BMPs, TGFβs and integrins in muscle and germ cell development in mice

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Cover: Posterior part of an E9.5 mouse embryo stained for alkaline phosphatase activity, showing primordial germ cells as individual cells migrating out of the hindgut.

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BMPs, TGFβs and integrins in muscle and germ cell development in mice

BMPs, TGFβs en integrins in de ontwikkeling van spier- en kiemcellen in de muis

(met een samenvatting in het Nederlands)

Proefschrift

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Voor Bernard &
Para a minha familia toda

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chapter 1

introduction

Transforming growth factor-β signalling pathway

Members of the transforming growth factor-β (TGFβ) superfamily include TGFβs, nodal, activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and Müllerian inhibiting substance/anti-Müllerian hormone (MIS/AMH). A large variety of cell types in different organisms (from flies to humans) express at least several of these secreted growth factors. In mice, TGFβ superfamily members have major roles during embryogenesis, as evidenced by the observation that deficiency in genes of the TGFβ superfamily or genes belonging to the TGFβ signal transduction pathway result in many cases in early embryonic lethality (reviewed by Goumans and Mummery, 2000). Interestingly, these secreted proteins have different functions, such as regulating cell migration, adhesion, proliferation, differentiation or apoptosis and moreover the same TGFβ family member can both stimulate or block the same process depending for example on the cell type, stage of development or even the concentration of ligand available (reviewed by Massagué, 2000; Nilsen-Hamilton, 1990)

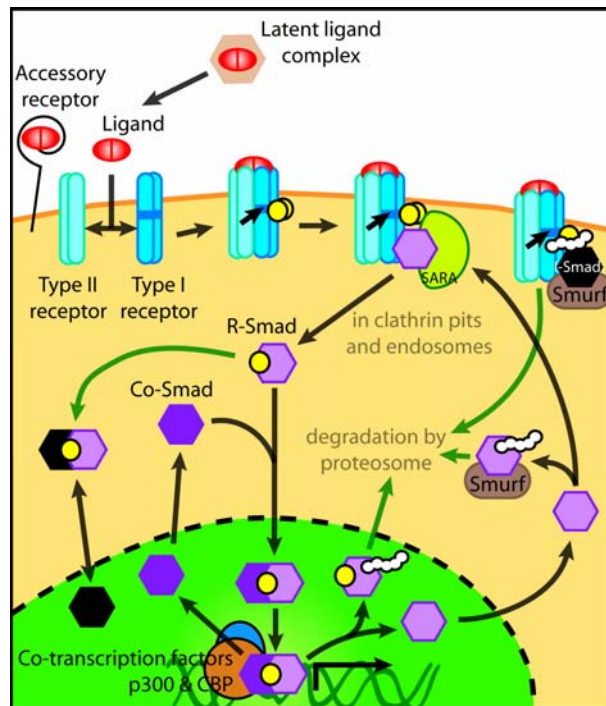

Fig.1. Schematic diagram of the TGFβ signal transduction pathway from the cell membrane to the nucleus. The arrows indicate the signal flow. The green arrows indicate the degradation pathway via the proteosome. Phosphate groups are represented by yellow circles and ubiquitin by white circles. Abbreviations are explained in the text. Adapted from Shi and Massagué, 2003.

The TGFβ superfamily consists of about 50 members (34 members in humans), all containing a hydrophobic signal characteristic of secretory polypeptides and six conserved cysteins that form a rigid cysteine knot. They are secreted as dimers in large latent complexes unable to signal. In this inactive form, they bind to extracellular matrix (ECM) proteins present at the surface of target cells and upon proteolytic cleavage active TGFβ dimers can be released (reviewed by Böttner et al., 2000). The active TGFβ dimers are then able to bind their transmembrane serine/threonine kinase type II and type I receptors, sometimes facilitated by an accessory receptor, forming heteromeric complexes that are internalized. The type II receptor, constitutively phosphorylated at several serine residues, is then able to transphosphorylate the type I receptor its the GS domain, present in the juxtamembrane region of the receptor. The active type I receptor in turn phosphorylates a group of proteins known as receptor regulated (R-)Smads. The R-Smads are kept in the cytosol by accessory/scaffolding proteins, including SARA, that present the R-Smads to the active type I receptor (Fig. 1). Once activated (phosphorylated at the two most C-terminal serine residues), the R-Smads bind to the common mediator (Co-)Smad, Smad4 and translocate to the nucleus, probably mediated by importins and nucleoporins. In the nucleus, the Smad-complex together with other transcription factors, besides the essential p300 and CBP co-activators or co-repressors, bind to specific motifs in promoter regions of target genes and regulate transcription. To terminate the signal, the Smads can be ubiquitinated and targeted to the proteasome for degradation, a process that can be mediated by Smurfs. Alternatively, Smads may be dephosphorylated by still unidentified phosphatases and shuttled back to the cytoplasm (reviewed by Shi and Massagué,

2003). Although the TGFβ signalling pathway might seem rather linear and simple, there is a growing list of molecules able to modulate each step of the pathway, involved in inhibitory loops, and even Smad-independent transcription pathways, together responsible for the complexity of the responses triggered by the TGFβ family members mentioned above (Balemans and van Hul, 2002; Derynck and Zhang, 2003).

Major differences between BMP and TGFβ signalling

The BMP (BMPs/GFDs/AMH) and TGFβ (TGFβs/activins/nodal) subfamilies interact differently with their receptors. BMP subfamily members exhibit high affinity for the type I receptor and low affinity for the type II receptor, whereas TGFβ subfamily members, on the other hand, interact exclusively with the type II receptor. However, both type I and II receptor are required for signal transduction. Furthermore, although the steps of the signalling pathway are similar, specific BMP and TGFβ subfamily members use different type II receptors, type I receptors and Smads to signal and are antagonized by different molecules (Fig. 2) (reviewed by Shi and Massagué, 2003).

As shown in Fig.2, specific BMP subfamily members (BMPs, GFDs, AMH) interact with BMP type II receptor (BMPRII), activin type II receptors (ActRIIA and ActRIIB) or AMH type II receptor (AMHRII) in combination with activin receptor-like kinase (ALK)2, ALK3 and ALK6 to activate Smad1, Smad5 and Smad8. The TGFβ subfamily members (TGFβs, activins, nodal) bind TGFβ receptor II (TβRII), ActRIIA and ActRIIB in combination with ALK4, ALK5 and ALK7 to activate Smad2 and Smad3. However, note that TGFβ via ALK1 activates Smad1, Smad5 and Smad8, whereas for example GDF1 via ALK4/7 activates Smad2 and Smad3. Direct binding of all R-Smads (except Smad2) and Co-Smads to DNA is rather weak but sequence specific. However, Smad3 and Smad4 preferentially bind to GTCT-containing sequences, whereas BMP R-Smads interact with GC-rich containing sequences (reviewed by ten Dijke et al., 2003).

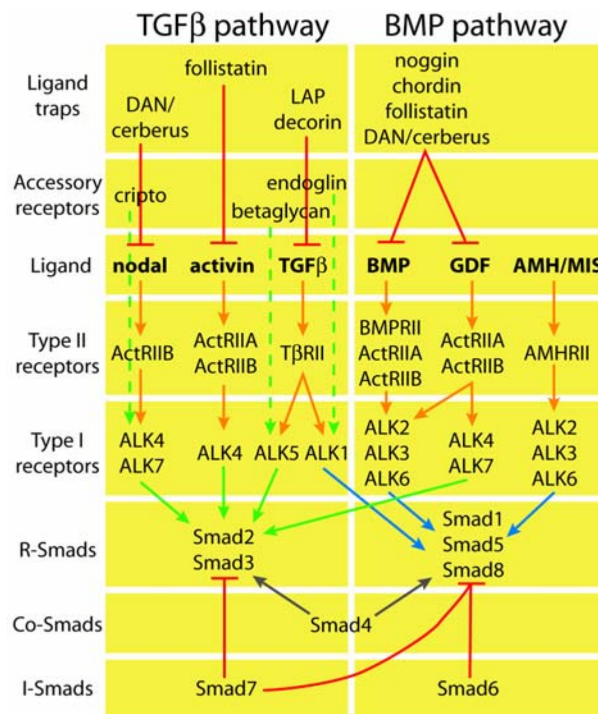

Fig. 2. Signalling specificity of the TGFβ superfamily in vertebrates. Orange arrows indicate the relationship between ligands, type I and type II receptors, dark green and dark blue arrows indicate signalling through the different Smads and gray arrows the association with Smad4, light green arrows shows the relation between several accessory receptors and the TGFβ pathway and in red the relation with several inhibitory factors. Adapted from Shi and Massagué, 2003 and Moustakas et al., 2001.

Various extracellular antagonists, including noggin and chordin, modulate BMP signalling exclusively, while DAN/cerberus and follistatin also modulate TGFβ signalling via nodal and activin, respectively. TGFβ itself can in the latent complex remain bound to the latent-associated polypeptide (LAP) or be trapped by the proteoglycan decorin. Other molecules that play important roles modulating BMP/TGFβ signalling, include the pseudoreceptor BAMBI, a molecule structurally similar to type I receptor that titrates BMP/TGFβ ligand away, while several accessory receptors, like betaglycan, endoglin and cripto present ligand to the receptors promoting TGFβ signalling (Fig. 2).

Still at the level of the receptors, negative regulation can occur by binding of inhibitory (I-)Smads, Smad6 and Smad7, to the active type I receptors thereby preventing phophorylation of the R-Smads (Fig. 2). Smad7 blocks both BMP and TGFβ signalling, while Smad6 preferentially blocks BMP signalling. Additionally, Smad6 can also bind activated BMP R-Smads directly preventing complex forming with Smad4, while Smad7 targets activated TGFβ receptors for ubiquitination and degradation via Smurfs (Fig. 1). Interestingly, Smad7 is an early target of the TGFβ signalling pathway, resulting in a negative feedback loop (reviewed by Shi and Massagué, 2003).

During embryonic development, members of the BMP and TGFβ signalling pathways are involved in many processes. However, ablation of genes of the BMP pathway in general results in early abnormal phenotypes and the mouse mutant embryos die before or soon after gastrulation (see below) primarily from defects in mesoderm formation; examples include *Bmp2*, *Bmp4*, *Bmp5/Bmp7*, *BmpRII*, *ActRIIA/ActRIIB*, *Alk2*, *Alk3*, *Smad4*, *Smad1* and *Smad5* mutant embryos (Ying et al., 2001; Winnier et al., 1995; Solloway and Robertson, 1999; Beppu et al., 2000; Song et al., 1999; Gu et al., 1999; Mishina et al., 1995; Sirard et al., 1998; Tremblay et al., 2001; Hayashi et al., 2002; Chang et al., 1999). Deletion of *Tgfβ1*, *TβRII*, *Alk5*, *Alk1* and *endoglin*, all genes belonging to the TGFβ pathway (Dickson et al., 1995; Oshima et al., 1999; Larsson et al., 2001; Oh et al., 2000; Arthur et al., 2000; Li et al., 1999) cause primarily defects in vascularization of the visceral yolk sac (see below). In humans, mutations in genes coding for specific members of the BMP and TGFβ signalling pathways result in a broad range of diseases, from congenital heart disease or anomalies in the intestinal tract to hereditable vascular disorders and cancer (reviewed by Miyazono et al., 2001; Waite and Eng, 2003).

The TGFβ signalling pathway and connective tissue growth factor

Connective tissue growth factor (CTGF) belongs to the CCN (CTGF, CYR61, NOV) superfamily of secreted growth factors and is associated with wound repair, tumorigenesis, artherosclerosis and fibrotic disorders of the skin, kidney, lung, liver and pancreas (Shakunaga et al., 2000; Wenger et al., 1999; di Mola et al., 1999; Igarashi et al., 1993, 1998; Oemar et al., 1997; Frazier and Grotendorst, 1997; Ito et al., 1998; Lasky et al., 1998; Paradis et al., 1999). *In vitro* at least, CTGF is able to promote ECM production but also to bind ECM proteins via its thrombospondin type I domain (Frazier et al., 1996; Duncan et al., 1999; Lam et al., 2003). Interestingly, via its von Willebrand type C domain, a cysteine-rich sequence similar to that found in chordin, CTGF has recently been shown to promote TGFβ1 binding to its receptors, while inhibiting BMP binding to BMP receptors (Abreu et al., 2002). Although CTGF is not directly associated with the cell membrane, it can bind to the ECM and therefore it might function as an accessory receptor (or inhibitor), presenting (or titrating away) ligands to their respective receptors. On the other hand, CTGF is an early target of TGFβs (and BMPs) in different fibroblastic cell types, again *in vitro* (Moussad and Brigstock, 2000; Nakanishi et al., 1997); besides its function in ECM remodelling, it may therefore also be involved in a feedback loop modulating the TGFβ signalling pathway. In agreement with this, a TGFβ-response element has been identified in the *Ctgf* promoter suggesting a direct effect (Grotendorst et al., 1996; Holmes et al., 2001) although possibly through a Smad independent mechanism (Denton and Abraham, 2001).

During mouse embryonic development, CTGF expression is mainly restricted to skeletal, cardiovascular and neural tissues. Moreover, deficiency in *Ctgf* leads to perinatal death due to abnormal skeletogenesis, in particular caused by defective ECM remodelling and growth plate angiogenesis (Ivkovic et al., 2003). *Ctgf* mutant embryos exhibit reduced levels of matrix metalloproteinase-9 (MMP9). MMPs have important roles in degradation of ECM proteins but also in cleaving growth factors and their binding proteins thereby modulating specific signalling pathways (Sternlicht

and Werb, 2001). Interestingly, MMP9 is known to activate latent TGFβ (D'Angelo et al., 2001; Yu and Stamenkovic, 2000), thus providing an alternative mechanism through which CTGF may regulate TGFβ signalling.

The TGFβ signalling pathway and β1 integrin

The integrin family is formed by molecules that, like CTGF, interact with components of the ECM. However, in contrast to CTGF, integrins are transmembrane receptors, formed by non-covalently bound α- and β-subunits. Integrins are involved in cell-cell adhesion and are considered the major adhesion receptors connecting cells to the ECM. In vertebrates, β1 integrin is the most widespread subunit, able to associate with the largest number of α-subnunits (reviewed by Danen and Sonnenberg, 2003). Mice deficient in β1 integrin die around E5.5 and exhibit defects in inner cell mass development and failure of the primitive endoderm migration (Stephens et al., 1995; Fässler and Meyer, 1995). In the mouse, two isoforms of β1 integrin, β1A and β1D were identified and shown to be non-redundant during mouse development: replacement of β1A by β1D is embryonic lethal while removal of β1D results in survival to adulthood (Baudoin et al., 1998).

Integrins and the TGFβ pathway interact at several levels. Firstly, there is evidence that αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 integrins interact with the RGD (Arg-Gly-Asp, an integrin binding sequence present in ECM proteins) motif of LAP, mediating activation of latent TGFβ, both *in vitro* and *in vivo* (Munger et al., 1999; Morris et al., 2003; Ludbrook et al., 2003). Secondly, integrins and TGFβ (Smad-independent) signalling synergize at the level of phosphorylation/activation of common downstream signalling molecules, including those belonging to the Ras/Erk MAPK signalling (reviewed by Danen and Sonnenberg, 2003; Derynck and Zhang, 2003). Interestingly, a recent report showed that it is through binding of TGFβ that integrins activate Ras/Erk MAPK signalling (Luettich and Schmidt, 2003). Another report suggested that integrin activation of the Ras/Erk MAPK signalling potentiates BMP Smad-dependent signalling (Suzawa et al., 2002). Thirdly, binding of integrins to RGD-peptides induced transcription of TGFβ (Ortega-Velasquez et al., 2003), while TGFβ on the other hand is known to upregulate expression of several integrins, including β1 integrin (Thibault et al., 2001; Li et al., 2003).

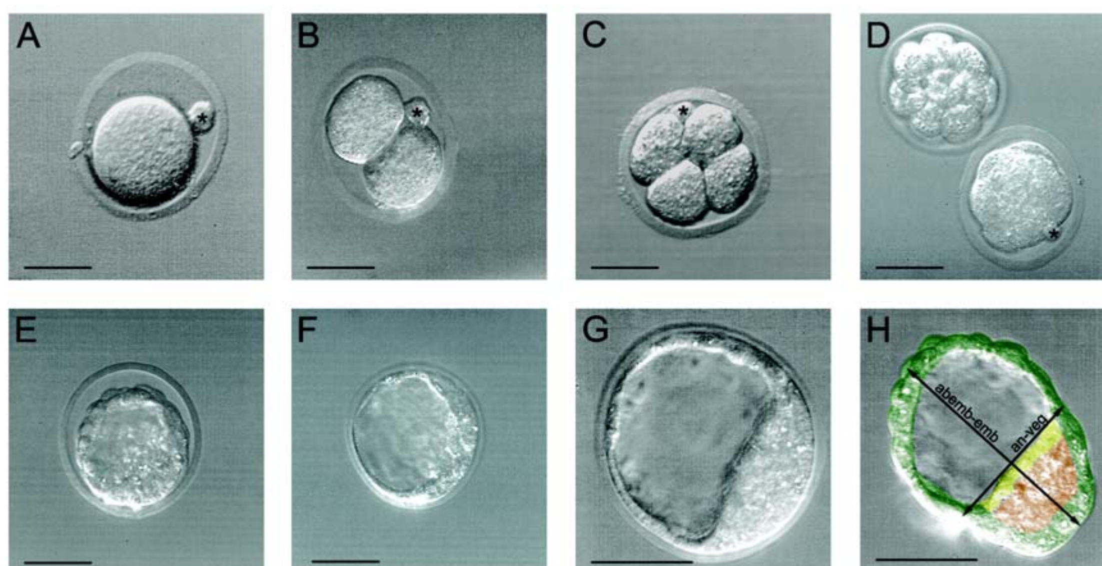

Fig. 3. Mouse pre-implantation development. The asterisk marks the second polar body. The late blastocyst consists of three cell subpopulations: the trophectoderm (green), the inner cell mass (orange) and the primitive endoderm (yellow). In the blastocyst three axes can be defined: the embryonic-abembryonic (abemb-emb), the animal-vegetal (an-veg) and a third axis on the same plane, but perpendicular to the an-veg axis. Photomicrographs are courtesy of B. Roelen. Scale bar: 40 μm.

Mouse embryonic pre-implantation development

Before implantation, the development of the mammalian embryo (Fig. 3) is characterised by a relative independence from the maternal environment. The fertilised egg, or 1-cell embryo, completes meiosis and is pushed towards the uterus by cilia in the oviduct, while remaining within a glycoprotein coat, the zona pellucida. The first cleavage takes place approximately 20 hours post-fertilisation, but divisions become more rapid thereafter. Transcription of the mouse embryonic genome starts at the 2-cell stage (Flach et al., 1982), after which maternal mRNAs are rapidly degraded. In addition, demethylation of the paternal and maternal genomes begins after fertilization leading later during implantation to epigenetic reprogramming (reviewed in Reik et al., 2001). At the mid-8-cell stage, the embryo undergoes "compaction", as increased E-cadherin-mediated adhesion between all blastomeres results in them flattening against each other, forming a smooth, spherical ball of cells, the morula. During the next cleavage, some blastomeres divide along the apical/basal axis, forming two polar outer cells, while others divide to form one outer and one inner blastomere (Fleming et al., 1987). Thus two different lineages become established in the morula (Fig. 3): (1) the outer cells form the first epithelium of the embryo, the trophectoderm; (2) the inner cells remain nonpolarised and pluripotent, giving rise to the inner cell mass (ICM). At the 32-cell stage, "cavitation" starts as the trophectoderm cells, now forming a tight junction seal separating the inner cells from the maternal environment, secrete fluid directionally into the morula. This leads to the formation of a fluid-filled blastocoel cavity and the embryo is designated a blastocyst (Fig. 3). As the blastocyst expands, the ICM cells become positioned at one end of this cavity. The blastocyst, up until this stage present in the oviduct reaches the uterus on embryonic day (E)3.5 and shortly thereafter "hatches" from the zona pellucida, but is initially not adhesive. It only becomes competent for attachment to the maternal tissue when the uterus reaches its receptive stage, which occurs at E4.5.

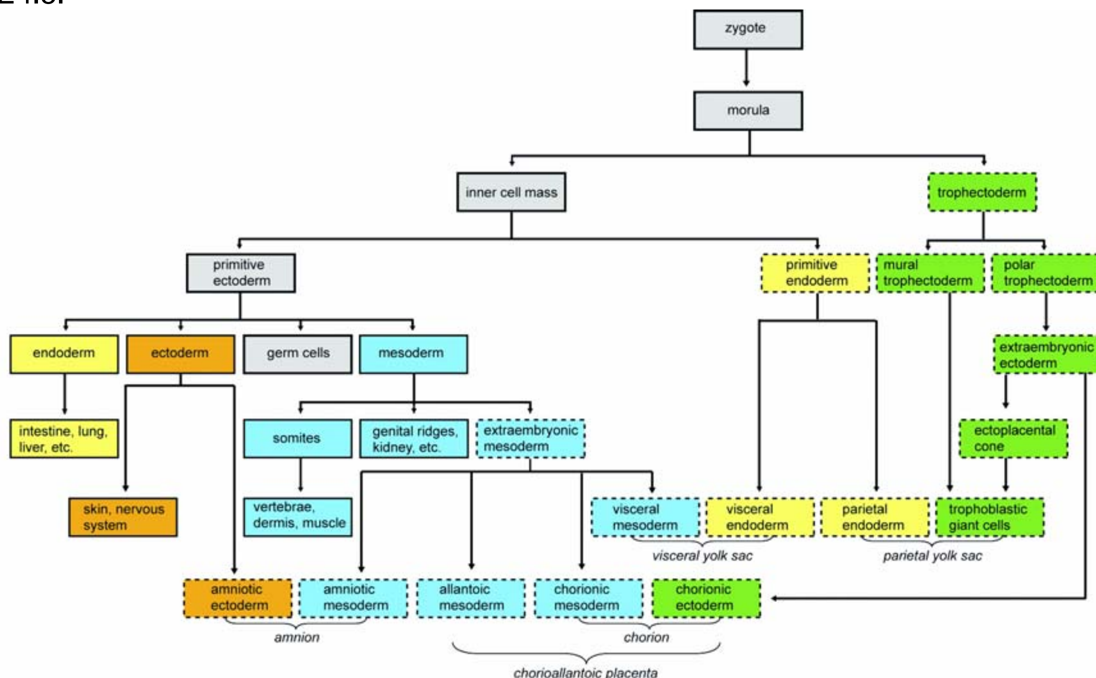

Fig. 4. Cell lineages in mouse development. Trophectoderm-derived tissues are depicted in green, endoderm-derived tissues in yellow, ectoderm-derived tissues in orange and mesoderm-derived in blue. The cell/tissues represented in grey are regarded as pluripotent. All extraembryonic tissues are enclosed by hatched lines, while embryonic tissues are enclosed by solid lines. Adapted from Hogan et al., 1994 and Gardner, 1983.

