS embryos were cultured on plastic coverslips coated with poly-L-lysine (50 μg/ml). Germ layers that had adhered firmly after 2 days were used further. mESCs were preplated to remove any feeders and 10 000 cells were added per 4-well to the cultured germ layers and cultured in DMEM containing 15% FCS. The cocultures were monitored every day for the presence of areas of beating muscle.

Immunofluorescence and immunohistochemistry

For immunofluorescence, cells were fixed 30 min with 4% paraformaldehyde (PFA) at room temperature (RT), rinsed several times in PBS, permeabilized for 8 min in 0.1% Triton X-100/PBS, rinsed in PBS, and blocked for 1 h at RT in 4% normal goat serum (NGS, DAKO) in PBS. The cells were then incubated with mouse α-alpha-actinin (1:800, Sigma) in 4% NGS/PBS for 1 h at RT, rinsed several times in 0.05% Tween 20/PBS, and subsequently incubated with goat α-mouse Cy3 (1:250, Jackson Immuno Research) in 4% NGS/PBS for 1 h at RT. After washing in 0.05% Tween 20/PBS, the coverslips containing the cells were mounted in Mowiol.

In the coculture experiment using Rosa embryos and mESCs, cells were analyzed for β-galactosidase activity. Briefly, immediately after fixation, the cells were washed with PBS and treated with a solution containing Xgal (500 mM $K_3Fe(CN)_6$, 500 mM $K_4Fe(CN)_6$, 1 M $MgCl_2$, 40 mg/ml Xgal in PBS) overnight at 37 °C. Thereafter, cells were stained as above with mouse α-alpha-actinin followed by biotinylated goat α-mouse (1:250, Life Sciences). Thereafter, the cells were incubated with ABC-complex (DAKO) for 1 h at RT, washed in PBS and Tris/HCl 50 mM (pH 7.5), and incubated with DAB (Fast 3,3′-diaminobenzidine tablet set, Sigma) until brown coloring showed up and embedded in Mowiol.

Embryos were embedded in paraffin and sectioned transversely (7 μm) using standard procedures. Immunostaining was performed as described (Chuva de Sousa Lopes et al., 2005) and adequate negative controls were performed (data not shown). The primary antibodies used were rabbit α-phosphorylated Smad1/5/8 (1:200, Cell Signaling), mouse α-beta-catenin (1:100, BD Biosciences), and rabbit α-phosphorylated JNK (1:200, Cell Signaling).

Acknowledgments

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