Cell lineage differentiation in the mouse

At the late blastocyst stage, the trophectoderm exists as two distinct cell populations, the polar trophectoderm that contacts the ICM and the mural

trophectoderm that contacts the blastocoel (Fig. 3). Additional to the trophectoderm and ICM, a third cell population becomes clearly visible, the primitive endoderm (or hypoblast) formed by the cells in the ICM facing the blastocoel. Both trophectoderm and primitive endoderm are extraembryonic lineages and do not contribute to the embryo proper. The remaining cells of the ICM form the primitive ectoderm (or epiblast). Two cell populations develop from the primitive endoderm (reviewed in Gardner, 1983): cells leaving the ICM as individual mesenchymal cells and migrating on the basal side of the mural trophectoderm eventually lining the blastocoel cavity, are termed parietal endoderm. Parietal endoderm cells are responsible for synthesising a thick basement membrane, *Reichert's membrane*, deposited between them and the trophectoderm. Reichert's membrane is a transient structure unique to rodents and bats that not only contributes to the regulation of nutrient and waste exchange between embryo and mother, but also protects the embryo against the immune system of the mother. Together, the parietal endoderm, trophectoderm and Reichert's membrane are designated the parietal yolk sac (Fig. 4), the structure that directly contacts the maternal uterine tissue. Primitive endoderm cells that remain in contact with the ICM, differentiate into epithelial visceral endoderm, which initially remains in contact with the ICM/epiblast and extraembryonic ectoderm (Fig. 4,5). The anterior visceral endoderm (AVE) plays an important role in determining the anterior-posterior axis of the developing embryo (reviewed in Beddington and Robertson, 1999). As gastrulation begins, epiblast cells give rise to the *definitive endoderm* of the embryo proper displacing the visceral endoderm towards the extraembryonic ectoderm. Meanwhile, extraembryonic mesodermal cells derived from the posterior primitive streak (Fig. 5) colonise the basal side of the visceral endoderm, differentiating into extraembryonic mesothelium and blood islands. These cells together with the VE and its basement membrane form the visceral yolk sac (Fig. 4,5). The visceral yolk sac is the first site of haematopoiesis in the conceptus providing blood cells to the early embryo as the heart begins rhythmic contraction around E8.5, but this "extraembryonic" blood (with primitive nucleated red blood cells) is then gradually replaced by blood produced by the foetal liver and aorta-gonad-mesonephros region (Baron et al., 2003). The extraembryonic mesoderm also contributes to the *chorion* (with the extraembryonic ectoderm), the *amnion* (with the embryonic ectoderm) and the *allantois* located at the posterior side of the embryo that grows into the exocoelomic cavity and eventually develops into the umbilical cord (Fig. 4,5).

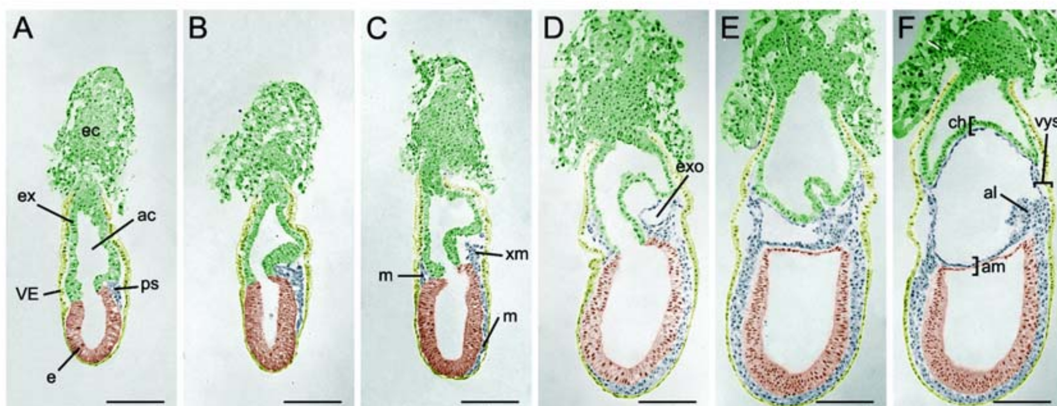

Fig. 5. Development during gastrulation (E6.5–E7.5). The tissues coloured green, the extraembryonic ectoderm and ectoplacental cone, are derived from the trophectoderm. The tissues in yellow are derived from the primitive endoderm (the visceral endoderm) and epiblast cells that passed through the streak generating the definitive endoderm. The tissues coloured orange are derived from the inner cell mass and remain ectoderm. The tissues coloured blue are formed during gastrulation and represent primitive streak and mesoderm-derived tissues, excluding the primordial germ cells present at the basis of the allantois (see Fig. 6). Abbreviations: ac, proamniotic canal; al, allantois; am, amnion; ch, chorion; e, epiblast; ec, ectoplacental cone; ex, extraembryonic ectoderm; exo, exocoelom; m, nascent mesoderm; ps, primitive streak; VE, visceral endoderm; vys, visceral yolk sac; xm, extraembryonic mesoderm. Scale bar: 100 μm.

Primordial germ cell development

Primordial germ cells (PGCs) are the cells that form the gametes, the only cells in the body that undergo meiosis and transmit the genetic information of the species from generation to generation (Fig. 6). In mice, the PGCs are allocated around E7.2 at the base of the allantois and posterior region of the primitive streak, visible as a scattered population of about 45 cells. PGCs can be identified by their high level of tissue non-specific alkaline phosphatase (TNAP) activity (Fig. 7) or by staining with an antibody recognizing PGC7/Stella or the marker for pluripotency Oct4 (Chiquone, 1954; Ginsburg et al., 1990; Yeom et al., 1996; Saitou et al., 2002; Sato et al., 2002). Until the onset of gastrulation at E6.5, epiblast cells are still not committed to any lineage and can enter the germ line when placed in the proximal epiblast region (Tam and Zhou, 1996), but thereafter no additional recruitment to the germ line occurs. This restriction in pluripotency is also clear from the expression pattern of Oct4, which is present in all epiblast cells, but after gastrulation becomes restricted to the PGC population (Yeom et al., 1996). In agreement with these studies, Lawson and Hage (1994) identified the proximal epiblast region adjacent to the extraembryonic ectoderm using clonal analysis as the region of the embryo at E6.0 that later becomes fated to a PGC and extraembryonic mesoderm fate. Several members of the BMP signalling pathway are known to be involved in PGC (and extraembryonic mesoderm) specification, including BMP4, BMP2, BMP8b, Smad1 and Smad5 (Ying et al., 2000; Winnier et al., 1995; Ying et al., 2001; Tremblay et al., 2001; Hayashi et al., 2002; Chang and Marzuk, 2001). In addition, a second signal emanating from the nascent posterior extraembryonic mesoderm might regulate PGC allocation. Recently, E-cadherin, Lim1 and Hnf3β have been identified as part of that second signal (Okamura et al., 2003; Tsang et al., 2001).

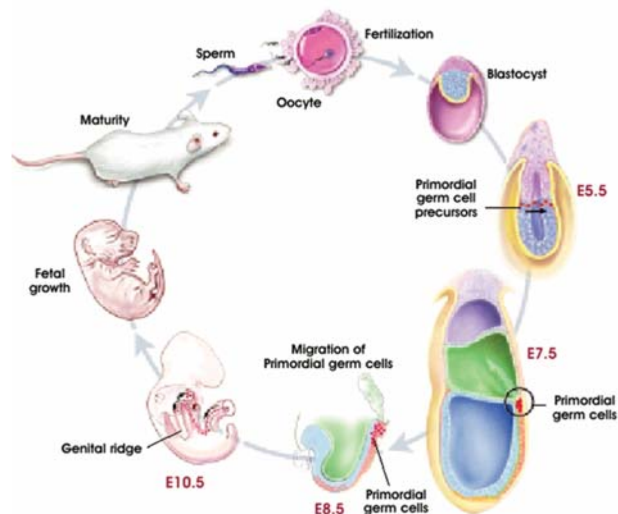

Fig. 6. Development of mouse primordial germ cells during the mouse life cycle. Tissues depicted in blue are ICM (blastocyst), epiblast (E5.5) and ectoderm (E7.5-E8.5); tissues depicted in pink/magenta are trophectoderm derived extraembryonic tissues; tissues depicted in yellow are primitive and definitive endoderm derived. PGCs and PGC precursors are depicted in red. From "Stem cells: Scientific progress and future research directions" NIH, 2001.

From E8.0 to E9.5, the PGCs ingress and migrate through the hindgut, a definitive endoderm derived tissue (Fig. 6,7). During this period, PGCs exhibit a motile morphology (being elongated and extending processes) and have been shown to migrate actively *in vivo* (Gomperts et al., 1994; Molyneux et al., 2003). A number of genes have been identified as important during migration through the hindgut, including *Fgf8*, *germ cell deficient* and the c-kit/Steel (receptor/ligand) pathway (Sun et al., 1999; Pellas et al., 1991; De Miguel et al., 2002). Moreover, *in vitro* TGFβ1 has been shown to function as a chemoattractant for PGCs isolated at E8.5 and E10.5 (Godin and Wylie, 1991). Between E9.0 and E9.5, the PGCs start leaving the hindgut into the adjacent mesenchyme to reach the gonadal ridges by E10.5-E11.5 (Fig. 7). This abrupt change in migration "direction" is probably associated with changes in cell adhesion. Laminins, β1 integrins and proteoglycans are though to play a role during this development phase (Anderson et al., 1999; García-Castro et al., 1997). Recently,the SDF1/CXCR4 (ligand/receptor) pathway has been shown to be essential for homing of PGCs to the gonadal ridges, but not required for directed migration (Ara et al., 2003; Molyneaux et al., 2003).

Once the PGCs reach the gonadal ridges (Fig. 7), they lose their motile morphology and round up, being often referred to as gonocytes. Furthermore, female PGCs reactivate their silent X-chromosome (Monk and MacLaren, 1981). Around E11.5, PGCs have totally demethylated genomes, but methylation imprints are re-established in a sex-specific manner with paternal methylation imprints acquired early and maternal specific methylation obtained late during gametogenesis (reviewed in Reik et al., 2001). In the genital ridges, the PGCs increase the expression of germ-cell specific genes, inclusing *vasa*, *germ cell nuclear antigen 1* and *germ cell-less* (Toyooda et al., 2000; Enders and May, 1994; Kimura et al., 1999), but also Scp3, a meiotic gene (Di Carlo et al., 2000). Both male and female PGCs undergo two or thee additional rounds of mitosis and around E12.5 enter the pre-meiotic stage. In contrast to male germ cells that undergo mitotic arrest, female germ cells enter meiosis and are arrested in diplotene around the time of birth (reviewed by McLaren et al., 2003). PGCs increase their population from the estimated 45 cells at E7.5 to about 25 000 cells at E13.5, and therefore replicate 8 times with an average division time of 16 hours (Tam and Snow, 1981).

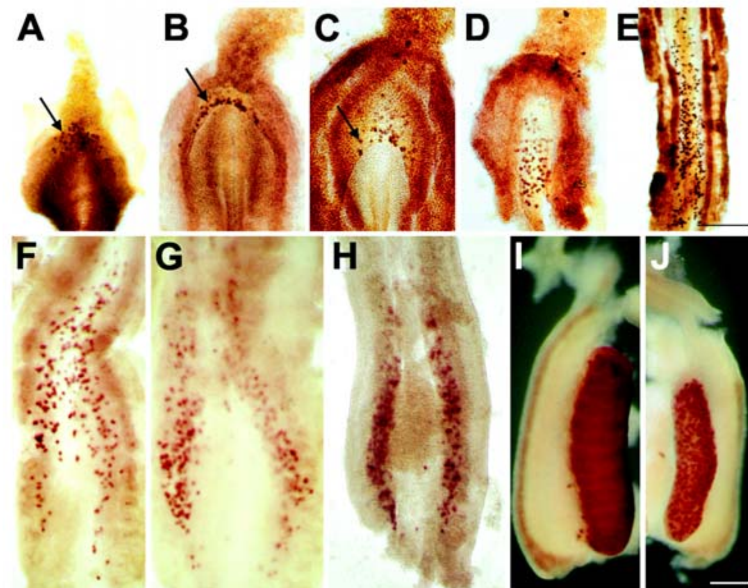

Fig. 7. Migration of PGCs from the allantois base to the gonads. PGCs (black arrow) **are visualised as brown individual c**ells after staining for alkaline phosphatase activity (Chiquone, 1954; Ginsburg et al., 1990) at E7.5 at the base of the allantois and at 6 somites (E8.0), 9 somites (8.0-E8.5) and 11 somites (E8.5) stage migrating through the developing hindgut. Between E9.5 a nd E10.5, PGCs leave the hindgut to the body wall and mesenterium, as it forms and expands, and ingress into the gonadal ridges. At E12.5, male (I) and female (J) gonads are already distinguishable. Top is posterior and bottom is anterior. Scale bar: 200 µm (A-E and F-J).

Skeletal and cardiac muscle development

Skeletal and cardiac progenitors arise from different populations of mesoderm, respectively paraxial mesoderm and lateral plate mesoderm. Note that extraembryonic mesoderm and PGCs ingress through the posterior part of the primitive streak, while the lateral and paraxial mesoderm ingress through the middle and anterior part of the primitive streak, respectively (Kinder at al., 2001).

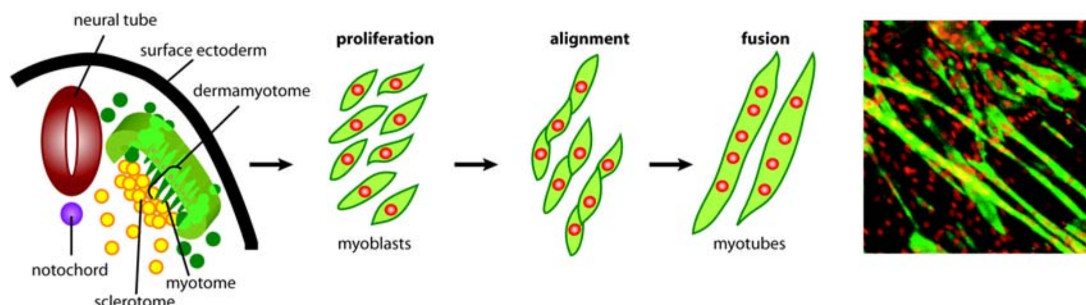

Fig. 8. Differentiation of skeletal muscle. Somites are formed by the sclerotome and dermomyotome which gives rise to the myoblasts and dermis. Myoblasts delaminate and migrate to places where they will develop the musculature of the body. They then stop proliferating, align and fuse to form multinucleated myotubes that assemble to form muscle fibers. Photograph of C2C12 cells, a satellite muscle derived cell line 4 days after differentiation towards skeletal muscle, immunostained for myosin heavy chain (green), a sarcomeric protein and with ToPro3 nuclear staining (red).

The paraxial mesoderm gives rise to the somites, segmented blocks of mesoderm cells that develop on each side of the neural tube. The somites undergo epithelialization and in response to signals from the neural tube and notochord, the somites differentiate forming two distinct regions: the dermomyotome and the sclerotome (Fig. 8). The sclerotome develops into the majority of the bones and cartilage of the body, while both myogenic and dermal progenitors are derived from the dermomyotome. Myogenic progenitors, also known as myoblasts, express the muscle-specific transcription factors Myf5 and MyoD (reviewed by Buckingham, 2001). Once specified, these cells delaminate from the somites and migrate to sites of muscle formation. There, some myoblasts irreversibly exit the cell cycle, align with each other and fuse forming multinucleated myotubes. During development, there are two waves of myogenesis involving two distinct populations of myoblasts. Between E11.5 and E15.5 in the mouse, primary myotubes are formed through the fusion of primary (embryonic) myoblasts, while secondary (fetal) myoblasts remain proliferative. After primary myotubes have stopped forming, secondary myoblasts progressively enter the muscle differentiation programme. They use the primary myotubes as a scaffold to attach to and fuse with each other, giving rise to secondary myotubes, a process that starts at E15.5 in the mouse and goes on until well after birth (Stickland, 1982; Wigmore and Dunglison, 1998). In adult mice, myogenesis can still occur triggered by skeletal muscle damage. During the process of regeneration, satellite muscle cells, which constitute a reservoir of undifferentiated muscle precursor cells, are recruited to form new myofibres (Seale and Rudnicki, 2000).

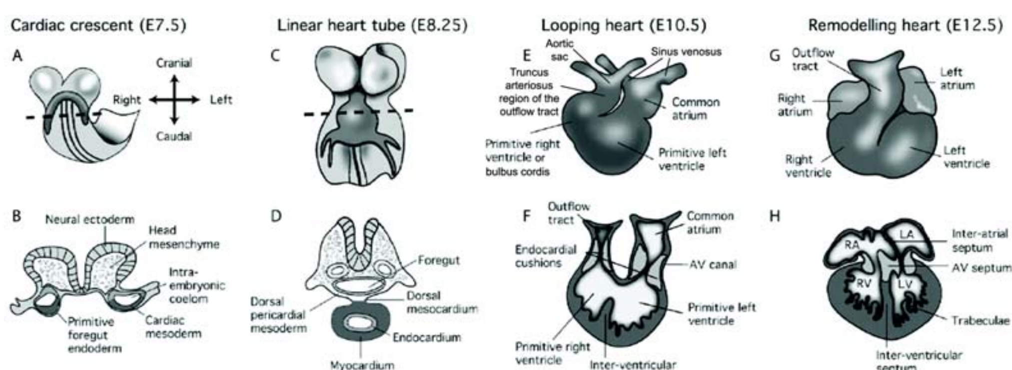

Fig. 9. Embryonic heart muscle development. Schematic drawings of both whole mount and heart sections during the major steps of mouse heart development. Dark shading represents myocardial tissue. AV, atrioventricular channel; LA, left atrium; LV, left ventricle. Adapted from Solloway and Harvey, 2003.

The lateral plate mesoderm (at each side of the embryo) migrates during gastrulation from the posterior side of the primitive streak to an anterior-proximal position in the embryo. There, the paired heart-forming fields converge to form the cardiac crescent (Fig. 9). Interestingly, there is a concerted movement of presumptive cardiac mesoderm with the overlying foregut endoderm (Lawson and Pederson, 1987; Tam and Beddington, 1992; Tam et al., 1997), suggesting that there may be interactions between these two tissues. However, the presumptive cardiac mesoderm is not committed to the cardiac lineage until it reaches the heart-forming region after migration. Still in association with the foregut endoderm, the paired cardiac progenitors move ventrally, joining at the ventral midline, creating a linear heart tube around E8 (Fig. 9). At this stage, the heart is formed by a distinct endocardium and myocardium and elongates anteriorly and posteriorly. At E8.5, the bilateral symmetric heart tube undergoes a process called cardiac looping morphogenesis, during which the different heart chambers acquire their shape, position and left-right asymmetry. From E12.5 until birth, the form of the heart is retained, but it undergoes a tremendous increase in size. It is during this last

developmental period that the process of septation and valvulogenesis takes place, remodelling the heart with respect to the chambers connection and separation (reviewed by Solloway and Harvey, 2003).

Placentation

During the first half of gestation, mice embryos rely on a yolk sac placenta, formed by tissues derived from the trophectoderm and primitive endoderm for nutrition and survival (Fig. 10). During this period exchanges with the maternal environment occur by diffusion. However, mice develop a chorio-allantoic placenta around E8.5, when the allantois, elongated across the exocoelomic cavity, makes close contact with the chorionic plate, the chorion flattened against the ectoplacental cone. This event is called chorio-allantoic fusion. Over the next days, chorionic trophoblast cells and foetal blood vessels undergo extensive branching, referred to as "vascular invasion" of the chorion, forming the placental labyrinth layer (Fig. 10). However, the chorionic plate remains as a defined structure, adjacent to the highly vascularised placental labyrinth. In the labyrinth, the chorionic trophoblast cells form two separate cell layers around the lumen of the maternal blood sinus. Interestingly, these distinct concentric cell layers are formed from the fusion of two or more chorionic trophoblast cells into a multinucleated cell type, the syncytiotrophoblast. Note that just as the syncytioytophoblasts of the placenta, the myotubes that form skeletal muscle (described above) are also multinucleated cells.

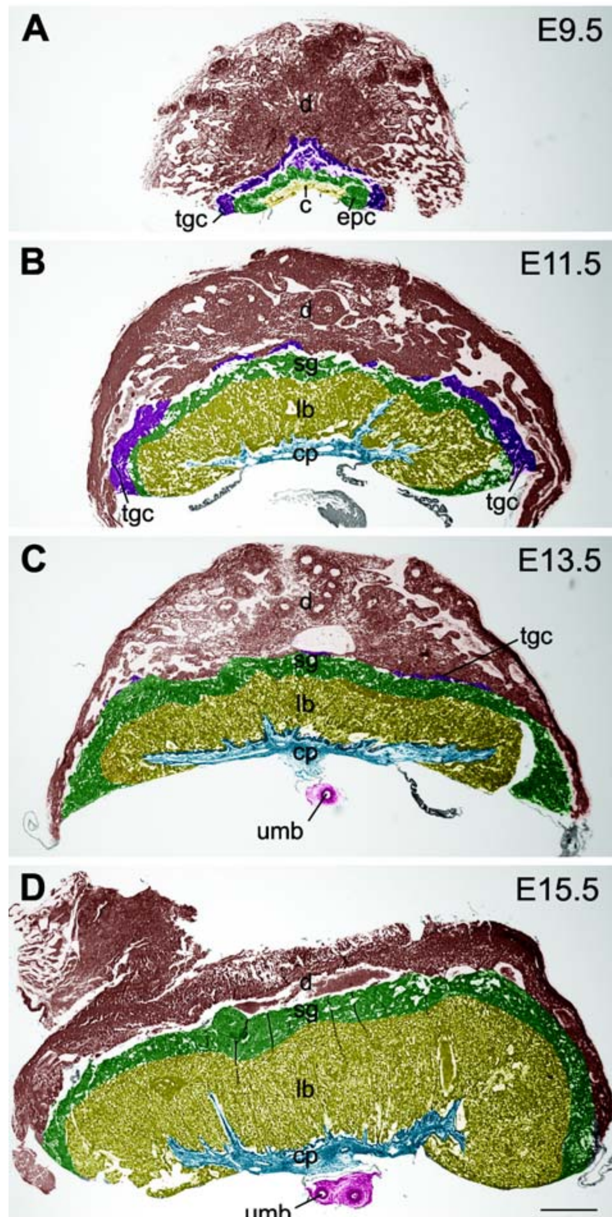

Fig. 10. Placentation. (A) An increasing number of trophoblast giant cells (magenta) and ectoplacental cone cells (green) form the placenta of the E9.5 embryo. The allantois vasculature sprouts into folds in the chorion (yellow). (B-D) Two days later, the labyrinth (yellow) has developed greatly between the spongiotrophoblast layer (green) and the chorionic plate (blue). The trophoblast giant cells (magenta) become localised at the border of the placenta. Note that the umbilical cord is also visible (pink). Abbreviations: c, chorion; cp, chorionic plate; d, deciduum; epc, ectoplacental cone; lb, labyrinth; sg, spongiotrophoblast; tgc, trophoblast giant cells; umb, umbilical cord. Scale bar: 500 µm.

Large maternal arterial blood vessels cross the spongiotrophoblast layer, a compact non-syncytial cell layer located between the trophoblast giant cells (forming the outer layer of the placenta at the interface with the maternal deciduum) and the placental labyrinth (Fig. 10). Interestingly, the maternal endothelium is progressively removed and as the maternal blood enters the placental labyrinth the small branches formed by the syncytiotrophoblast cell layers become the maternal "blood vessels" themselves (Adamson et al., 2002). Transport of gases and nutrients thus occurs in the placental

labyrinth from the maternal blood through the syncytiotrophoblast cells and foetal blood vessels into the foetal blood, while embryonic waste products are transported in the opposite direction. The chorio-allantoic placenta starts functioning around E10.0 and reaches full maturation at E17.5, meeting the ever-growing demands of the foetus (reviewed in Rossant and Cross, 2001).

Aim of this thesis

Although the TGFβ/BMP signalling pathway has been studied for over 20 years, many aspects of the signalling are still poorly understood. Recently, two novel sets of tools have been developed which will help us gain new insights in the TGFβ/BMP signalling pathway. One of those set of tools are antibodies that specifically recognize the C-terminal phosphorylated form of either TGFβ or BMP R-Smads, providing accurate information of where and when TGFβ/BMP activated the Smad-dependent signalling pathway (Persson et al., 1998). We made use of those antibodies to map TGFβ/BMP signalling during early mouse development (Chapter 2 and 3). The other set of tools consist of transgenic mice that carry either a specific TGFβ responsive or BMP responsive promoter coupled to LacZ or GFP (Neptune et al., 2003; Monteiro et al., unpublished results). Although these reporter mice do not give information about immediate TGFβ/BMP signalling, because the reporter molecules have to be produced and have a half life of several days, these reporter mice provide clear evidence for transcriptional activation as result of the TGFβ or BMP signalling. Using these transgenic reporter mice, we were able to map TGFβ/BMP signalling transcriptional activation during PGC migration (Chapter 4). Furthermore, we studied in detail the role of the BMP signalling pathway in particular via ALK2 in the formation of PGCs (Chapter 3) and the role of the TGFβ signalling pathway via ALK5 in the migration of PGCs (Chapter 4).

A major function of TGFβ is to regulate the expression of genes involved in the formation and remodelling of the extracellular matrix (ECM). We focus on two of these genes, β1integrin and CTGF and investigated their putative role(s) in the development of PGCs and the heart, respectively, during mouse development (Chapter 5 and 6). In addition, we studied the relationship between CTGF and the TGFβ/BMP signalling pathway after experimentally induced myocardial infarction in mice (Chapter 6). At least in rats, CTGF and TGFβ are related to the development of fibrosis post-infarction. By contrast, in mice little is known about the role of these two molecules during heart remodelling and fibrosis post-infarction, however characterization of the molecular signals involved during these processes in mice may prove to be extremely useful in combination with the use of mouse genetic models to develop new therapies for ischemic heart diseases, which are an important cause of mortality in western societies. Our results challenge the prevailing model of the relationship between TGFβ signalling and CTGF post-myocardial infarction.

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chapter 2

distribution of phosphorylated Smad2 identifies target tissues of TGFß ligands in mouse development

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Distribution of phosphorylated Smad2 identifies target tissues of TGFβ ligands in mouse development

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Abstract

Transforming growth factor β (TGFβ) and related family members control the development of tissues by regulating cell proliferation, differentiation, migration and apoptosis. They transmit signals to the nucleus via phosphorylation of Smad proteins. Here, we used an antibody specifically recognising phosphorylated Smad2 (PSmad2) to identify tissues that have received signals of TGFβ family members acting via Smad2, e.g. TGFβs, activins and nodal. At embryonic day (E)5.5–E8.5, punctuated nuclear PSmad2 staining was scattered throughout the embryo. At E10.5–E12.5, specific zones of the neural tube and brain, ganglia, premuscle masses and precartilage primordia exhibited pronounced nuclear staining, while tissues undergoing epithelial–mesenchymal interactions showed prominent cytoplasmic staining. Interestingly, in the endocardium and most endothelial cells PSmad2 is not detected at E10.5–E12.5, although at E8.5 these cells were stained. These data document the cells that may have received a TGFβ-like stimulus and illustrate, for the first time, the dynamic regulation in space and time of phosphorylated Smad2 during mouse development.
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Keywords: Smad2; TGFβ; Mouse embryo

1. Results and discussion

Smads are important intracellular components of the TGFβ signalling pathway. Specific members of the TGFβ superfamily, including activins, TGFβs and nodal, induce Smad2 (and/or Smad3) phosphorylation through their respective type I receptors, termed activin receptor-like kinases, ALK4, ALK5 and ALK7.

Previous studies have described the distribution of Smad2 mRNA (Waldrip et al., 1998; Tremblay et al., 2000; Dick et al., 1998) and protein (Flanders et al., 2001; Oxburgh and Robertson, 2002) during mouse development. Here, an antibody specifically recognising the phosphorylated C-terminus of Smad2 (Persson et al., 1998) was used to analyse the distribution of activated Smad2 (PSmad2) in mouse embryonic development.

The specificity of this antibody has been tested previously in cells overexpressing constitutively active (ca) ALKs (Persson et al., 1998; Goumans et al., 2002) and was confirmed here in mouse embryos, by both Western blotting (Fig. 1) and immunohistochemistry (Fig. 3A, B and data not shown).

At E6.0–E9.5, Smad2 transcripts are expressed ubiquitously (Waldrip et al., 1998; Tremblay et al., 2000). At E5.5–E7.5, we observed cells with nuclear and/or cytoplasmic PSmad2 staining randomly throughout embryonic and extraembryonic tissues (Fig. 2A–D). This widespread Smad2 activation is probably mediated by ALK4 (Gu et al., 1998) or ALK7 (Reissmann et al., 2001), but not ALK5 (Roelen et al., 1994). After the onset of gastrulation, some embryos showed prominent PSmad2 in the anterior visceral endoderm, surrounding both the embryonic and extraembryonic ectoderm (Fig. 2B, C). The PSmad2 expression differed from that of BMP activated Smads (Fig. 2F–H and Hayashi et al., 2002), confirming no cross-reactivity between the PSmad2 antibody and PSmad1.

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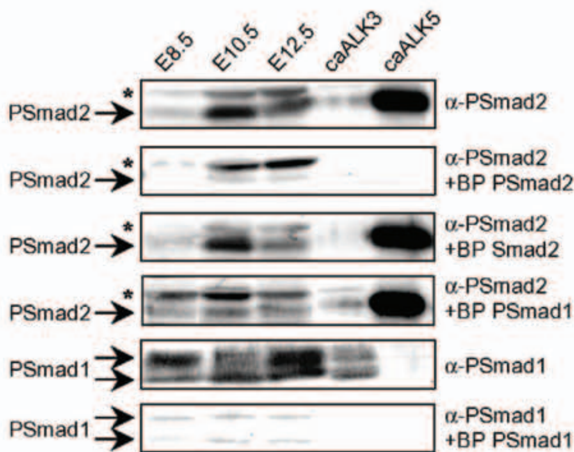

Fig. 1. PSmad antibody specificity. Using Western blot analysis, PSmad2 and PSmad1 were specifically detected in mouse embryos from E8.5 to E12.5. Lysate of COS-1 cells transfected (DEAE-dextran) with both flagged-Smad2 and caALK5 and lysate of HepG2 cells transfected (calcium phosphate) with caALK3, a BMP type I receptor, were used as controls. Recognition of PSmad2 by anti-PSmad2 was blocked by preincubation with cognate PSmad2 phosphopeptide, but not by the corresponding non-phosphorylated Smad2 peptide or the PSmad1 phosphopeptide. Anti-PSmad2 does not recognise PSmad3, a protein of smaller size than PSmad2 (not shown; Nakao et al., 1997; Faure et al., 2000). The PSmad1 antibody recognises the C-terminus of all BMP phosphorylated Smads (PSmad1, 5 and 8) and identifies the endogenous signal as a doublet (Faure et al., 2000). Recognition of PSmad1 by anti-PSmad1 was blocked by preincubation with PSmad1 phosphopeptide. The asterisk indicates a non-specific band. Preincubation was performed as described by Persson et al. (1998) using 5 μg/ml of peptide.

At E8.5, PSmad2 was detected predominantly in nuclei (Fig. 3), scattered throughout the embryonic mesenchyme, somites, body wall, surface ectoderm, blood vessels, blood, fore- and hindgut, endo- and myocardium of the heart. In the visceral yolk sac, PSmad2 positive cells were observed in both mesoderm and endoderm; this continued to E10.5 (Fig. 4G). In neural tissue, PSmad2 was restricted to the neuroepithelium of the prospective fore- and hindbrain (Fig. 3C), including rhombomeres (not shown), rostral extremity of the neural tube closure (Fig. 3D–F) and neural plate (Fig. 3F). At this stage, the expression of ALK4 and ALK7 has not been documented, but ALK5 is present in the neural tube and heart (Mariano et al., 1998).

By E10.5, PSmad2 was observed in the neuroepithelial layer of all brain cavities (Fig. 4D–F). At E12.5, nuclear PSmad2 was present distally in the hypothalamus and the mantel layer of the thalamus and epithalamus (Fig. 5C). Contrasting with the PSmad1 pattern described in the dorsal neural tube (Ahn et al., 2001), nuclear PSmad2 was detected both in the ependymal layer and motor neurons (ventral neural tube) at E10.5 (Fig. 4A), but only in the motor neurons by E12.5 (Fig. 5B). Furthermore, between E10.5 and E12.5, all cranial and dorsal root ganglia, notochord,

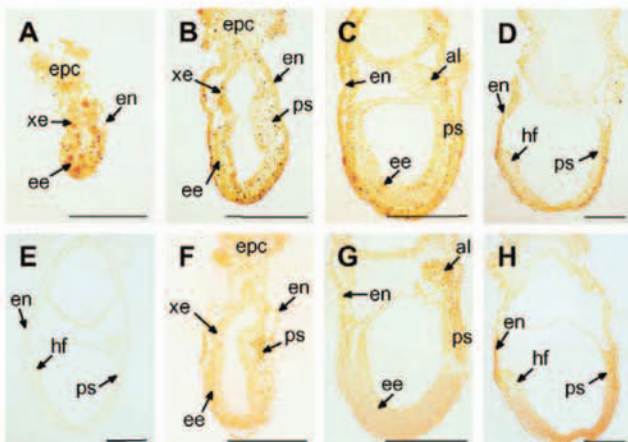

Fig. 2. Distribution of PSmad2 and PSmad1 in E5.5–E7.5 mouse embryos. Sagittal sections of E5.5 (A), E6.5 early streak (B, F), E7.0 late streak (C, G) and E7.5 early head fold (D, H) embryos are shown. PSmad2 positive cells were present throughout the embryo (A–D), while PSmad1 positive cells were restricted to the posterior mesoderm and, to a lesser extent, the endoderm surrounding both the proximal and extraembryonic ectoderm (F–H). E, E7.5 early head fold negative control (without first antibody). Abbreviations: al, allantois; ee, embryonic ectoderm; en, (visceral) endoderm; epc, ectoplacental cone; hf, headfold; ps, primitive streak region; xe, extraembryonic ectoderm. Scale bar: 0.2 mm.

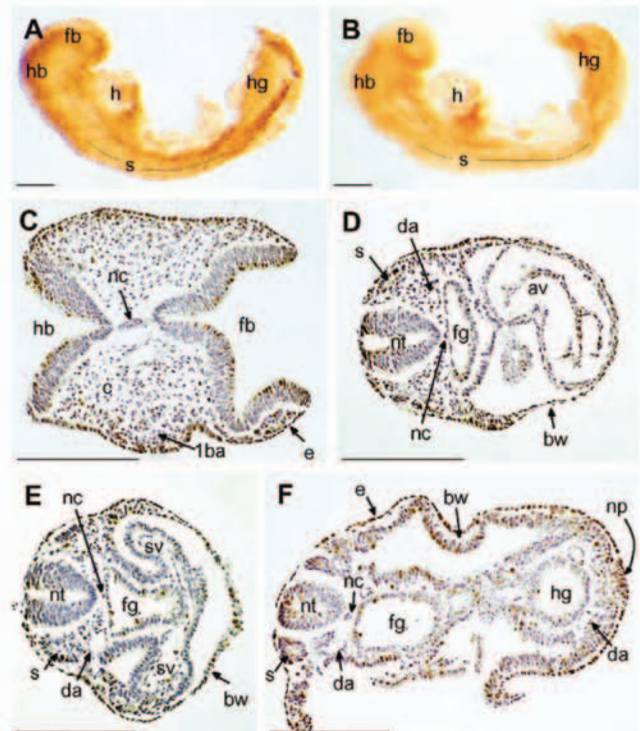

Fig. 3. Distribution of PSmad2 in E8.5 mouse embryos. Whole mount immunohistochemistry using PSmad2 antibody (A) and PSmad2 antibody prebound to its corresponding phosphopeptide (B). Transverse sections of E8.5 embryos stained with PSmad2 antibody are shown (C–F). Expression at E9.5 was similar to that at E8.5, with the exception that staining became detectable at low levels in the notochord, nasal epithelium and buccal cavity (data not shown). Abbreviations: 1ba, origin of the first branchial arch; av, atrio-ventricular canal of the heart; bw, body wall; c, cephalic mesenchyme; da, dorsal aorta; e, surface ectoderm; fb, prospective forebrain; fg, foregut; h, heart; hb, prospective hindbrain; hg, hindgut; nc, notochord; np, neural plate; nt, neural tube; s, somites; sv, sinus venosus of the heart. Scale bar: 0.2 mm.

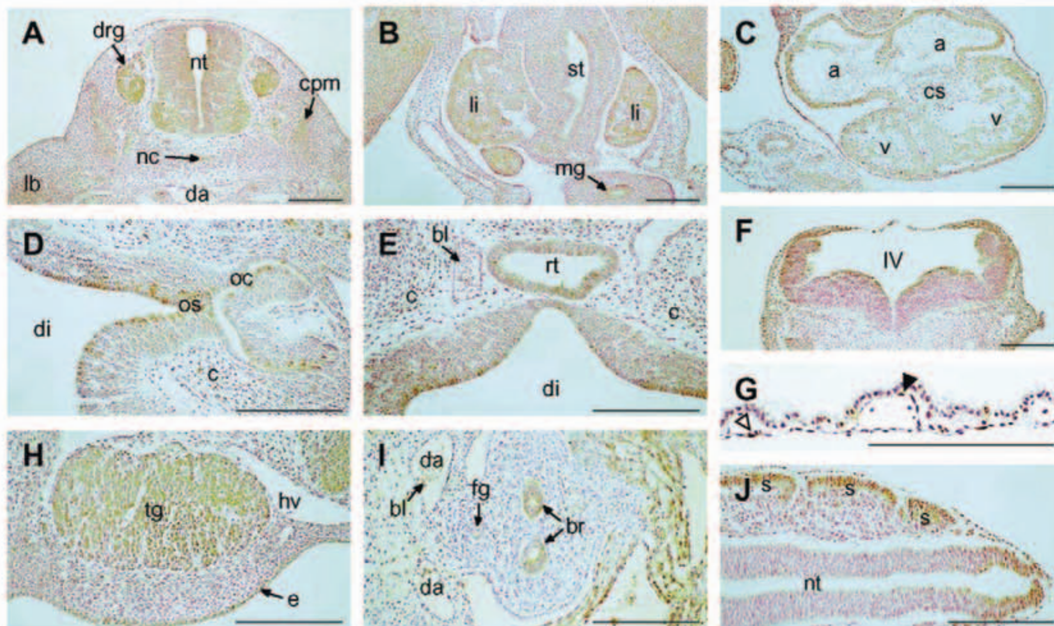

Fig. 4. Distribution of PSmad2 in E10.5 mouse embryos. Transverse sections of E10.5 embryos stained with PSmad2 antibody showing the neural tube (A), stomach (B), heart (C), eye (D), Rathke's pouch (E), brain (F), yolk sac containing positive endothelial (black arrowhead) and mesothelial cells (white arrowhead) (G), ganglion (H), lung (I) and tail (J). Abbreviations: a, atrium; bl, blood; br, main bronchus; c, cephalic mesenchyme; cs, endocardial cushion tissue; da, dorsal aorta; di, diencephalon; drg, dorsal root ganglion; e, surface ectoderm; fg, foregut; hv, primary head vein; lb, forelimb bud; li, liver; mg, midgut; nc, notochord; nt, neural tube; oc, optic cup; os, optic stalk; cpm, condensation of paraxial mesoderm; rt, Rathke's pouch; s, somites; st, stomach; tg, trigeminal (V) ganglion; v, ventricle; IV, fourth ventricle. Scale bar: 0.2 mm.

surface ectoderm, somites/premuscle masses, precartilage primordia, including Meckel's cartilage and cephalic and body mesenchyme were PSmad2 positive (Figs. 4, 5 and 7).

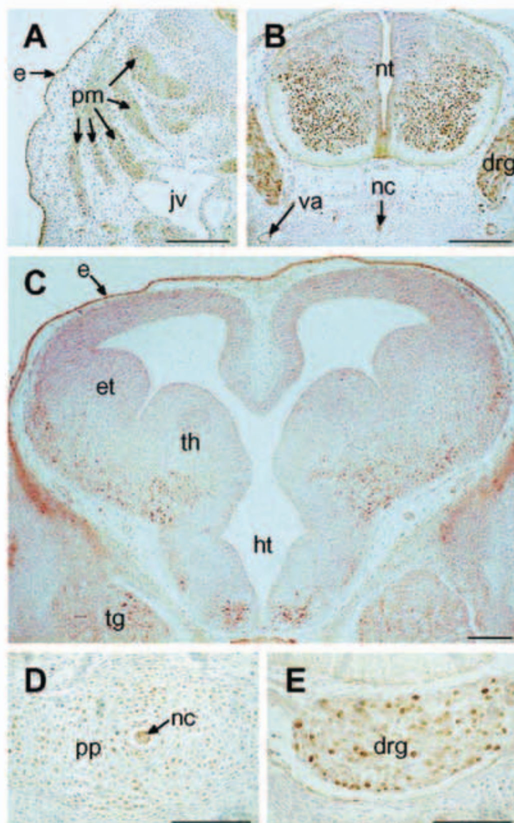

Fig. 5. Distribution of PSmad2 in neural and mesenchymal tissues in E12.5 embryos. Transverse sections of E12.5 embryos stained with PSmad2 antibody showing the premuscle masses (A), neural tube (B), brain (C), precartilage primordium and notochord (D) and dorsal root ganglia (E). Abbreviations: drg, dorsal root ganglion; e, surface ectoderm; et, epithalamus; ht, hypothalamus; jv, external jugular vein; nc, notochord; nt, neural tube; pm, premuscle mass; pp, precartilage primordium; tg, trigeminal (V) ganglion; th, thalamus; va, vertebral artery. Scale bar: 0.2 mm.

ALK5 and ALK4 have been described in all these tissues, except the notochord (Mariano et al., 1998; Iseki et al., 1995; Verschueren et al., 1995). ALK7 has been detected by RNase protection at E7.0, E11.0 and E15.0 but its tissue distribution not described (Reissmann et al., 2001).

In arteries, veins and capillaries, most endothelial cells had become PSmad2 negative by E10.5, although at E12.5, nuclear PSmad2 was observed in the vascular smooth muscle cell layer of arteries (Fig. 6A, B). In the heart, PSmad2 was not detected in cushion tissue and was no longer present in endocardium at E10.5–E12.5, whereas it was maintained in the myocardium (Figs. 4C and 6C, E, F), probably via ALK5 (Mariano et al., 1998), but not ALK4 (Verschueren et al., 1995).

Surprisingly at E12.5, some tissues showed high levels of cytoplasmic PSmad2 (Fig. 7). This was particularly evident in organs undergoing mesenchymal–epithelial interactions, such as lung (Fig. 7E), gut (Fig. 7B), pancreas (Fig. 7B), kidney (Fig. 7C), salivary glands (Fig. 7A), vibrissae (Fig. 7G) and tooth primordia (Fig. 7H). Although some nuclear PSmad2 was observed in the mesenchymal layers of some of

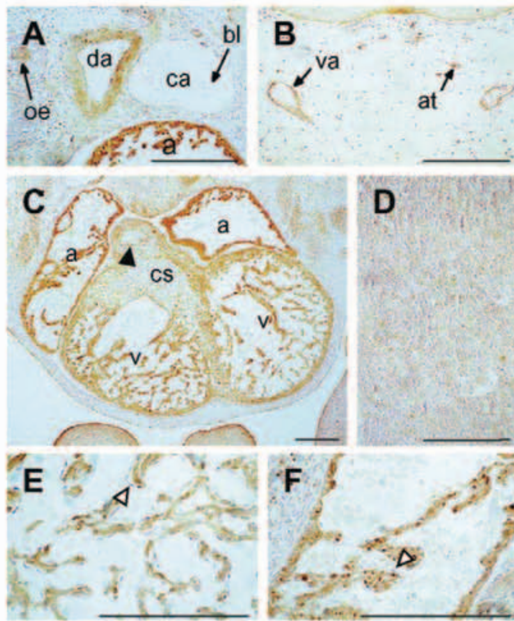

Fig. 6. Distribution of PSmad2 in the cardiovascular system and liver in E12.5 embryos. Transverse sections of E12.5 embryos stained with PSmad2 antibody showing arteries and veins (A, B), the heart at the level of the aortic valve (black arrowhead) (C), liver (D) and the ventricle (E) and atrium (F) at higher magnification. Note that both the endocardial cushion tissue and the endocardium are negative (white arrowheads). Abbreviations: a, atrium; at, arterioles; bl, blood; ca, anterior cardinal vein; cs, endocardial cushion tissue; da, dorsal aorta; oe, oesophagus; v, ventricle; va, vertebral artery. Scale bar: 0.2 mm.

these structures (lung, stomach, midgut and pancreas), all epithelia contained marked cytoplasmic PSmad2. TGFβ/activin ligands and receptors are present in those organs, often in both the epithelium and mesenchyme (Mariano et al., 1998; Heine et al., 1987; Fitzpatrick et al., 1990; Pelton et al., 1990, 1991; Verschueren et al., 1995; Feijen et al., 1994). Since Smad2 phosphorylated in its C-terminus rapidly accumulates in the nucleus (Heldin et al., 1997), cytoplasmic PSmad2 staining was unexpected. Kretzschmar et al. (1999) have shown that Ras activation leads to phosphorylation of MAP kinase sites present in the linker domain of (P)Smad2, preventing its nuclear accumulation in epithelial cells. Thus, activation of the Ras pathway in epithelia, but also at other sites, could be responsible for the observed cytoplasmic localisation of type I receptor activated Smad2. In gonadal ridges, where both ALK4 and ALK5 are expressed (Verschueren et al., 1995; Richards et al., 1999), PSmad2 was also predominantly cytoplasmic (Fig. 7F).

In the liver, PSmad2, probably activated by ALK5 (Mariano et al., 1998), but not ALK4 (Verschueren et al., 1995), was widespread at E10.5 (Fig. 4B) and scattered at E12.5 (Fig. 6D).

Finally, it is interesting to note that the pattern of nuclear localisation of Smad2 changes between E8.5 and E12.5, reflecting the dynamic character and widespread use of the TGFβ signalling pathway during mouse development (Table 1).

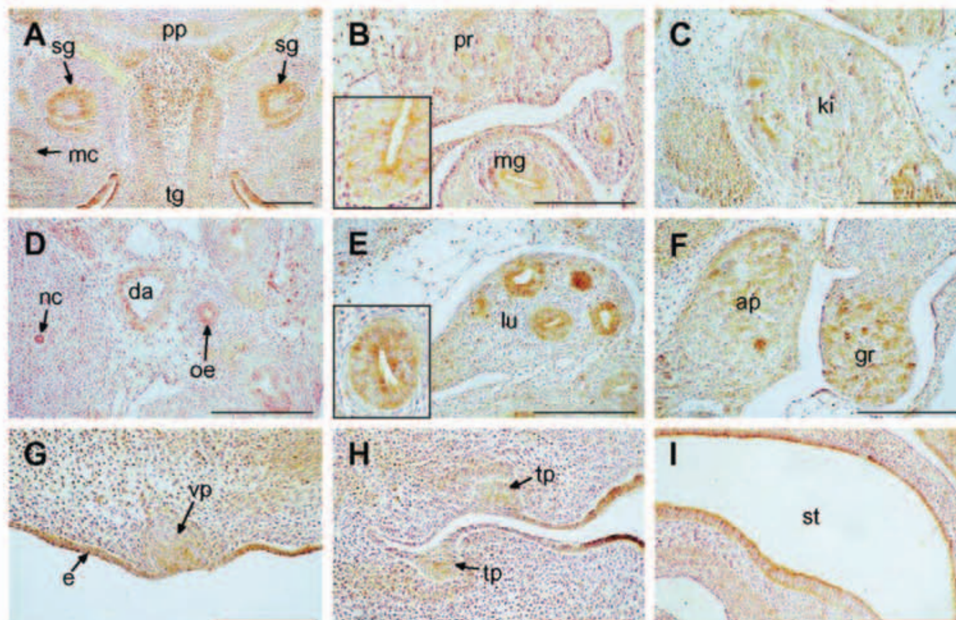

Fig. 7. Distribution of PSmad2 in tissues undergoing epithelial–mesenchyme transition in E12.5 embryos. Transverse sections of E12.5 embryos stained with PSmad2 antibody showing the salivary glands (A), pancreas and midgut (B), kidney (C), oesophagus (D), lung (E), adrenal primordium and genital ridge (F), vibrissa primordium (G), tooth primordia (H) and stomach (I). Insets show the midgut (B) and lung bronchus (E) at higher magnification. Abbreviations: ap, adrenal primordium; da, dorsal aorta; e, surface ectoderm; gr, genital ridge; ki, kidney; lu, lung; mc, pecartilage primordium of Meckel's cartilage; mg, midgut; nc, notochord; oe, oesophagus; pp, precartilage primordium; pr, pancreas; sg, salivary gland; st, stomach; tg, tongue; tp, tooth primordium; vp, vibrissa primordium. Scale bar: 0.2 mm.

Table 1
Tissue distribution of PSmad2 in the mouse[a]

Organ/tissue	E8.5	E10.5	E12.5
Surface ectoderm	$++^{n}$	++	++
Brain	$++^{*n}$	$++^{*}$	$++^{*n}$
Neural tube	$++^{*n}$	$++^{*n}$	$++^{*n}$
Notochord	–	+	++
Nasal epithelium		+	$++^{c}$
Lens (pit)		+	nd
Dorsal ganglia		++	++
Cranial ganglia		++	++
Cephalic mesenchyme	$+^{n}$	+	+
Body mesenchyme	$+^{n}$	+	+
Somites/premuscle mass	$+^{n}$	++	++
Precartilage primordia			++
Genital ridges		nd	$++^{c}$
Liver		$++^{c}$	$+^{c}$
Heart	$+^{n}$	++	$++^{*n}$
Blood vessels	$+/-^{n}$	+/−	$++^{*n}$
Blood	$+^{n}$	+/−	–
Visceral yolk sac	$+^{n}$	$+^{n}$	nd
Lung		$++^{*c}$	$+^{*c}$
Salivary glands			$++^{c}$
Oesophagus/foregut	$+^{n}$	+/−	$++^{*c}$
Buccal cavity		+	+
Stomach		+	$+^{*c}$
Midgut/hindgut	$+^{n}$	+	$+^{*c}$
Pancreas			$+^{*c}$
Mesonephros		nd	$+^{*c}$
Kidney			$+^{*c}$
Adrenal primordia			$++^{c}$
Vibrissa primordia			$++^{*c}$
Tooth primordia			$++^{*c}$

[a] PSmad2 is present both in the cytoplasm and nucleus, otherwise indicated by n (mainly nuclear) or c (mainly cytoplasmic). *, restricted to specific regions (see text); +/−, low expression; +, moderate expression: ++, strong expression; −, not detected; nd, not determined.

2. Experimental procedures

2.1. Embryos

F2 E5.5–E12.5 embryos were from (C57BL/6 × CBA)$_{F1}$ crosses.

2.2. Immunohistochemistry

For whole mount immunohistochemistry, embryos (E5.5–E8.5) were fixed in 2% paraformaldehyde (PFA), dehydrated, treated 5 h with 5% hydrogen peroxide in methanol, rehydrated, permeabilised with 0.1% Triton X-100 (Merck) and blocked overnight (o/n) in 4% normal swine serum (DAKO) in 0.05% Tween (Merck). After o/n incubation with rabbit anti-PSmad2 or anti-PSmad1 (Persson et al., 1998), embryos were washed in 0.05% Tween, treated o/n with biotin-conjugated goat anti-rabbit IgG (DAKO), then with the ABComplex/HRP (DAKO), followed by the Fast 3,3′-diaminobenzidine tablet set (DAB, SIGMA), fixed in 2% PFA/0.1% gluteraldehyde, embedded in plastic and sectioned as in Lawson et al. (1999).

Paraffin sections of E10.5–E12.5 embryos were treated as above, but using 1.2% hydrogen peroxide for 15 min. PowerVision™ Poly-HRP-Conjugates (ImmunoVision Technologies) was used as secondary antibody with DAB Embryonic tissues were denoted according to Kaufman (1992).

2.3. Western blotting

Protein isolation for Western blotting was described by Faure et al. (2000). Analysis was as described in Larsson et al. (2001), using ~190 μg of total lysate.

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chapter 3

BMP signalling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo

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BMP signaling mediated by ALK2 in the visceral endoderm is necessary for the generation of primordial germ cells in the mouse embryo

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Abstract

Deletion of various bone morphogenetic proteins (BMPs) and their downstream Smads in mice have clearly shown that BMP signaling is essential for the formation of primordial germ cells (PGCs). However, the molecular mechanism through which this takes place is still unclear. Here, we demonstrate that BMP4 produced in the extraembryonic ectoderm signals through ALK2, a type I BMP receptor, in the visceral endoderm (VE) to induce formation of PGCs from the epiblast. Firstly, embryonic day (E)5.5-E6.0 embryos cultured on fibronectin formed PGCs in the presence of VE, but not in its absence. Secondly, *Alk2* deficient embryos completely lacked PGCs and the heterozygotes had reduced numbers, resembling *Bmp4* deficient phenotypes. Thirdly, expression of constitutively active ALK2 in the VE, but not in the epiblast, was sufficient to rescue the PGC phenotype in *Bmp4* deficient embryos. In addition, we show that the requirement for the VE at E5.5-E6.0 can be replaced by culturing embryos stripped of VE on STO cells, indicating that STO cells provide or transduce signals necessary for PGC formation that are normally transmitted by the VE. We propose a model in which direct signaling to proximal epiblast is supplemented by an obligatory indirect BMP dependent signal via the VE.

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Introduction

In most metazoan animals, the development of primordial germ cells (PGCs) is crucial for the survival of the species, since these are the cells that form the gametes that transmit the genetic material to future generations. In species such as *Drosophila*, *C. elegans*, *Xenopus*, zebrafish and chick, the PGC lineage can be followed from the egg or the two-cell stage onwards due to the segregation of germ-cell determinants (reviewed by McLaren, 2003). By contrast, in the mouse only at embryonic day (E)7.2 does a founding population of approximately 45 cells descendent from E6.2-E6.5 proximal epiblast (embryonic ectoderm) cells become lineage restricted to the germ line (Lawson and Hage, 1994). Moreover, at E6.5, both proximal and distal epiblast cells are still competent to become PGCs if positioned posteriorly in the proximal epiblast (Tam and Zhou, 1996; Yoshimizu et al., 2001). Interestingly, although allocated by different mechanisms, the PGC lineage is one of the first exclusively embryonic lineages to be established in the mouse as it is also in the other species mentioned.

To date, the repertoire of signals involved in PGC induction has only been identified in part. Lawson et al. (1999) demonstrated that the signal transduction cascade activated by bone morphogenetic protein 4 (BMP4) produced by the extraembryonic ectoderm is necessary for the generation of PGCs and allantois. In addition, other BMPs (BMP8b and BMP2) have been shown to be of importance for establishing normal numbers of PGCs (Ying et al., 2000; Ying et al., 2001a). Recently, Fragilis/mil-1 was detected in the proximal epiblast at E6.25-E6.5 (Tanaka and Matsui, 2002; Saitou et al., 2002) where progenitors of PGCs (among others) become specified (Lawson and Hage, 1994). Strikingly, Fragilis/mil-1 is downregulated in *Bmp4* deficient embryos (Saitou et al., 2002) and may thus be involved in the specification of germ cells, although this is currently unclear.

BMPs belong to the TGFβ superfamily of secreted growth factors. At the cell surface of target cells, BMP dimers bind type I (activin receptor-like kinase (ALK)2, ALK3 or ALK6) and type II (BMPR-II, ActR-IIA or ActR-IIB) BMP receptors forming heteromeric complexes. The type II BMP receptor phosphorylates the type I BMP receptor, allowing it to phosphorylate the BMP receptor-regulated Smads (Smad1, 5 or 8). Upon activation, these Smads bind to Smad4 and translocate from the cytoplasm to the nucleus where they regulate transcription of BMP target genes (for a detailed review see Shi and Massagué, 2003).

Using gene ablation in mice, Smad1 and Smad5 have been described as important for induction of PGCs (Tremblay et al., 2001; Hayashi et al., 2002; Chang and Matzuk, 2001). Embryos lacking *BmpRII* or *ActR-IIA* -/-; *ActR-IIB* -/- double homozygotes fail to form mesoderm entirely, while single mutants for *ActR-IIA* or *ActR-IIB*, although exhibiting a number of defects, develop to term (Beppu et al., 2000; Matzuk et al., 1995; Oh et al., 1997; Song et al., 1999). All three known type I BMP receptors have been deleted in mice, but only *Alk2* and *Alk3* mutants exhibit an embryonic lethal phenotype (Gu et al., 1999; Yi et al., 2000; Mishina et al., 1995). Similar to *BmpRII* deficient embryos, *Alk3* deficient embryos implant and form a proamniotic cavity but fail to gastrulate, whereas *Alk2* deficient mice are arrested slightly later in development. The *Alk2* mutation shows incomplete penetrance and some *Alk2* homozygotes generate embryonic mesoderm, although extraembryonic mesoderm is never observed. This is reminiscent of the *Bmp4* mutant phenotype on some genetic backgrounds (Winnier et al., 1999). In addition, *Alk2* homozygous embryos show abnormal visceral endoderm with a visible constriction at the embryonic-extraembryonic boundary. ALK2 mRNA was detected at E6.0 by RT-PCR (Roelen et al., 1994) and ALK2 was detected at E6.5 by immunofluorescence specifically in the visceral endoderm surrounding the epiblast, but not in the epiblast itself (Gu et al., 1999).

Although BMPs produced by the extraembryonic tissues are required for PGC formation, there is no evidence that BMPs signal directly to the proximal epiblast cells inducing a number of them to become PGCs. This important unsolved question results in part from the difficulty in detecting BMP protein *in vivo*, but also because of the diversity of *in vitro* conditions used by different groups to study the formation of PGCs, including the use of different feeder cells (which are themselves sources of signals) or feeder-free conditions i.e. fibronectin, different mouse strains and embryos or epiblasts of slightly different ages, therefore to relate the work of others to our own, we used embryos of different developmental stages and cultured them in parallel on feeder cells and fibronectin.

Here, we have studied how BMP signaling mediates the formation of PGCs in mouse embryos in culture. Firstly, we demonstrate for the first time that at E5.5-E6.0, the presence of the visceral endoderm is necessary for formation of PGCs. Secondly, we show that PGCs are absent in *Alk2* deficient mouse embryos and that *Alk2* heterozygotes contained a significantly reduced number, reminiscent of the observations in *Bmp4* mutant mice (Lawson et al., 1999). Furthermore, at E6.0-E6.5, expression of constitutively active Alk2 (which triggers BMP signaling in the absence of ligand) in the visceral endoderm of *Bmp4* deficient embryos was sufficient to restore the ability of the *Bmp4* -/- embryos to form PGCs. Together, our results indicate that there is a novel signal activated by BMP4 via ALK2 in the visceral endoderm that is required for proximal epiblast cells to adopt a PGC fate.

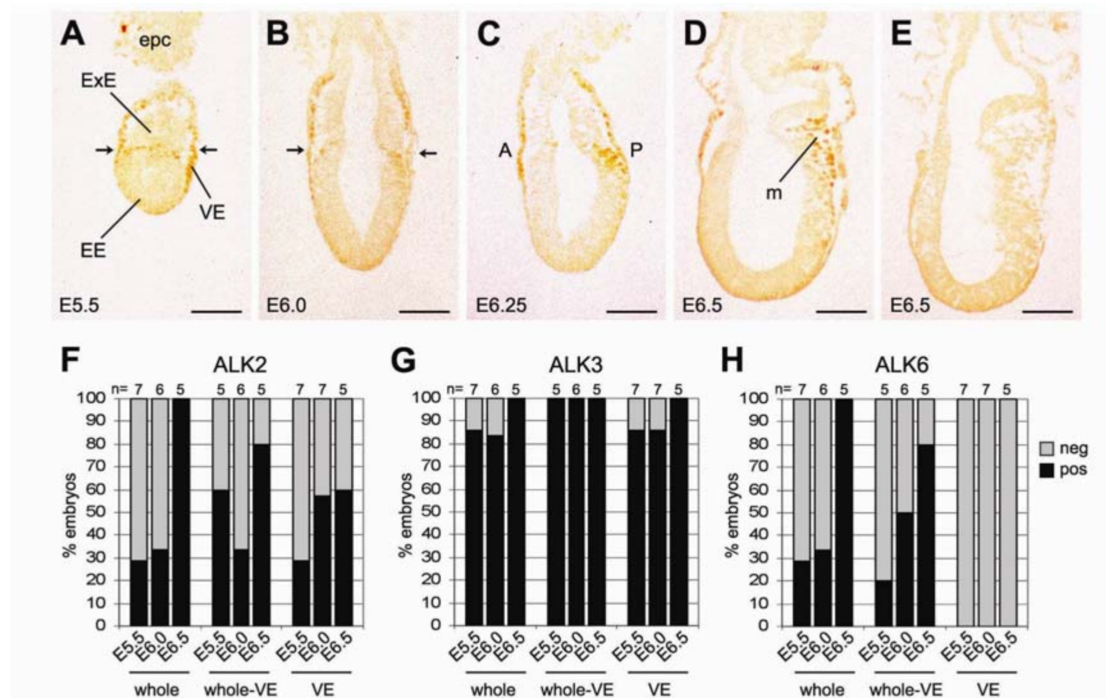

Fig. 1. BMP signaling and expression of BMP type I receptors in early postimplantation mouse embryos. A-E, activated (C-terminal phosphorylated) BMP receptor-regulated Smads (PS1) were detected by immunohistochemistry in the VE and proximal epiblast (arrow) of E5.5 (A) and E6.0 (B) embryos. As gastrulation begins, in addition to prominent PS1 staining in the VE, staining was also evident in the posterior part of the embryo at E6.25 (C) and at E6.5 in the primitive streak and nascent mesoderm (D). E, E6.5 embryo used as negative control (the PS1 antibody was omitted). Abbreviations: A, anterior; EE, epiblast; epc, ectoplacental cone; ExE, extraembryonic ectoderm; m, nascent mesoderm; P, posterior. Scale bar: 50 m. F-H, percentage of embryos (% embryos) expressing mRNA for the different BMP type I receptors, ALK2 (F), ALK3 (G) and ALK6 (H) obtained by RT-PCR in intact embryos (whole), in embryos with the VE removed (whole-VE) and the isolated VE, at E5.5, E6.0 and E6.5. n is de number of independent samples analyzed. The primer combinations used had similar sensitivities (data not shown). GAPDH mRNA was detected in all samples, confirming that RNA isolation and reverse transcription were successful (data not shown).

Results

BMP signaling in proximal epiblast and visceral endoderm during early postimplantation mouse development

Expression of type I and type II BMP receptors and receptor-regulated Smads has been detected as early as E6.0-E7.0 in the mouse embryo (Hayashi et al., 2002; Roelen et al., 1994; Roelen et al., 1997a; Mummery and van den Eijnden-van Raaij, 1999; Dewulf et al., 1995). However, little is known about their expression at earlier stages, even though BMP4 and BMP8b mRNA are present in the extraembryonic ectoderm (ExE) at E5.5 (Lawson et al., 1999; Ying et al., 2000), BMP2 mRNA is detected in the visceral endoderm (VE) at E5.0 (Coucouvanis and Martin, 1999) and the BMP signaling they initiate is thought to be necessary to induce epiblast cells to become PGCs (Yoshimizu et al., 2001).

We detected active BMP signaling in cells of the proximal epiblast at E5.5 (Fig. 1A) using an antibody that specifically recognizes the active (C-terminal phosphorylated) form of the BMP receptor-regulated Smads (PS1, Persson et al., 1998), suggesting that at E5.5 BMPs from the ExE signal directly to epiblast cells. In addition, cells of the VE also exhibited distinctive nuclear PS1 staining (Fig. 1A). At E6.0, BMP signaling was maintained and as gastrulation began (E6.25-E6.5), prominent PS1 was also evident in the cells that formed the primitive streak and nascent mesoderm at the posterior side of the embryo (Fig. 1B-D), as also shown by Hayashi et al. (2002).

To determine which BMP type I receptors mediated the observed activation of BMP signaling both in the VE and the proximal epiblast during early postimplantation stages (E5.5-E6.5), we performed RT-PCR on whole embryos, embryos from which the VE had been removed (whole-VE) and the isolated VE. ALK2 and ALK3 mRNA was detected in whole embryos, isolated VE and VE-stripped embryos at all stages, but ALK3 mRNA was detected at higher frequency (Fig. 1F-G). ALK6 mRNA was detected in VE-stripped embryos, but never in the isolated VE (Fig. 1H).

At E5.5-E6.0, the VE is necessary for induction of PGC formation

To date, the role of the VE in the induction of PGCs has been unclear, in part because in studies on the formation of PGCs, embryos have usually been cultured *in vitro* in the absence of VE, but on a layer of fibroblast feeder cells to facilitate attachment to the substratum. Under these culture conditions, isolated E6.0-E6.25 epiblasts containing no VE and no ExE can develop PGCs (Yoshimizu et al., 2001; Ying et al., 2001b). To define the role of the VE in the induction of PGCs, we cultured E5.5-E6.5 embryos either intact (whole) or with the VE removed (but retaining the ExE) on coverslips coated either with fibroblast feeder cells (STO/SNL cells) or directly on fibronectin (FN). The explants were subsequently analyzed for alkaline phosphatase (AP) activity (Fig. 2). Even though PGCs are not the only AP positive cell population in explants (and embryos), their typical morphology and AP staining pattern, in particular at the cell surface, and the characteristic dark cytoplasmic "spot", allowed us to identify them clearly and unambiguously as PGCs (Chiquone, 1954; Ginsburg et al., 1990). Independent confirmation of their identity was nevertheless obtained (see next section).

We observed that when cultured on STO cells, explants derived from E5.5-E6.0 embryos did indeed generate AP positive PGCs, even when stripped of VE (Fig. 2C,D,H,I). Moreover, although the number of PGCs in the explants was variable, both intact and VE-stripped embryonic explants developed similar numbers of PGCs in culture ($P>0.4$). Explants derived from whole E5.5-E6.0 embryos (containing intact extraembryonic tissues) cultured on FN developed similar numbers of PGCs to explants cultured on STO cells ($P>0.4$). Strikingly, VE-stripped embryos isolated at E5.5-E6.0 did not develop PGCs when cultured on FN-coated coverslips instead of feeder cells, despite similar overall development of the explants (Fig. 2A,B,E,F) and

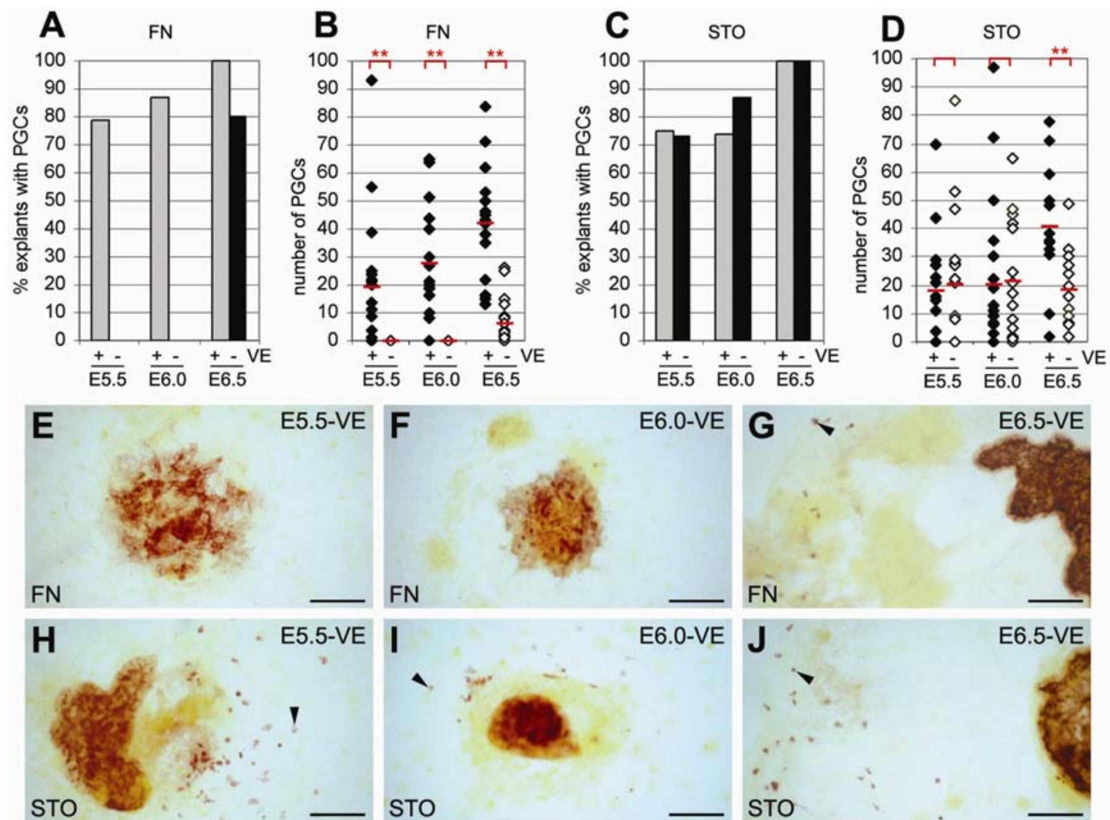

Fig. 2. Formation of PGCs after culture of E5.5-E6.5 embryos either on STO cells or fibronectin. A, percentage (%) of explants derived from intact or VE-stripped E5.5-E6.5 embryos cultured on FN that developed PGCs as determined by AP staining. B, numbers of PGCs detected in explants (n=15-20) cultured on FN depicted in A. C, % of explants derived from intact or VE-stripped E5.5-E6.5 embryos cultured on STO cells that developed PGCs as determined by AP staining. D, numbers of PGCs detected in explants (n=15-19) cultured on STO cells depicted in C. The Student's t-test was used for statistical analysis (** P 0.01) and averages are depicted by a red stripe. E-J, explants derived from VE-stripped E5.5-E6.5 embryos cultured on FN and STO cells after AP staining. Arrowheads indicate PGCs. Scale bar: 150 m.

expression of Esx1 mRNA, a marker of ExE-derived tissues (see next section). These results strongly indicate that in the absence of feeder cells, in addition to the ExE, the VE is in fact required for the formation of PGCs at E5.5-E6.0.

Explants derived from E6.5 embryos no longer appeared to require either VE or feeder cells, and the majority of the VE-stripped embryos cultured on FN formed PGCs (Fig. 2A,G,J), although their number was significantly lower than the number of PGCs in explants derived from intact embryos (Fig. 2B,D). This reduced number of PGCs may be caused by the fact that E6.5-VE embryos cultured both on FN and STO cells had relatively less time to adapt to the culture conditions after the removal of the VE before PGC allocation than either E5.5-VE or E6.0-VE (cultured on feeder cells), so that fewer PGCs formed.

We thus clearly demonstrated that at E5.5-E6.0, the presence of the ExE was not sufficient for induction of PGCs from the epiblast, but that the additional presence of VE was required. However, culturing VE-stripped embryos on a fibroblast feeder layer instead of FN restored the potential of the epiblast cells to generate PGCs. The feeder cells (together with the ExE) thus appeared to allow formation of PGCs by providing either direct signals to the epiblast or ExE or indirectly by promoting the *de novo* formation of endoderm.

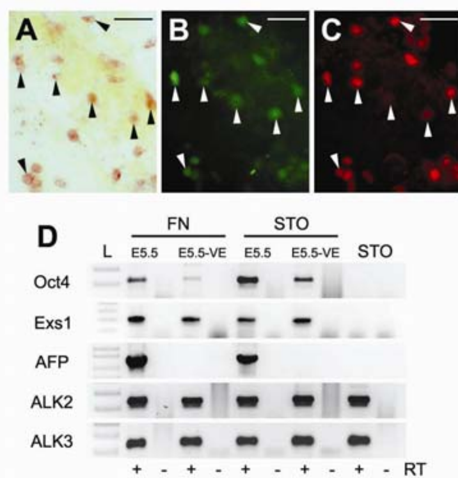

Fig. 3. STO cells support the formation of PGCs, but not by inducing de novo endoderm formation. A-C, most AP-positive cells identified as PGCs (A) coexpressed Oct4 (B) and PGC7/Stella (C) as demonstrated for Oct4ΔPE:gfp heterozygous E6.0 embryos cultured without VE on STO cells. PGCs are indicated by arrowheads. Scale bar: 40 m. D, genes expressed in E5.5 explants cultured whole or without VE (-VE) either on STO cells or FN were studied by RT-PCR independently in 3-6 explants per group. Oct4 mRNA was present in explants where PGCs were observed, but it was also detected in half of the E5.5-VE derived explants analyzed cultured on FN (3 out of 6). Esx1 mRNA was detected in all explant groups analyzed in 40-100% of the explants, but not on STO cells. α-fetoprotein (AFP) mRNA was only present in explants cultured whole (containing VE). ALK2 and ALK3 mRNA was detected in all samples analyzed, including STO cells. Contamination by genomic DNA did not occur as indicated by amplification of each sample without reverse transcription (- RT). L, DNA ladder.

Influence of the STO feeder cell layer on explant culture

Since the unambiguous identification of PGCs in culture is essential for the conclusions of the present study, we sought independent means of confirming that cells with AP staining and distinctive morphology were indeed PGCs. Using *Oct4ΔPE:gfp* transgenic embryos (Yeom et al., 1996), we showed that the majority of the AP positive cells (approximately 90%) identified as PGCs in E6.0-VE derived explants cultured on STO cells, co-expressed Oct4 (driven by its distal promoter element) and/or PGC7/Stella (Saitou et al., 2002; Sato et al., 2002), both independent PGC markers (Fig. 3A-C). We confirmed the presence of Oct4 transcripts by RT-PCR in explants derived from E5.5 embryos and strikingly also found that some E5.5-VE derived explants (3 out of 6 explants) cultured on FN expressed Oct4 mRNA (Fig. 3D). Because these explants had no recognizable PGCs, they may have been slightly younger embryos that developed less well in culture and retained Oct4 positive pluripotent epiblast cells. Furthermore, we detected expression of Esx1 mRNA, a marker for ExE-derived tissues normally upregulated *in vivo* at E8.5 (Li et al., 1997) in all groups of E5.5 explants in at least 40% of the explants analyzed, but not in STO cells alone (Fig. 3D). This indicated that the absence of PGCs observed in VE-stripped E5.5 derived explants (but not in explants from intact embryos) cultured on FN was not the result of the possible degeneration of the ExE, but is specifically due to the absence of the VE; it also showed that the kinetics of Esx1 mRNA upregulation in explants is comparable to that expected from *in vivo* reports.

To establish whether the PGC inducing capacity of feeder cells during the culture of embryos was indirect and the result of *de novo* endoderm differentiation, we performed RT-PCR to detect expression of α-fetoprotein (AFP) mRNA, a marker of VE (Dziadak and Adamson, 1978) but also expressed transiently in definitive gut endoderm at E8.0-E9.0 (Gualdi et al., 1996; Kuo et al., 1997) and subsequently in the liver, a definitive endoderm derivative (Watt et al., 2001). After culture either on FN or STO cells, we detected AFP transcripts in explants derived from intact E5.5 embryos as expected, but not in E5.5-VE derived explants (Fig. 3D). These results suggest that STO cells did not induce or support the formation of endoderm from VE-stripped embryos.

Interestingly, STO cells expressed mRNA for ALK2 and ALK3, the two BMP type I receptors detected in the VE (Fig. 3D), although neither BMP4 nor BMP2 transcripts were detected (data not shown). STO cells showed no or weak PS1 staining, but when treated with 100 ng/ml of BMP4, nuclear PS1 staining was evident (data not shown), indicating activation of the BMP signaling pathway. Therefore, STO cells could be supporting formation of PGCs by translating BMP signals generated in

the ExE of the embryos cultured. To test this hypothesis, we isolated E5.5-E6.5 embryos obtained from crossings of *Bmp4* heterozygous mice, cut them longitudinally in two halves and cultured them on STO cells in the presence or absence of exogenous BMP4. From all explants derived from E5.5-E6.5 *Bmp4* heterozygous and wildtype littermates, 40-83% contained PGCs, independently of the presence of exogenous BMP4 (Fig. 4A-C). Furthermore, the number of PGCs was not significantly different between BMP4 treated and untreated explant groups ($P \geq 0.2$), suggesting that addition of exogenous BMP4 is not sufficient to increase the number of PGCs formed, even at E5.5.

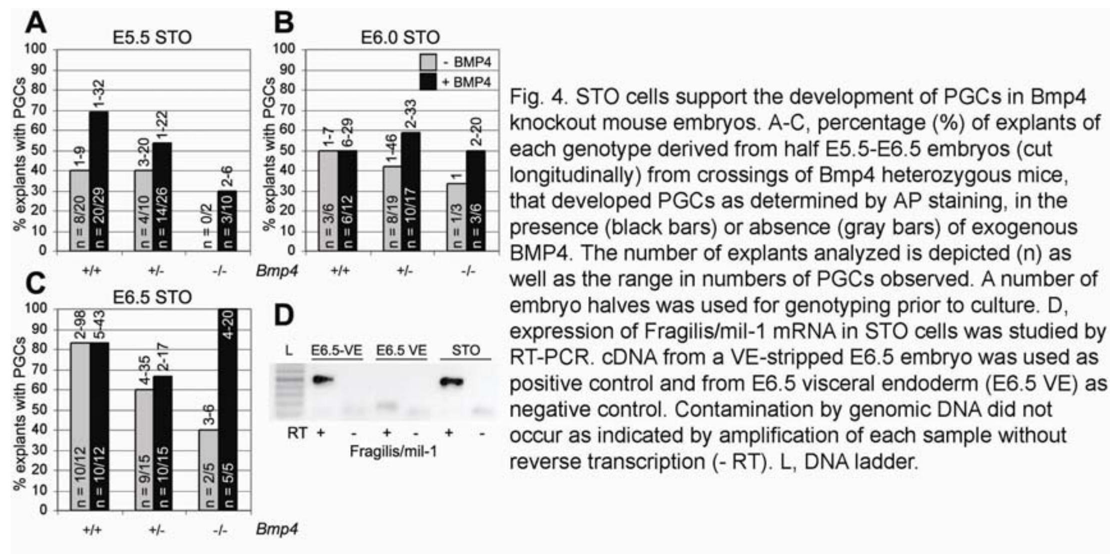

Fig. 4. STO cells support the development of PGCs in Bmp4 knockout mouse embryos. A-C, percentage (%) of explants of each genotype derived from half E5.5-E6.5 embryos (cut longitudinally) from crossings of Bmp4 heterozygous mice, that developed PGCs as determined by AP staining, in the presence (black bars) or absence (gray bars) of exogenous BMP4. The number of explants analyzed is depicted (n) as well as the range in numbers of PGCs observed. A number of embryo halves was used for genotyping prior to culture. D, expression of Fragilis/mil-1 mRNA in STO cells was studied by RT-PCR. cDNA from a VE-stripped E6.5 embryo was used as positive control and from E6.5 visceral endoderm (E6.5 VE) as negative control. Contamination by genomic DNA did not occur as indicated by amplification of each sample without reverse transcription (- RT). L, DNA ladder.

Surprisingly, a number of *Bmp4* deficient explants were able to generate PGCs when cultured on STO cells, even in the absence of exogenous BMP4 (Fig. 4B,C). This clearly indicated that STO cells provided more than just an appropriate substratum or transduction of BMP4 from the ExE, but probably expressed signal(s) similar to those downstream of BMP4 in the embryo important for the formation of PGCs. STO cells thus have an intrinsic ability to trigger ≥E6.0 *Bmp4* -/- epiblast cells to adopt a PGC fate, together with possible signals from the *Bmp4* -/- ExE or VE cells, i.e. BMP8b and BMP2, respectively. In this respect, it is of note that we detected Fragilis/mil-1 transcripts in STO cells by RT-PCR (Fig. 4D). This transmembrane molecule acts downstream of BMP4 and is present before gastrulation throughout the epiblast and co-localizes during gastrulation with the precursor population from which the PGCs arise (Saitou et al., 2002; Tanaka et al., 2002).

ALK2 is necessary for PGC formation *in vivo*

The unexpected role of the VE in formation of PGCs under feeder-free conditions and the observation that active BMP signaling was present at E5.5-E6.0 in the VE, particularly at the junction between extraembryonic and embryonic ectoderm, led us to investigate whether ALK2, one of the BMP type I receptors detected in the VE at these developmental stages could be involved in formation of PGCs.

We counted PGCs in embryos lacking *Alk2*, after AP staining and failed to identify any PGCs in these mutants at E7.5-E8.0, although their wildtype littermates (3-7 somites) contained on average 44 PGCs (Fig. 5). More interestingly, the *Alk2* heterozygous embryos contained a reduced number of PGCs (26 on average) compared to wildtype littermates ($P<0.05$), phenocopying in this respect *Bmp4* heterozygous embryos (Lawson et al., 1999). These results indicate that ALK2 is necessary for the formation of PGCs.

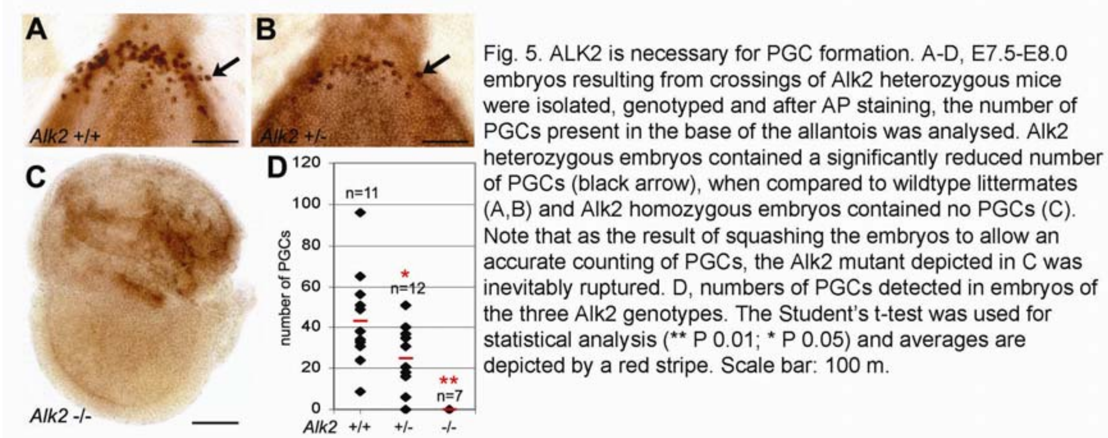

Fig. 5. ALK2 is necessary for PGC formation. A-D, E7.5-E8.0 embryos resulting from crossings of Alk2 heterozygous mice were isolated, genotyped and after AP staining, the number of PGCs present in the base of the allantois was analysed. Alk2 heterozygous embryos contained a significantly reduced number of PGCs (black arrow), when compared to wildtype littermates (A,B) and Alk2 homozygous embryos contained no PGCs (C). Note that as the result of squashing the embryos to allow an accurate counting of PGCs, the Alk2 mutant depicted in C was inevitably ruptured. D, numbers of PGCs detected in embryos of the three Alk2 genotypes. The Student's t-test was used for statistical analysis (** P 0.01; * P 0.05) and averages are depicted by a red stripe. Scale bar: 100 m.

Expression of constitutively active ALK2 in the VE of *Bmp4 -/-* embryos induces formation of PGCs

Although we showed that *in vitro* the presence of the VE between E5.5-E6.0, on the one hand and that *in vivo* the BMP type I receptor ALK2 on the other is necessary for formation of PGCs, it was still unclear whether it is ALK2 activation of the BMP signaling pathway in the VE that causes proximal epiblast cells to adopt a PGC fate. To test this hypothesis, we infected E5.5-E6.0 and E6.5 embryos generated from crossings of *Bmp4* heterozygous mice with adenovirus expressing constitutively active (ca)*Alk2* (which triggers BMP signaling in the absence of ligand) and cultured the embryos on FN. Firstly, however we infected intact E5.5 and E6.5 embryos with adenovirus expressing *LacZ* and showed that punctuated β-galactosidase staining (X-gal) was detected exclusively in the VE (Fig. 6A). Further, we confirmed by Western blot analysis that infection of HepG2 cells with adenovirus encoding ca*Alk2* (ca*Alk2*-virus) induced phosphorylation of BMP receptor-regulated Smads (PS1) as did exposure to exogenous BMP4 (Fig. 6B). Infection of intact E5.5 and E6.5 *BRE:LacZ* transgenic embryos, containing a BMP responsive element coupled to *LacZ* (Monteiro et al., unpublished results) with ca*Alk2*-virus indicated exactly where activation of the BMP signaling pathway occurred. After one day of culture, E5.5 *BRE:LacZ* transgenic embryos showed no detectable X-gal staining, but when infected with ca*Alk2*-virus a number of VE cells became X-gal positive (data not shown). Most E6.5 embryos had a well developed exocoelomic cavity surrounded by extraembryonic mesoderm after one day of culture. Using E6.5 *BRE:LacZ* transgenic embryos, we observed activation of BMP signaling in particular in the mesoderm but infection with ca*Alk2*-virus resulted in ectopic activation of BMP signaling in a punctuated fashion in the VE as expected, in addition to BMP signaling in the mesoderm and some epiblast cells (Fig. 6C). Together, these data indicate that adenoviral infection is effective in E5.5-E6.5 intact embryos, occurred in the VE and expression of caALK2 resulted in activation of BMP signaling in embryos *in vitro*.

After infection with ca*Alk2*-virus, we found clearly identifiable PGCs in explants derived from E5.5-E6.0 *Bmp4* homozygous knockout embryos at similar frequencies as in heterozygous and wildtype littermates (Fig. 6D), whereas in contrast to the majority of heterozygous and wildtype littermates, only 25% (1 in 4) ca*Alk2* infected explants derived from E6.5 *Bmp4* homozygous embryos formed PGCs (Fig. 6D). Our results indicate that activation of the BMP signaling pathway via caALK2 in the VE of *Bmp4* deficient embryos is sufficient to induce the formation of PGCs around E6.0-E6.5 (when the product encoded by the adenovirus becomes expressed), but not after that (at E7.0) when PGCs are about to become allocated.

To investigate whether activation of the BMP signaling pathway via caALK2 directly in the epiblast (or ExE) could lead to the formation of PGCs, we also infected

wildtype E5.5-E6.0 and E6.5 embryos with and without VE with ca*Alk2*-virus and cultured the embryos *in vitro* on FN. PGCs were readily observed in infected explants derived from whole embryos, but not in infected explants derived from VE-stripped embryos (Fig. 6E), although the embryos could be effectively infected (Fig. 6A). These results clearly indicate that overexpression of caALK2 in the epiblast (or ExE) of VE-stripped wildtype embryos is not sufficient to induce formation of PGCs and therefore the rescued *Bmp4* -/- embryos developed PGCs due to the overexpression of caALK2 specifically in the VE.

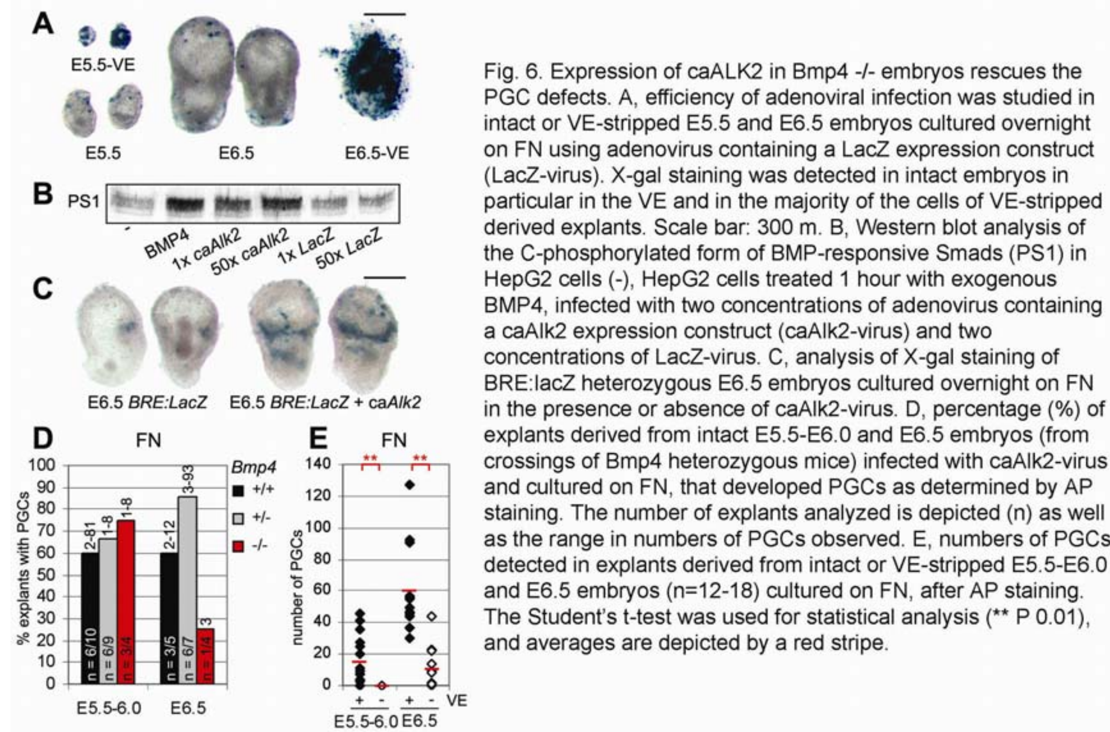

Fig. 6. Expression of caALK2 in Bmp4 -/- embryos rescues the PGC defects. A, efficiency of adenoviral infection was studied in intact or VE-stripped E5.5 and E6.5 embryos cultured overnight on FN using adenovirus containing a LacZ expression construct (LacZ-virus). X-gal staining was detected in intact embryos in particular in the VE and in the majority of the cells of VE-stripped derived explants. Scale bar: 300 m. B, Western blot analysis of the C-phosphorylated form of BMP-responsive Smads (PS1) in HepG2 cells (-), HepG2 cells treated 1 hour with exogenous BMP4, infected with two concentrations of adenovirus containing a caAlk2 expression construct (caAlk2-virus) and two concentrations of LacZ-virus. C, analysis of X-gal staining of BRE:lacZ heterozygous E6.5 embryos cultured overnight on FN in the presence or absence of caAlk2-virus. D, percentage (%) of explants derived from intact E5.5-E6.0 and E6.5 embryos (from crossings of Bmp4 heterozygous mice) infected with caAlk2-virus and cultured on FN, that developed PGCs as determined by AP staining. The number of explants analyzed is depicted (n) as well as the range in numbers of PGCs observed. E, numbers of PGCs detected in explants derived from intact or VE-stripped E5.5-E6.0 and E6.5 embryos (n=12-18) cultured on FN, after AP staining. The Student's t-test was used for statistical analysis (** P 0.01), and averages are depicted by a red stripe.

We also addressed the question of whether BMP4 acted exclusively on the VE and if this signal was sufficient to induce the formation of PGCs from epiblast cells under feeder-free conditions. Therefore, we cultured the distal part of E6.0 wildtype embryos (containing both epiblast and VE) in the presence of exogenous BMP4, ca*Alk2*-virus or both and analyzed the number of PGCs after culture on FN, but PGCs were not observed (n=17-19; data not shown). Thus, we were unable to show that BMP4 (or caALK2) acts exclusively via the VE at E6.0-E6.5, but we suggest that although both BMP4 and ALK2 are necessary for the formation of PGCs, they are not sufficient to induce PGCs ectopicaly and the presence of the ExE may be also required.

Discussion

ExE and VE are both essential for PGC allocation at E5.5-E6.0

Of the three BMP ligands known to be involved in the development of PGCs, BMP4 and BMP8b are produced in the ExE at E5.5-E7.5 (Lawson et al., 1999; Ying et al., 2000), while BMP2 is expressed slightly earlier at E5.0-E7.5 in the VE (Coucouvanis and Martin, 1999). Thus a role for the VE as well as the ExE in PGC formation has been suggested but never fully elucidated. One reason for this is that most studies on the generation of PGCs *in vitro* have been performed by culturing embryos on a layer of feeder cells.

We observed the appearance of PGCs in E5.5-6.0 VE-stripped embryos (epiblast and ExE) cultured on STO cells, but not in parallel cultures on FN, indicating

that in the absence of feeder cells, E5.5-E6.0 epiblast cells are not able to develop PGCs independently of signals provided by both extraembryonic tissues, VE and ExE. This suggests that although BMP signals generated in the ExE at E5.5-E6.0 are necessary to induce PGCs in the epiblast, *in vitro* they are clearly not sufficient and the presence of the VE is also required. This observation has not been documented previously.

After E6.0, epiblast cells of VE-stripped embryos were capable of generating PGCs, although the numbers of PGCs observed were significantly reduced compared to explants derived from intact embryos, suggesting that the VE is still important at that stage, but no longer strictly necessary. In agreement with our data, Yoshimizu et. al. (2001) observed that when cultured on FN, isolated E6.0 epiblasts were unable to develop PGCs, while 40% of the explants derived from E6.25 epiblasts contained PGCs. Together these results demonstrate that under feeder-free culture conditions, ≤E6.0 isolated epiblast cells are not able to form PGCs autonomously, but require external signals provided not only by the ExE, but also by the VE.

ALK2 functions as a type I receptor for BMP4

Activated BMP receptor-regulated Smads (PS1) were detected at E5.5-E6.0 in the proximal epiblast and in the VE. Smad1, 5 and 8 are generally activated by BMPs, but they can also be activated by TGFβ via ALK1, a type I receptor (Chen and Massagué, 1999), but not by nodal (Kumar et al., 2001). However, ALK1 mRNA is only expressed from E6.5 onwards (Roelen et al., 1997b), in contrast to ALK2, ALK3 and ALK6 mRNA, which we showed were expressed earlier in the embryo. Both ALK3 and ALK6 are well-characterized type I receptors that translate signals from BMPs of both the DPP class (BMP2 and 4) and 60A class (BMP5, BMP6, BMP7, BMP8a and BMP8b). ALK2 on the other hand is known to translate signals from BMPs of the 60A class, but the relationship with the DPP class is less clear. Although BMP2 has been shown to bind ALK2 and activate the BMP signaling pathway (Liu et al., 1995), it has not been formally shown that BMP4 also has this capacity.

In this study, we have provided the first evidence that ALK2 functions *in vivo* as a type I receptor for BMP4 (and possibly BMP2). Firstly, we demonstrated that *Alk2* -/- embryos form no PGCs, while in *Alk2* +/- embryos the number of PGCs was reduced when compared to wildtype littermates. This reflected exactly the findings in mutant *Bmp4* mice, where *Bmp4* +/- embryos had reduced number of PGCs and *Bmp4* -/- lacked PGCs entirely (Lawson et al., 1999). Secondly, we were able to rescue formation of PGCs in *Bmp4* -/- embryos by expressing caALK2 in the VE, which mimicked activation of the BMP pathway, but were not able to rescue formation of PGCs by expressing caALK2 in VE-stripped wildtype embryos. These data suggest a role for ALK2 specifically in the VE, transducing BMP signals necessary for formation of PGCs.

It is however not clear whether this effect is restricted to caALK2 or whether caALK2 downstream intracellular targets are distinct from those of caALK3 and caALK6. Nevertheless, we demonstrated that ALK2 does play a crucial role in the formation of PGCs and that in its absence, neither ALK3, the other BMP type I receptor detected in the VE at E5.5-E6.0 nor ALK6 are capable of supporting allocation of PGCs. We have no evidence for which BMP type II receptor partners ALK2 in the visceral endoderm, although all BMP type II receptors are functional before gastrulation (Beppu et al., 2000; Song et al., 1999). In addition, Smad5 mRNA was not detected in the VE at E6.5 (Chang et al., 1999), while Smad1 is clearly expressed in the VE before gastrulation and more importantly deficiency in *Smad1* results in complete absence of PGCs (Hayashi et al., 2002), similarly to deficiency in either *Alk2* or *Bmp4*.

Indirect BMP4 signaling controls PGC formation at E5.5-E6.5

Based on the data reported here, we propose that a still unknown signal, regulated by BMP4 (and probably BMP2) activation of ALK2 in the VE is involved in directing proximal epiblast cells towards a PGC (and possibly allantois) fate, at E5.5-E6.0 (Fig. 7). This would explain why embryos without VE but containing the ExE (and therefore able to produce BMP4) were unable to form PGCs when cultured under feeder-free conditions.

Bmp2 -/- mutants form a reduced number of PGCs (Ying et al., 2001a) and more importantly *Bmp2* +/-; *Bmp4* +/- double heterozygotes had fewer PGCs when compared to single *Bmp4* or *Bmp2* heterozygous embryos (Ying et al., 2001a), suggesting that BMP4 and BMP2 have additive effects in the induction of PGCs. This observation is in agreement with the proposed role of BMP4 in the VE, where BMP2 is produced. Interestingly, additive effects were not observed between either BMP2 or BMP4 with BMP8b (Ying et al., 2000; Ying et al., 2001a) and therefore we suggest that BMP8b signals independently of BMP4/2, probably directly to the proximal epiblast via Smad5 (and Smad1) (Fig. 7). In contrast to the absence of PGCs in *Bmp4*, *Alk2* and *Smad1* deficient embryos, at least 50% of *BMP8b* or *Smad5* deficient embryos contained PGCs, although in reduced numbers (Lawson et al., 1999; Hayashi et al., 2002; Ying et al., 2000; Chang and Matzuk, 2001).

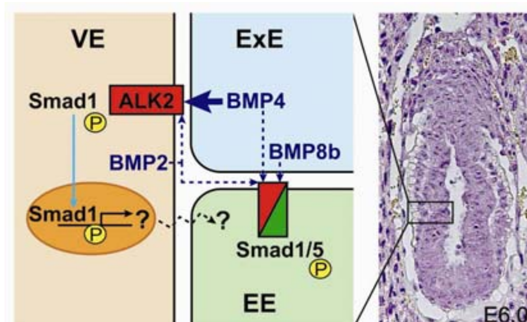

Fig. 7. BMP signaling and the formation of PGCs. At E5.5-E6.0, BMP4 secreted by the ExE binds to BMP receptor complexes consisting of BMP receptor type II and ALK2 in the VE which results in Smad1 phosphorylation and transcription regulation of BMP target genes. Among these target genes, an unknown factor(s) (?) directs proximal epiblast cells towards a PGC (and possibly allantois) fate. BMP2 produced by the VE possibly signals together with BMP4 in the VE and both may also signal in parallel in the proximal epiblast. BMP8b signals independently of both BMP2 and BMP4 and probably acts directly from the ExE on the proximal epiblast via ALK2 (red) or possibly ALK3 (green).

PGCs are completely absent in *Bmp4* deficient embryos. The observation that activation of BMP signaling in the VE (by caALK2) was sufficient to induce PGCs in *Bmp4* -/- embryos at E6.0-E6.5 indicates that the BMP4-ALK2 mediated signal produced by the VE that we propose is important for generation of PGCs in the embryo. Although it could be argued that adenovirus could infect both VE cells and some epiblast cells, we demonstrated that *caAlk2*-infected VE-stripped embryos formed no PGCs, showing that the induction of PGCs in *Bmp4* -/- mutants is specifically due to overexpression of caALK2 in the VE, mimicking there activation of BMP signaling.

The work presented here clearly indicates that BMP4 signaling via ALK2 in the VE induces indirectly the development of PGCs from epiblast cells. But although necessary, both BMP4 and ALK2 are not sufficient to induce ectopic PGCs in the distal epiblast in the absence of ExE at E6.0, suggesting that there is probably a second signal needed produced by the ExE e.g. BMP8b, to induce epiblast cells to differentiate to PGCs (Fig. 7).

The role of feeder cells in PGC development *in vitro*

The outcomes of experiments using different feeder cells to study the development of PGCs are not equivalent. Yoshimizu et al. (2001) cultured E6.25 proximal epiblasts on STO and on SI/SI$_4$ cells and obtained different results both with respect to the percentage of explants with PGCs (62% vs. 35%, respectively) and the number of PGCs (ranging from 1-45 vs. 4-7, respectively). This suggested that STO

cells in particular provided important external support for the development of PGCs in culture.

Ying et al. (2001b) were unable to detect PGCs by culturing E6.0-E6.25 *Bmp4* -/- embryos on transgenic Bmp4-producing COS7 cells. Therefore, we were surprised by the observation that E6.5 *Bmp4* -/- embryos cultured on STO cells formed PGCs. Here, we demonstrate that STO cells induced the formation of PGCs not by inducing endoderm formation, nor by transducing BMP signals produced by the ExE. It is more likely that STO cells produce either a similar signal to the postulated BMP4-ALK2 mediated signal generated in the VE, or a downstream signal similar to that produced by proximal epiblast cells themselves. This would also explain why E5.5-E6.0 VE-stripped embryos developed PGCs when cultured on STO cells, but not on FN. Presumably, COS7 cells produce this signal less efficiently.

STO cells express leukemia inhibitor factor, steel factor and mast-cell growth factor, among other diffusible factors known to stimulate PGC proliferation and survival in culture (Dolci et al., 1991; Godin et al., 1991). Furthermore, we detected Fragilis/mil-1 mRNA by RT-PCR in STO cells. Together, these factors could provide an environment that is permissive not only for proliferation and survival, but also for the induction of PGCs in culture. Although it is still unclear whether Fragilis/mil-1 is necessary for the formation of PGCs, the observation that it acts downstream of BMP4 in the epiblast and its co-localization with PGCs as they form (Tanaka and Matsui, 2002; Saitou et al., 2002), makes it a good candidate to explain the PGC-inductive properties of STO cells, in particular in *Bmp4* -/- embryos.

Interestingly, STO cells are able to support the development of PGCs from VE-stripped E5.5-E6.0 embryos, isolated E6.0 whole or proximal epiblasts (Yoshimizu et al., 2001), but not distal E6.0 epiblasts (Yoshimizu et al., 2001). Thus, the signals produced by STO cells are not sufficient to induce ectopic PGCs.

At E5.5-E6.0, the epiblast needs BMP4 to develop (PGCs) in culture

By developing in close contact with STO cells, E6.0-E6.5 *Bmp4* -/- embryos formed PGCs. However, PGCs did not emerge from E5.5 *Bmp4* -/- explants cultured on STO cells, but were observed when those explants were cultured on STO cells and BMP4 was added to the culture medium, suggesting that the signals produced by STO cells are not sufficient to induce PGCs in cultured embryos prior to E6.0, but additional BMP signals may be necessary to support general development and subsequently PGCs may form. We observed formation of PGCs in VE-stripped E5.5 embryos cultured on STO cells probably because additional to the STO-produced signals, BMPs are being produced in the ExE. Similarly, Hayashi et al. (2002) cultured E5.5-E5.75 epiblasts on STO cells, but were only able to observe PGCs when BMP4 was added to the culture medium. Moreover, they observed that E5.5-E5.75 *Smad1* -/- mutant epiblasts did not develop PGCs, even in the presence of exogenous BMP4 on STO cells. In agreement with the results mentioned above, Toyooka et al. (2003) recently showed that germ cells were formed in coaggregates of embryonic stem (ES) cells with transgenic Bmp4-producing feeders, including STO cells, immediately after 1 day of culture, but germ cells were not observed in aggregates of ES cells only cultured in the presence of exogenous BMP4 or coaggregates of ES cells with STO cells.

All together, the results indicated that at E5.5-E6.0 BMP signals i.e. BMP4 are necessary not only specifically for the generation of PGCs but probably for the general development and differentiation of E5.5-E6.0 epiblast cells in culture, as also suggested by Pesce et al. (2002).

Material and methods

Isolation of embryos and culture of embryonic explants

The genetic background of the *Alk2*^{tm1} mice (Gu et al.,1999) was BL6/129 and of both wildtype and *Bmp4*^{tm1blh} mice (Lawson et al., 1999) was BL6/CBA. *Oct4ΔPE:gfp* transgenic mice were generated as described by Anderson et al. (1999) on a BL6/CBA background. *BRE:LacZ* transgenic mice were generated using the BRE promoter sequence (Korchynskyi and ten Dijke, 2002) coupled to *LacZ* on a 25% BL6/75% 129 and will be published separately (Monteiro et al., unpublished).

The mice were kept in a normal or reversed light/dark cycle and the noon of day (12:00) of the vaginal plug was designated E0.5 or E0.0, respectively. Uteri isolated from mice killed by cervical dislocation were kept in cold Dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 7.5% fetal calf serum (FCS) and 10 mM HEPES. E5.5, E6.0 and E6.5 embryos were isolated using tungsten needles to remove both the Reichert's membrane and the ectoplacental cone, and staged according to Yoshimizu et al. (2001).

For the culture of embryonic explants, we used (1) whole embryos, consisting of intact epiblast, ExE, VE and, at E6.5, primitive streak and nascent mesoderm; (2) whole embryos cut longitudinally (through the axis of bilateral symmetry, if this was morphologically discernible) using glass needles, but containing parts of all tissues described above; (3) embryos containing both an intact epiblast and ExE plus, depending on the stage, the primitive streak and nascent mesoderm, but with the VE layer removed. To remove the VE layer, whole embryos were placed for 4 minutes at 4^0C in a droplet of pancreatin/trypsin solution (3% pancreatin (Applichem) in PBS and 2.5% trypsin (Invitrogen) in HBSS without calcium (Invitrogen) mixed 4:1). Thereafter, the embryos were placed in DMEM containing 15% FCS and the VE was removed mechanically using tungsten needles. After the dissection of two embryos/explants, these were placed immediately in DMEM containing 15% FCS, at 37^0C and 7.5% CO_2. Each individual embryo/explant was cultured on a glass coverslip either covered with a confluent layer of mitomycin C-treated STO/SNL feeder cells ($7.5x10^4$ cells/well) or coated with FN (20 μg/ml, Harbor BioProducts) in 4-well culture plates (NUNC). Explants derived from E5.5 embryos were cultured for 4 days and E6.0 and E6.5 embryos for 3 days. When used, BMP4 (R&D systems) was added to the culture medium at 100 ng/ml.

Analysis of primordial germ cells

PGCs were identified and counted on the basis of AP activity and morphology using a 20x objective (Axioplan, Zeiss) as described by Lawson et al. (1999). Student's t-test was used to analyze the differences in the number of PGCs between the different groups of embryos/explants.

Immunohistochemistry

PS1 was detected in wildtype E5.5-E6.5 embryos using rabbit anti-PSmad1 antibody (Persson et al., 1998) by whole mount immunohistochemistry as described (Chuva de Sousa Lopes et al., 2003).

After the removal of the VE layer, heterozygous *Oct4ΔPE:gfp* E6.0 embryos were cultured for 3 days on STO cells (as described above) and used for detection of PGC7/Stella and Oct4. After fixation in 4% paraformaldehyde/PBS (PFA) for 2 hours at 4^0C, the explants were washed three times in PBS, permeabilized 8 minutes with 0.1% Triton X-100 (Merck), washed three times in PBS, blocked with 4% normal goat serum (Dako) with 0.05% Tween (Merck) for 1 hour at room temperature (RT), incubated 1 hour at RT with rabbit anti-PGC7/Stella antibody (Sato et al., 2002) diluted 1:2000 in blocking solution, washed three times in 0.05% Tween and incubated 1 hour at RT with Cy3-conjugated goat anti-rabbit antibody (Jackson

Immuno Research Laboratories, Inc) diluted 1:250 in blocking solution. After staining, the explants were immediately analyzed for GFP positive cells (Oct4) and Cy3 positive cells (PGC7/Stella) using epifluorescent microscopy (Axioplan, Zeiss). Thereafter, the explants were washed three times in PBS, kept for 1 hour in 70% ethanol at 4^0C, washed 3 times in distilled water and treated with AP-staining solution, as above.

Genotyping of embryos and embryonic explants

Samples were boiled in 10 μl 0.1% W1 (Sigma) 5 minutes and after the addition of 10 μl proteinase K (2 mg/ml, Invitrogen) incubated overnight (o/n) at 55^0C, then boiled 5 minutes and the DNA amplified by PCR. The *Bmp4* mutation was detected with primers 5'-CCAGACTAGTCCATCACAATG-3' and 5'-TTGAGGTGATC AGCCAGTGGA-3', and amplification conditions 94^0C 5 minutes, 40 cycles of 94^0C 15 seconds, 60^0C 30 seconds and 72^0C 45 seconds, followed by 72^0C 5 minutes. Thereafter, the amplified product was digested o/n with *SfiI* (214+227bp wildtype and 441bp knockout). Neomycin primers were 5'-GATTGCACGCAGGTTCTC-3' and 5'-G ATGTTTCGCTTGGTGGTC-3' and amplification conditions 94^0C 5 minutes, 35 cycles of 94^0C 30 seconds, 58^0C 30 seconds and 72^0C 30 seconds, followed by 72^0C 10 minutes to distinguish *Alk2* heterozygous from wildtype embryos.

RT-PCR

Individual embryos, individual explants and STO cell culture were frozen in liquid nitrogen and stored at -80^0C. mRNA was isolated using Dynabeads® mRNA DIRECT™ Micro kit (Dynal). Half of each sample was used for RT-PCR by standard procedures and the other half used as control for DNA contamination. Amplification conditions for ALK2, ALK3 and GAPDH cDNA were 94^0C 2 minutes, 2 cycles of 94^0C 15 seconds, 60^0C 30 seconds and 72^0C 5 minutes, followed by 94^0C 2 minutes. Thereafter, the cDNA-magnetic beads were removed with a magnetic particle concentrator (Dynal MPC®-E), resuspended in cold 1M TRIS/HCl pH7.5 and re-used, while the rest of the solution was further used for amplification (94^0C 2 minutes, 40 cycles of 94^0C 15 seconds, 60^0C 30 seconds and 72^0C 45 seconds, followed by 72^0C 7 minutes). Conditions for amplification of ALK6, Oct4, Esx1, AFP and Fragilis/mil-1 cDNA were similar, except for the annealing temperatures which were 55^0C, 55^0C, 55^0C, 58^0C and 62^0C respectively. Hemi-nested PCR of 30 cycles was used to detect ALK3, ALK2 and ALK6 cDNA. The primers used for ALK2 were 5'-GGAGTAATGATCCTTCCTGTGC-3', 5'-TCTTACACGTCATCTTCCCCTG-3' and 5'-ACCACCGAGAGGATGATAAGGC-3'; for ALK6 were 5'-ACGGAGCAGTGATGAGT GTCT-3', 5'-TTCTGGGTTCCTCTGTGTCTG-3' and 5'-TCTGAACTCACTGGGCAG TAG-3'; for GAPDH were 5'-AGCCAAAAGGGTCATCATCTCC-3' and 5'-CGAAGGT GGAAGAGTGGGAGTT-3'; for Fragilis/mil-1 were 5'-ATCCTTTGCCCTTCAGTGCT GC-3' and 5'-TTCAGGACCGGAAGTCGGAATC-3' and primers for ALK3, Oct4, Esx1 and AFP have been described by Roelen et al. (1997a), Levenberg et al. (2002), Inoue et al. (2002) and Goumans et al. (1999), respectively.

Adenoviral infection, cell culture and Western blot analysis

Intact or VE-stripped E5.5-E6.0 and E6.5 embryos were infected o/n with adenoviruses expressing constitutively active (ca)*Alk2* or *LacZ* (Fujii et al., 1999), refreshed and cultured on FN-coated coverslips at 37^0C and 5% CO_2. $0.5x10^7$, $1x10^7$ and $1.5x10^7$ virus particles per 250 μl were used for individual E5.5, E6.0 and E6.5 embryos, respectively. Embryonic explants were subsequently fixed for 2 hours in 4% PFA at 4^0C, washed 3 times in PBS and either stained for X-gal using standard procedures after o/n culture or stained for AP activity as described above after 3 or 4 days in culture.

The human hepatic cell line HepG2 (ATCC HB-8065) was maintained in DMEM supplemented with 10% FCS at 37^0C and 7.5% CO_2. For adenovirus infection, 30000 cells/cm^2 were seeded in 12-wells (Greiner bio-one) grown o/n and either infected with $1x10^7$ and $50x10^7$ virus particles in 2 ml medium o/n or treated with 100 ng/ml BMP4 for 1 hour. Thereafter, protein isolation for Western blotting was performed using RIPA (Faure et al., 2000) and 35 ug total lysate separated by 12.5% SDS-PAGE and transferred to Hybond-C Extra nitrocellulose (Amersham Biosciences). The membrane was washed in TBST (0.05M TRIS/HCl pH7.5, 0.14M NaCl, 0.1% Tween), blocked in 3% skimmed milk/TBST for 30 minutes at RT, washed in TBST, incubated with rabbit anti-PSmad1 antibody (1:1000) in TBST o/n at 4°C, washed in TBST, incubated with HRP-conjugated goat anti-rabbit antibody (1:5000, BD Biosciences) in TBST 1 hour at RT, washed in TBST and analyzed with ECL kit (Amersham Biosciences) followed by exposure to X-ray film (Fuji).

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chapter 4

transforming growth factor β signalling and the development of primordial germ cells in the mouse

in preparation

Transforming growth factor β signaling and the development of primordial germ cells in the mouse

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Abstract

The transforming growth factor β (TGFβ) signaling pathway has shown to be involved in controlling the migration of primordial germ cells (PGCs) to the gonadal ridges *in vitro*. We investigated the role of the TGFβ signaling pathway *in vivo*, both analyzing the migration of PGCs in embryos deficient in TGFβ signaling, via the type I receptor ALK5 and analyzing the expression of active (C-terminal phophorylated) Smad2, a TGFβ effector protein. Furthermore, we investigated transcriptional activation mediated by TGFβ in PGCs in transgenic reporter mice containing a TGFβ responsive sequence coupled to green fluorescent protein. Our results show that TGFβ neither controls migration or proliferation of PGCs until they reach the gonadal ridges. However, TGFβ may control the mitotic arrest of PGCs that occurs once the PGCs colonize the gonadal ridges.

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Introduction

In mammals, the primordial germ cells (PGCs) have an extra gonadal origin. They are allocated during gastrulation around embryonic day (E)7.2 at the base of the allantois, where a founder population of about 45 cells can easily be identified on the basis of staining for tissue non-specific alkaline phosphatase (TNAP) activity (Lawson and Hage, 1994; Ginsburg et al., 1990; Chiquoine, 1954). To reach the gonadal ridges, the PGCs first occupy the definitive endoderm (Anderson et al., 2000), migrate through the hindgut and ingress the dorsal body wall before dorsal mesentery formation. They continue to leave the hindgut as the mesentery forms and expands and move laterally into the gonadal ridges (E9.0-E10.5) (Molyneaux et al., 2001). By E11.5, most PGCs have entered the gonadal ridges. While migrating, the PGCs proliferate so that they are about 26 000 when they finally enter mitotic arrest in the gonads (Tam and Snow, 1981). The mechanism controlling PGC migration is not fully understood. Although most of the migratory process appears active and directional, a significant number of ectopic PGCs are observed not only scattered along the migratory route, but also in the surface ectoderm of the tail and the hindlimb bud mesoderm.

Experiments on cultured PGCs suggest two different mechanisms that may contribute to regulate PGC migration, one chemotactic and the other based on a gradient of extracellular matrix (ECM) (Godin et al., 1990; Godin and Wylie, 1991; Alvarez-Buylla and Merchant-Larios, 1986; Ffrench-constant et al., 1991; García-Castro et al., 1997). Indirect evidence for involvement of an ECM gradient *in vivo* was provided by Anderson and colleagues (1999), who showed that PGCs lacking the ECM receptor β1 integrin did not colonize the gonadal ridges efficiently. Recently, a specific requirement for the chemokine stromal cell-derived 1 (SDF1) and its receptor CXCR4 for proper colonization of the gonadal ridges has been demonstrated (Molyneaux et al., 2003; Ara et al., 2003). However, The SDF1/CXCR4 is not involved in the earlier phases of PGC migration.

In vitro, transforming growth factor β1 (TGFβ1) has been shown to mimic the chemotropic effect of E10.5 gonadal ridges observed for PGCs isolated from E8.5 embryos, in a dose dependent manner. Moreover, a neutralizing antibody against TGFβ1 blocked the chemotropic effect from the gonadal ridges, suggesting that indeed TGFβ could be involved in the homing of PGCs (Godin and Wylie, 1991). However, these *in vitro* experiments were all performed on STO feeder cells and there is no direct evidence that E8.5 PGCs express TGFβ receptors so that it remains unclear whether the observed TGFβ effect was direct on the PGCs or indirect via the feeders. Nevertheless, even though at E8.5 the gonadal ridges have not yet formed and the PGCs have just entered the hindgut, at E10.5 the dorsal body wall does express TGFβ1 (Godin and Wylie, 1991), suggesting that TGFβ1 could play a role regulating migration of PGCs towards the gonadal ridges *in vivo*.

TGFβ1 is the prototype of the TGFβ superfamily of secreted growth factors, which also includes among others TGFβ2, TGFβ3 and bone morphogenetic proteins (BMPs). TGFβ1-3 signal through serine/theonine kinase transmembrane receptors, requiring both the type II (TβRII) and type I (ALK5 and, specifically in endothelial cells, ALK1) receptors to initiate a cellular response. Upon activation of ALK5, TGFβ receptor regulated (R-)Smads, Smad2 and Smad3 are phosphorylated in their C-terminal sequence and translocate to the nucleus where they modulate transcription of TGFβ target genes (reviewed by Shi and Massagué, 2001).

The purpose of this study was to determine whether TGFβ signaling contributed to PGC migration directly *in vivo*. Furthermore, we also investigated whether BMP signaling contributed to migration control of PGCs, because recently phosphorylated (BMP) R-Smads and transcripts of the BMP receptors were observed in isolated E11.5 PGCs (Pesce et al., 2002). We used antibodies that specifically recognize the active form (C-terminal phosphorylated) of the TGFβ R-Smad, PSmad2 or the BMP R-Smads, PSmad1/5/8 and the recently generated transgenic

reporter mice containing *LacZ* or green fluorescent protein (*gfp*) reporter genes driven by promoter sequences specifically responsive to TGFβs (CAGA-reporter) or BMPs (BRE-reporter) R-Smads (Neptune et al., 2003; Monteiro et al., unpublished results). In these mice, expression of the reporter indicates the tissues and cells where transcriptional activation as result of either TGFβ or BMP signaling has taken place. We observed active TGFβ, but not BMP, signaling in PGCs at E10.5 and E12.5. In addition, the distribution of PGCs was analyzed in *Alk5* deficient mice. The results showed that while *Alk5* deficient embryos had similar numbers of PGCs as their wildtype littermates, their PGCs seem to migrate more efficiently to the gonadal ridges and therefore TGFβ seems not to function as a chemoattractant *in vivo*.

Materials and Methods

Mouse strains and embryo isolation

Wildtype mice were maintained on a BL6/CBA background, *Tgfbrl*$^{+/-}$ (or *Alk5*$^{+/-}$) mice (Larsson et al., 2001), CAGA:*gfp* mice (Neptune et al., 2003) on a BL6 background and BRE:*LacZ* mice (Monteiro et al., unpublished results) on a 25% BL6/25% CBA/50% 129 background. Embryos were isolated in cold Dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 7.5% fetal calf serum (FCS) and 10 mM HEPES. Wildtype and *Alk5*$^{+/-}$ embryos were fixed overnight (o/n) in 4% paraformaldehyde in PBS (PFA) and embedded in paraplast (Sigma) using standard procedures. *Alk5* genotyping was performed as described (Larsson et al., 2001).

PGC staining

BRE:*LacZ* and CAGA:*gfp* males were crossed with wildtype females. The resulting E9.0-E10.5 embryos were dissected as described (Lawson et al., 1999) or the gonads of E12.5 embryos isolated, before fixation for 2 hours in 4% PFA at 4^0C. BRE:*LacZ* embryonic fragments were stained for β-galactosidase using standard procedures for 4 hours at 37^0C, while CAGA:*gfp* embryonic fragments were photographed using filters for GFP. All fragments were washed in cold PBS and stained as whole-mount for alkaline phosphatase (AP) activity (Lawson et al., 1999) and mounted in 70% glycerol. Sections of embryos resulting from *Alk5* heterozygote crossings or wildtype were stained for AP activity using ASMX/Fast Red TR (Sigma) following manufacturer's instructions, counterstained with hematoxylin, dehydrated and mounted in DePeX. In all cases, AP staining was performed maximally 3 days after embryo collection.

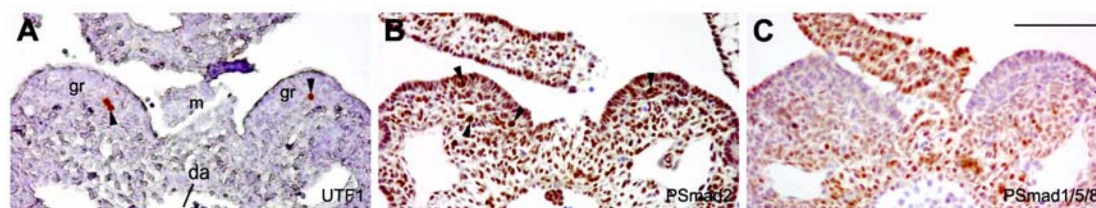

Fig. 1. PGCs are direct targets of TGFβ signaling during migration at E10.5. A-C, tranverse sections of E10.5 embryos were immunostained for UTF1 (A), PSmad2 (B) and PSmad1/5/8 (C). Black arrows indicate PGCs scattered through the gonadal ridges. Abbreviations: da, dorsal aorta; gr, gonadal ridges; m, mesenterium. Scale bar: 100 μm.

Immunohistochemistry

Sections of embryos were dewaxed, rehydrated and treated for 15 minutes at room temperature (RT) with 1.2% hydrogen peroxide in MeOH. Sections to be stained with rabbit anti-PSmad2 (Persson et al., 1998) and anti-UTF1 antibodies were boiled for 20 minutes in 10 mM sodium citrate pH6.0, sections to be stained

with rabbit anti-PSmad1/5/8 antibody (Cell Signaling) were boiled 20 minutes in 10 mM Tris/1mM EDTA pH9.0. Other antibodies used were anti-phospho p44/42 MAP kinase (Cell signaling) and anti-phospho p38 MAP kinase (Cell signaling). Sections were blocked for 30 minutes at RT in TNB blocking solution (provided by the TSA Biotin System^{TM}, Perkin Elmer), incubated o/n at 4^{0}C with the first antibody in TNB (diluted at 1:100, except anti-phospho p38 MAP kinase diluted at 1:1000), followed by 30 minutes incubation at RT with biotin-conjugated swine anti-rabbit IgG (DAKO) in TNB and treatment with the ABComplex/HPR (DAKO), the TSA Biotin System and Fast 3,3'-diaminobenzidine tablet set (DAB, Sigma). Sections were counterstained with hematoxylin, dehydrated and mounted in DePeX.

Results

At E9.0-E10.5, TGFβ signaling, but not BMP signalling, is active during the migration of PGCs from the hindgut to the gonadal ridges

Although TGFβ1 as been described as a chemoattractant molecule for migratory PGCs *in vitro*, its role *in vivo* has never been clarified. Using immunohistochemistry, we show that indeed at E10.5, PGCs exhibit nuclear staining for PSmad2, indicating that TGFβ signaling is active in the PGCs (Fig. 1). At E10.5, PGCs are entering the gonadal ridges and can be morphologically distinguished from the somatic cells surrounding them due to the typical large round nucleus as shown using the marker for pluripotency UTF1 (Fig. 1A). Making use of transgenic CAGA:*gfp* TGFβ reporter mice (Neptune et al., 2003), we expected to show transcriptional activation of the GFP reporter as a result of activation of TGFβ signaling specifically in the PGCs along the hindgut, mesenterium, body wall and the gonadal ridges. However, at E10.5 no GFP was detected in the embryo proper and visceral yolk sac (data not shown). This was unexpected because embryos deficient in either TGFβ1 on specific genetic backgrounds, TβRII or ALK5 die around E10.5, indicating not only that TGFβ signaling is occurring, but more importantly that TGFβ signaling is necessary for proper development during this developmental stage (Dickson et al., 1995; Oshima et al., 1996; Larsson et al., 2001). We generated transgenic CAGA:*lacZ* TGFβ reporter mice and will analyze PGC development in those reporter mice.

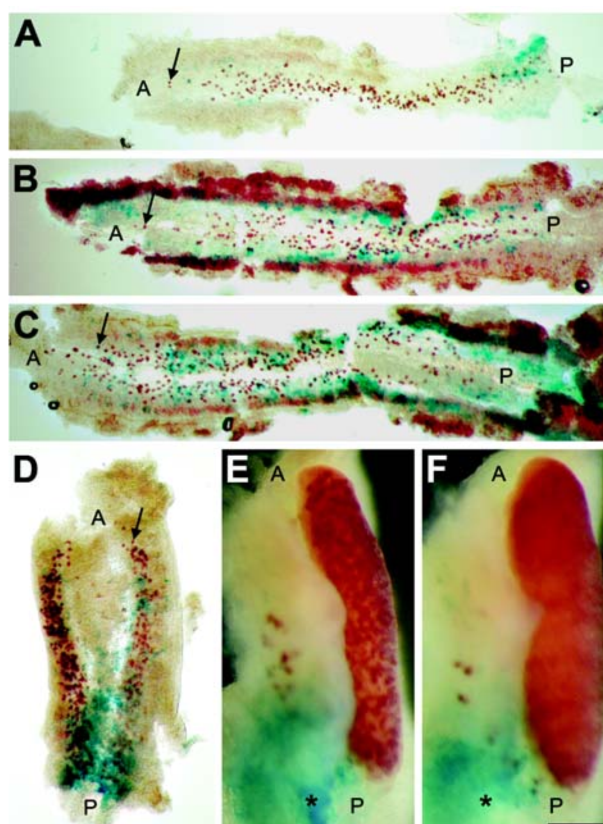

Fig. 2. PGCs are not direct targets of BMP signaling between E9.0 and E12.5. A-D, the posterior (hindgut) region of BRE:LacZ embryos was stained for alkaline phosphatase activity (PGCs, black arrow) and β-galactosidase (BMP responsive cells). At E9.25 (A), E9.5 (B), E9.75 (C) and E10.5 (D), these two cell populations do not colocalize. E-F, at E12.5, female (E) and male (F) gonads are distinguishable and PGCs are not direct targets of BMP signaling, in contrast to the metanephros primordium (asterisk). Abbreviations: A, anterior; P, posterior. Scale bar: 200 μm.

At E10.5, PGCs are not receiving BMP signaling, as no nuclear PSmad1/5/8 was detected (Fig. 1C). Transcriptional activation resulting from BMP signaling was

analyzed in transgenic BRE:*LacZ* BMP reporter mice (Monteiro et al., unpublished results) in particular in the PGCs. In contrast to the extensive β-galactosidade staining observed for example in the developing metanephros, positioned caudally from the gonadal ridges (Fig. 2), no staining was observed in the PGCs between E9.0 and E12.5 (Fig. 2). Together, these results suggest that BMPs do not directly signal to the PGCs during migration and colonization of the gonadal ridges.

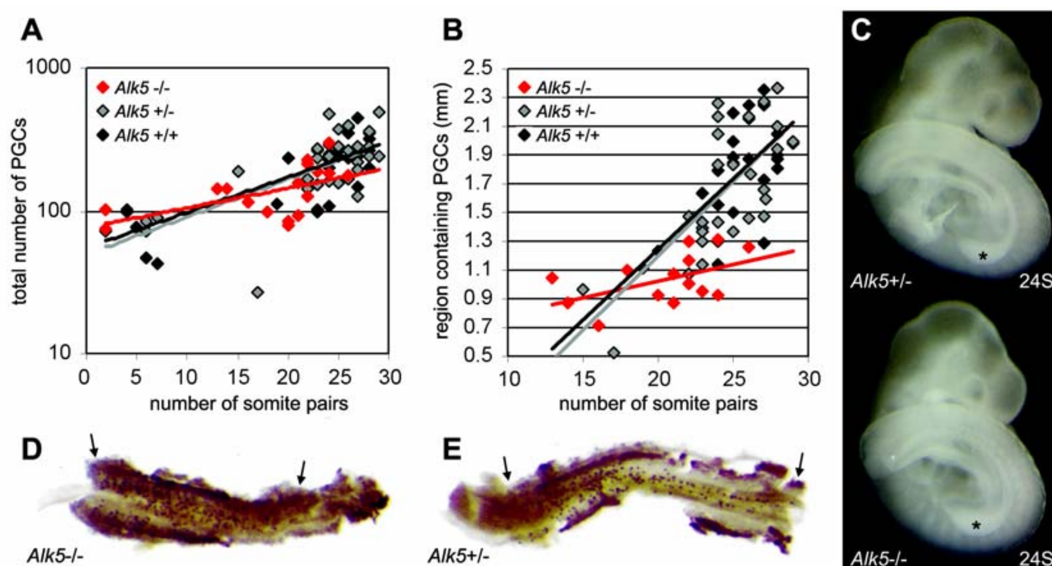

Fig. 3. PGC distribution in Alk5 deficient embryos until E10. A, linear regression analysis of the total number of PGCs counted as whole-mounts versus the number of somite pairs, in embryos from Alk5+/- intercrosses. The regression equations are expressed as y= a+bx, for log PGC number (y) on somite number (x) at the mean values of x and y: wildtype 2.262= 1.692+0.0264(21.6); heterozygote 2.280= 1.741+0.0247(21.8); homozygote 2.134= 1.883+0.0138(18.2). B, linear regression analysis of the posterior (hindgut) region in lenght containing PGCs, from the most anterior to the most posterior versus the number of somite pairs, in embryos from Alk5+/- intercrosses. The regression equations are expressed as y= a+bx, for posterior (hindgut) region in lenght (y) on somite number (x) at the mean values of x and y: wildtype 1.746= -0.719+0.0983(25.1); heterozygote 1.683= -0.817+0.1037(24.1); homozygote 1.032= 0.023+0.0983(21.5), P<0.01. C, two littermate embryos of different genotypes (Alk5+/- and Alk5-/-) both with 24 somite pairs. The asterisk marks the forelimb bub. D-E, posterior (hindgut) region of the embryos depicted in C after staining for alkaline phosphatase activity. Black arrows indicate the most anterior and most posterior PGC.

Alk5 deficient embryos exhibited abnormal distribution of PGCs

The distribution of PGCs was analyzed in detail in *Alk5* deficient embryos until E10, when a large percentage of the mutants are still morphological normal, to see whether PGC migration is dependent of TGFβ signaling. We observed that the total number of PGC was similar in wildtype and *Alk5* deficient littermates during early development (3 to 30(S) somites), indicating that TGFβs do not have an anti-mitogenic effect on PGCs *in vivo* (Fig. 3A), in contrast to reports *in vitro* (Godin and Wylie, 1991; Richards et al., 1999). Furthermore, the distribution of PGCs was similar in wildtype and *Alk5* deficient littermates until E9.0 (20S) (data not shown). However thereafter, between E9.0 and E9.5 (20S-30S), even though the majority of *Alk5* deficient embryos analyzed were morphologically similar to wildtype littermates (Fig. 3C), the region in length containing PGCs was significantly shorter in mutant embryos (Fig. 3D,E). Comparing littermates with identical numbers of somite pairs, we observed that *Alk5* deficient PGCs migrate closer together and seem to leave the hindgut earlier than wildtype PGCs. Wildtype PGCs are very dispersed and still occupy posterior regions of the hindgut while the most anterior PGCs migrate to the mesenterium and body wall; the majority of *Alk5* deficient PGCs remain compact leaving the hindgut together. Surprisingly, migration of PGCs to the gonadal ridges seems to occur more efficiently in the absence of TGFβ signaling (Fig 3D,E), suggesting that *in vivo* TGFβs do not regulate homing of PGCs.

At E10.5, no morphologically normal *Alk5* deficient embryos could be recovered. However, neither the distribution nor the total number of PGCs in *Alk5* heterozygous was significantly different from wildtype embryos (Fig. 4A,B), suggesting that only the complete absence of ALK5 affected PGC behavior.

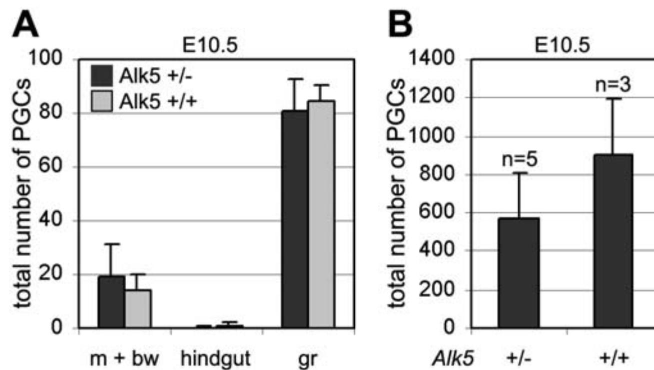

Fig. 4. PGC distribution in Alk5 heterozygous embryos at E10.5. A-B, the total number of PGCs was counted in sections of E10.5 Alk5+/- (n=5) and Alk5+/+ (n=3) littermates (A). PGCs were found predominantly in the gonadal ridges (gr), but also in the mesenterium and dorsal body wall (m + bw) and hindgut.

At E12.5, TGFβ Smad dependent/independent signaling in the PGCs

Although TGFβs do not have an anti-proliferative effect during PGC migration, they could regulate mitotic arrest at E12.5, as the PGCs loose their migratory morphology, round up and stop proliferating. Therefore, we studied the activation of TGFβ signaling in PGCs at E12.5. We observed that PGCs were positive for PSmad2, showing faint but clear PSmad2 nuclear staining (Fig. 5B). TGFβ is also known to activate Smad-independent pathways through the phosphorylation of mitogen-activated protein kinases (MAPKs), including p38 MAPK and Erk MAPK (Derynck and Zhang, 2003). These TGFβ Smad-independent pathways are related to rapid changes in cell morphology and could play a role during this phase of PGC development. However, using immunohistochemistry we were unable to detect phosphorylated p38 MAPK in the PGCs, while abundant nuclear staining was detected in the somatic cells surrounding these cells (Fig. 5D). Similarly, phosphorylated Erk MAPK was absent from the PGCs (data not shown). We also investigated BMP signaling by means of PSmad1/5/8 nuclear staining, but no active BMP signaling was detected in the PGCs, while interestingly prominent staining was observed in the tissue surrounding the Müllerian duct.

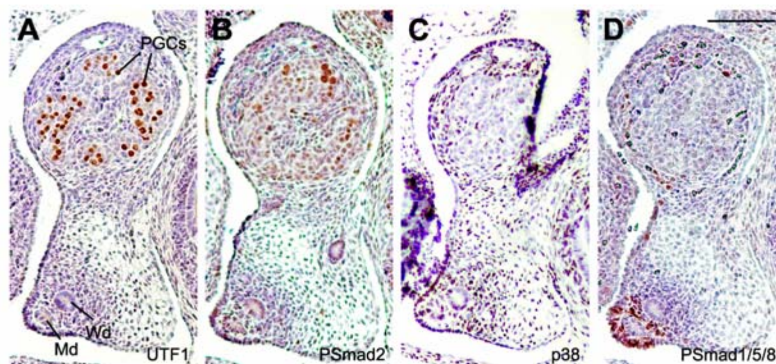

Fig. 5. PGCs are direct targets of TGFβ signaling at E12.5. A-D, tranverse sections of a male E12.5 embryo immunostained for UTF1 (A), PSmad2 (B), p38 MAPK (C) and PSmad1/5/8 (D). Abbreviations: PGCs, primordial germ cells; Md, Müllerian duct; Wd, Wolffian duct. Scale bar: 100 µm.

Discussion

TGFβ signaling does not play a role during PGC migration

At E9.5, active TGFβ Smad2-dependent signaling was observed in the migrating PGCs. However, PGCs were identified on the basis of morphology and no direct co-localization of PSmad2 and a PGC marker was shown. Using CAGA:*gfp*

reporter embryos, we expected to demonstrate that PGCs were indeed a direct target of TGFβ signaling. However, those reporter mice had no detectable levels of GFP at all at E9.5 and E10.5. Furthermore, we isolated the hindgut region of E8.5 embryos, containing the PGCs and cultured them overnight in the presence of exogenous TGFβ1, mimicking the culture conditions used by Godin and Wylie (1991), to test the possibility that only in the presence of 25ng/ml of TGFβ1 the PGCs would activate the TGFβ pathway. After culture, no GFP positive cells were observed at all, while large numbers of PGCs were detected after AP staining. We suggest that the CAGA:*gfp* reporter mice are not suitable to analyze TGFβ signaling activation in mouse embryos during the first half of gestation and therefore generated new transgenic lines of CAGA:*lacZ* reporter mice (Bowman et al., unpublished results). The activation of the TGFβ pathway will be analyzed specifically in the PGCs to clarify if direct TGFβ signaling is observed during PGC migration.

Interestingly, in the absence of *Alk5* (and therefore of TGFβ signaling) PGCs seem to reach the gonadal ridges more efficiently then wildtype PGCs. The observation that PGCs were distributed through a shorter length in the posterior part of the *Alk5* mutant embryos may be explained by the fact that in those mutants the expansion of the posterior part of the embryo is delayed, suggested by a relatively lower number of somites pairs present in the *Alk5* mutants analyzed, compared to heterozygote and wildtype littermates. Nevertheless, the number of PGCs observed in the Alk5 mutants is similar to wildtype littermates and therefore we suggest that TGFβ signaling via ALK5 does not play a significant role either in the migration or the proliferation behaviour of the PGCs *in vivo*, in contrast to reports from studies *in vitro* (Godin and Wylie, 1991; Richards et al., 1999).

BMP signaling does not play a role during PGC migration

The role of BMP signaling in the formation of PGCs has been well established (Lawson et al., 1999; Ying et al., 2000; Ying et al., 2001; Tremblay et al., 2001; Hayashi et al., 2002; Chang and Matzuk, 2001). Recent reports have shown that BMP receptors and active BMP R-Smads were present in E11.5 PGCs (Pesce et al., 2002), suggesting a putative role for BMP signaling during later stages of PGC development. Our results using immunohistochemistry for PSmad1/5/8 and the analysis of PGCs in the BRE:*lacZ* reporter embryos strongly suggested that no direct BMP signaling occurred in PGCs from E9.5 until E12.5. BMP8b is known to be involved in spermatogenesis postnatally (Zhao et al., 1996), therefore as a proof of principle that the CAGA:*lacZ* reporter line is indeed faithfully showing tissues where BMP signaling is active, newborn and adult testis will be analyzed.

TGFβ signaling and PGC mitotic arrest

In E11.5 embryos, PGCs expressed TβRII and Alk5 (Richards et al., 1999) and could therefore respond to TGFβ1 signals. At E12.5, PGCs exhibited faint nuclear PSmad2 and therefore TGFβ Smad-dependent signaling could be involved in the mitotic arrest of PGCs that occurs during this developmental stage. Additional experiments will be performed to clarify this observation, including the analysis of the recently generated CAGA:*lacZ* reporter mice.

TGFβ Smad-independent pathways although still poorly characterized are related to rapid morphological changes associated with cytoskeletal reorganization, in contrast to the TGFβ Smad-dependent pathway that directly modulates transcription (Derynck and Zhang, 2003). Once in the gonadal ridges, the PGCs undergo a clear change in morphology from an elongated typical migratory cell phenotype to a round cell phenotype. The activation of two TGFβ Smad-independent effector proteins were analysed, p38 and Erk MAPK, however no signal was detected in PGCs, indicating that at least the pathways activated by these MAPKs are not involved in the changes in morphology observed in the PGCs around E12.5.

However, we can not exclude that other TGFβ Smad-independent signaling pathways control this process.

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chapter 5

knock-in of integrin β1D affects primary but no secondary myogenesis in mice

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Knock-in of integrin β1D affects primary but not secondary myogenesis in mice

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SUMMARY

Integrins are extracellular matrix receptors composed of α and β subunits involved in cell adhesion, migration and signal transduction. The β1 subunit has two isoforms, β1A ubiquitously expressed and β1D restricted to striated muscle. They are not functionally equivalent. Replacement of β1A by β1D (β1D knock-in) in the mouse leads to midgestation lethality on a 50% Ola/50% FVB background [Baudoin, C., Goumans, M. J., Mummery, C. and Sonnenberg, A. (1998). *Genes Dev.* 12, 1202-1216]. We crossed the β1D knock-in line into a less penetrant genetic background. This led to an attenuation of the midgestation lethality and revealed a second period of lethality around birth. Midgestation death was apparently not caused by failure in cell migration, but rather by abnormal placentation. The β1D knock-in embryos that survived midgestation developed until birth, but exhibited severely reduced skeletal muscle mass. Quantification of myotube numbers showed that substitution of β1A with β1D impairs primary myogenesis with no direct effect on secondary myogenesis. Furthermore, long-term primary myotube survival was affected in β1D knock-in embryos. Finally, overexpression of β1D in C2C12 cells impaired myotube formation while overexpression of β1A primarily affected myotube maturation. Together these results demonstrate for the first time distinct roles for β1 integrins in primary versus secondary myogenesis and that the β1A and β1D variants are not functionally equivalent in this process.

Key words: β1 integrins, Knock-in, Myogenesis, Muscle mass, Cell migration, Placentation, Mouse

INTRODUCTION

Cell-extracellular matrix (ECM) interactions play a crucial role during embryonic development (DeSimone, 1994; Darribère et al., 2000). Cells interact with the ECM via a variety of receptors, the integrins being the most common (Hynes, 1992; Hynes, 1999). Integrins consist of heterodimer complexes of α and β subunits (van der Flier and Sonnenberg, 2001). The β1 subunit associates with at least 12 different α subunits and forms the largest and most abundantly expressed subfamily. In the mouse, it occurs as two highly homologous isoforms: β1A and β1D (van der Flier et al., 1995; Zhidkova et al., 1995). These are not functionally redundant; we have shown previously that replacement of β1A with β1D is lethal in embryos homozygous for the knock-in allele (*β1D ki/ki*) (Baudoin et al., 1998). Moreover, β1A and β1D have different binding affinities for the cytoskeletal proteins talin, filamin, α-actinin (Belkin et al., 1997) and the integrin linked kinase (ILK) (Hannigan et al., 1996). The stronger binding of β1D to talin and the observation that fibroblasts isolated from *β1D ki/ki* embryos show impaired migration in vitro (Baudoin et al., 1998), raises the possibility that cell migration might be affected when only β1D is expressed.

During mouse development, β1A and β1D expression is mainly non-overlapping: β1A is expressed ubiquitously, while β1D is detectable in the heart from E11.0, increasing sharply around birth, and in skeletal muscle from E17.5 (van der Flier et al., 1997; Brancaccio et al., 1998). In both cases β1D completely displaces β1A perinatally, suggesting distinct roles for each in striated muscle in vivo.

In vertebrates, myogenesis occurs sequentially, starting when proliferating myoblasts induce myogenic regulatory factors, such as Myf5, MyoD and myogenin, followed by an irreversible exit from the cell cycle, phenotypic differentiation and fusion of myoblasts to form multinucleated elongated myotubes (Buckingham, 2001). During mouse development,

there are two waves of myogenesis involving three distinct populations of myoblasts. Between E11.5 and E15.5 the formation of primary myotubes results from the fusion of primary (embryonic) myoblasts, while secondary (foetal) myoblasts remain proliferative. From E15.5, secondary myoblasts progressively enter the muscle differentiation programme. Finally, a third population of myoblasts (adult) develops and contributes to secondary myogenesis until well after birth and also gives rise to the quiescent satellite cells of adult muscle (Wigmore and Dunglison, 1998).

Antibody perturbation experiments have suggested a role for β1 integrins during several steps of myogenesis, including migration, differentiation and fusion (McDonald et al., 1995; Gullberg et al., 1998). However, in wild type/β1-null chimeric mice, β1-deficient myoblasts migrate, differentiate and fuse with wild-type myoblasts/myotubes (Fässler and Meyer, 1995). Thus the role of β1 integrins in myogenesis in vivo is presently not clear. In vitro studies show that exogenous β1D inhibits cell cycle progression in cultured C2C12 cells (Belkin and Retta, 1998), while exogenous β1A maintains proliferation in quail myoblasts when paired with a permissive α chain (Sastry et al., 1999). Together, these in vitro results suggest not only that integrin β1 may play a role in myogenesis, but that β1A and β1D might have different functions.

We studied in detail the role of β1A and β1D during myogenesis in vivo in the β1D knock-in mice, which were crossed into a less penetrant genetic background than used previously. This revealed a second period of lethality. Our results show that the replacement of β1A with β1D supports normal cell migration during development, but placentation is abnormal and the most likely cause of midgestational death. Furthermore, we show for the first time that β1 plays an important role during myogenesis in vivo, β1D being incapable of replacing β1A functionally during primary myogenesis.

MATERIALS AND METHODS

Animals and embryos

Generation of β1D knock-in mice on a mixed background (50% 129Ola/50% FVB) has been described previously (Baudoin et al., 1998). Here, heterozygous individuals were backcrossed four times onto a FVB background. Homozygous *β1D ki/ki* embryos were obtained from heterozygous crossings. The day of the vaginal plug was designated embryonic day (E) 0.5. Following cervical dislocation, embryos were collected from E8.5-18.5 or pregnancies were allowed to reach term. DNA isolated from tail biopsies or visceral yolk sacs was used for genotyping as described previously (Baudoin et al., 1998). For embryos sectioned in paraffin or paraplast, DNA was extracted from the embedded material using the TaKaRa DEXPAT™ kit.

Primordial germ cells

E10.5 embryos were fixed in 4% paraformaldehyde (PFA) for 2.5 hours at 4°C, embedded in Paraplast and stained for alkaline phosphatase (AP) activity (Lawson et al., 1999). Primordial germ cells (PGCs) in each embryo (n=8 for wild-type and n=6 for *β1D ki/ki* embryos) section were counted on the basis of the strong cytoplasmic AP activity and morphology (Chiquoine, 1954).

PGCs were isolated by flow cytometry as described by Abe et al. (Abe et al., 1996) and cDNA isolated by standard procedures. RT-PCR was performed on 2 ng cDNA and PCR conditions were 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds. Primers for integrin β1A (282 bp) and β1D (363 bp) were 5′-GGCAACAATGAAGCTATCGT-3′ and 5′-CCCT-CATACTTCGGATTGAC-3′; for Oct4, 5′-GGAGAGGTGAAAC-CGTCCCTAGG-3′ and 5′-AGAGGAGGTTCCCTCTGAGTTGC-3′ (Anderson et al., 1999); and for HPRT, 5′-GCTGGTGAAAAGGAC-CTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′ (Johansson and Wiles, 1995).

In situ hybridisation

Digoxigenin-labelled RNA probes were prepared from linearised plasmids (Sambrook et al., 1989). Whole-mount in situ hybridisation was performed as described by Henrique et al. (1995). mRNA was visualised using BM Purple (Roche). The expression of *Snail* (n=8 for *β1D ki/ki* and wild type or heterozygous) and *pax3* (n=2 for *β1D ki/ki* and heterozygous) were analysed in toto; some embryos were embedded in Technovit 8100™ and sectioned.

Histology

E18.5 embryos were fixed for 10 days in 4% PFA, washed for 10 days in PBS, 5 days in 0.83% NaCl, 10 days in 1:1 mix of 0.83% NaCl and 100% ethanol and stored in 70% ethanol, all at 4°C. After embedding in paraffin (Histowax), 7 μm sections were processed for Haematoxylin and Eosin staining.

Immunohistochemistry

E18.5 cleidomastoideus muscle was fixed in 2% PFA, dissected out and embedded in Tissue-Tek OCT compound. E14.5 placenta, E18.5 thoracic body wall, and E14.5, E16.5, E17.5 and E18.5 lower hindlimb muscles were embedded without fixation, but immediately frozen in liquid nitrogen-chilled isopentane. Transverse cryosections (5-10 μm) were fixed in 2% PFA and processed as described by Venters et al. (Venters et al., 1999). Some E14.5 placentae were fixed in 4% PFA and embedded in paraffin. Paraffin sections (7 μm) were processed as described previously (Zwijsen et al., 1999) and some were processed for Haematoxylin and Eosin staining.

Primary antibodies used were rabbit anti-EHS laminin (Sigma), rat anti-mouse endothelial glycoprotein (MECA32, DSHB), mouse anti-slow myosin heavy chain (slow MHC, NOQ7.1.1A) (Draeger et al., 1987), mouse anti-neonatal/fast MHC (MY-32, Sigma), rabbit anti-protein gene product 9.5 (PGP9.5) (Thompson et al., 1983), rat anti-muscle acetylcholine nicotinic receptor (mAb35; DSHB) and rabbit anti-phospho-histone H3 (UBI). Secondary antibodies were Alexa 488 (Molecular Probes) or TRITC-conjugated (Sigma) goat anti-rabbit IgG, FITC- or TRITC-conjugated goat anti-rat IgG (Sigma) and HRP-conjugated goat anti-mouse IgG (Southern Biotech). For the latter, diaminobenzidine was used as a substrate.

Myotube quantification

Primary and secondary myotubes were quantified by counting slow MHC-positive (primary) and slow MHC-negative (secondary) myotubes (Harris et al., 1989; Sheard and Duxson, 1996). Consecutive sections were labelled with the MY32 antibody, which stains all myotubes at E18.5.

In E18.5 body wall muscles, primary and secondary myotubes were counted per unit area of intercostal and serratus dorsalis muscles. The number of myotubes from the sternum to the vertebra in one section per individual (n=2 for wild type and n=3 for *β1D ki/ki*) was counted and the number of myotubes/mm^2 calculated.

In E18.5 cleidomastoideus and lower hindlimb muscles, primary and secondary myotubes were counted in 3-6 serial sections, from each individual (n=3 or 4 per genotype). The widest part of the hindlimbs and the red area of cleidomastoideus were used. Primary myotubes were counted in E14.5 lower hindlimb and the myotube diameter measured in extensor digitorum longus (EDL) muscle in at least 15 myotubes from each individual (n=3 per genotype). Primary

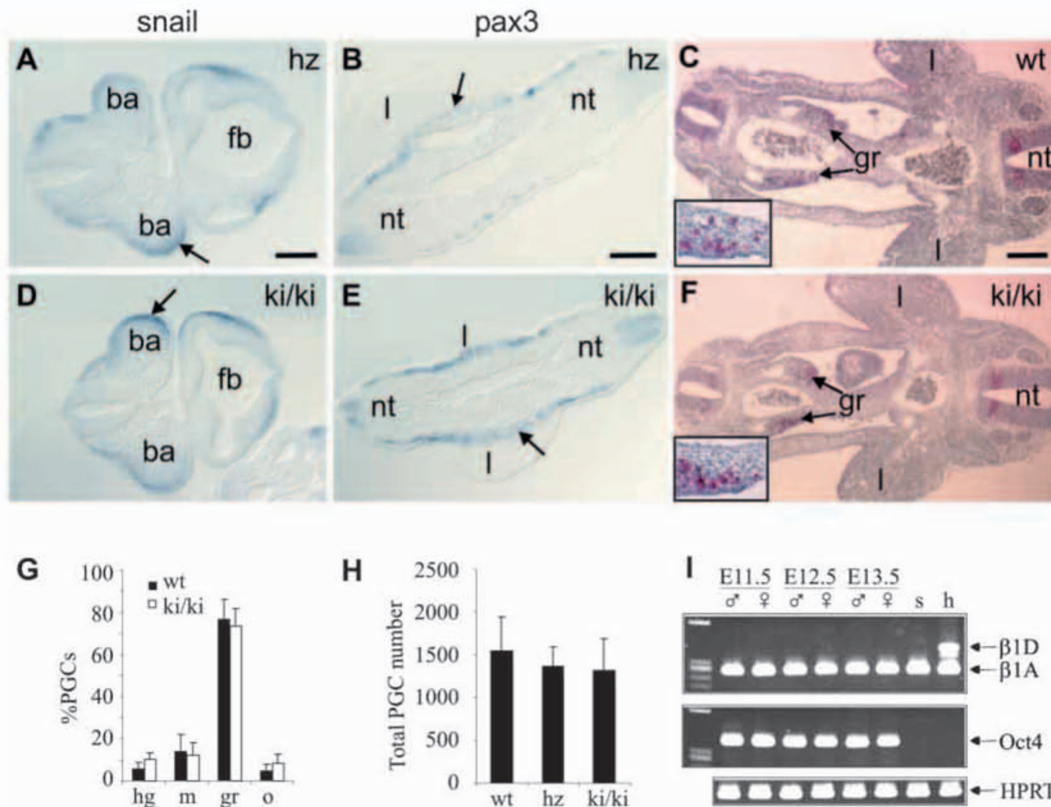

Fig. 1. Different migratory cell types behaved normally in *β1D ki/ki* embryos. (A-D) Transverse sections showing (A,D) *snail* expression in migratory neural crest cells (arrows) and (B,E) *pax3* expression in migratory limb muscle precursor cells (arrows) of E9.5 heterozygous (A,B) and *β1D ki/ki* (D,E embryos). (C,F) PGC distribution was analysed in transverse serial sections of E10.5 wild-type (C) and *β1D ki/ki* (F) embryos. An enlargement of the right gonadal ridge is shown. (G,H) At E10.5, PGCs were detected by AP staining in the hindgut (hg), mesenterium (m), gonadal ridges (gr) and ectopic regions (o) of *β1D ki/ki*, heterozygous and wild-type embryos in the quantities indicated. (I) RT-PCR detection of β1A and β1D in isolated PGCs from different stages. Oct4 is a PGC marker at these stages. HPRT is a loading control. ba, first branchial arch; fb, forebrain; gr, gonadal ridges; h, newborn heart; l, limb bud; nt, neural tube; s, E11.5 gonadal somatic tissue. Scale bars: 200 μm (A,B,D,E), 400 μm (C,F).

and secondary myotubes were also counted in E16.5 (n=1 per genotype) and E17.5 (n=2 per genotype) EDL muscle.

Differences in the numbers of myotubes were tested either by analysis of variance (body wall, E16.5 hindlimb) or nested analysis of variance. Differences in myotube diameter were tested by nested analysis of variance.

TUNEL assay

Apoptosis was analysed on cryosections of E14.5 placentae (n=2 or 3 per genotype), and E14.5, E16.5 and E17.5 (n=2, 1, 2 per genotype, respectively) hindlimb muscles using the Cell Death Detection Kit (Roche). TUNEL-positive nuclei were counted in the tibialis anterior, (TA), EDL and peroneus group (P) muscles using at least 5 serial sections per individual.

C2C12 differentiation

The mouse myoblast cell line C2C12 (ATTC CRL 1772) was grown in DMEM supplemented with 20% (v/v) foetal calf serum and high glucose (5 g/l). The C2C12/β1D and C2C12/β1A cells were generated by retroviral transduction with cDNA constructs for human β1A and β1D, as described previously (Gimond et al., 1999), but using 2×10^4 C2C12 cells and infection for 6 hours. Myogenic differentiation was induced as described by van der Flier et al. (van der Flier et al., 2002).

For immunofluorescence, cells were processed as described by van der Flier et al. (van der Flier et al., 2002), using mouse anti-sarcomeric MHC (MF20; DSHB) and FITC-conjugated goat anti-mouse IgG (Sigma). To-Pro 3 (Molecular Probes) was added to the last PBS wash. The number of MF20-positive cells (MHC^+, total myoblasts and myotubes) and respective nuclei were counted in three microscopic fields at 10× magnification and three to seven microscopic fields at 16× magnification. The fusion index (%) was calculated as the ratio of nuclei in myotubes (>3 nuclei) to the total number of nuclei in MHC^+ cells. Student's t-test was used to analyse the differences between wild-type and infected cells.

Western blotting was performed as in van der Flier et al. (van der Flier et al., 1997). Primary antibodies were mouse anti-p21 (UBI), rabbit anti-pRB (Santa Cruz), MF20, rabbit anti-connexin43 (Sigma), rabbit anti-β1Acyto (kind gift from U. Mayer) and mouse anti-β1Dcyto (2B1) (van der Flier et al., 1997). Secondary antibodies were HRP-conjugated sheep anti-mouse and donkey anti-rabbit (Amersham Pharmacia).

RESULTS

Two periods of lethality for *β1D ki/ki* mice

Embryos between E8.0 and E18.5 were collected and genotyped (Table 1). The genotypes showed a Mendelian

Table 1. Number of embryos of each genotype recovered from heterozygous crossings

Stage	+/+	+/*ki*	*ki/ki*
E8.0-E9.5	65^{1*} (26.4%)	105^{4*} (42.7%)	76^{1*} (30.9%)
E10.0-E11.5	93 (26.0%)	194^{4*} (54.0%)	70^{6*} (20.0%)
E12.0-E13.5	21 (20.0%)	59 (56.2%)	25^{1*}(23.8%)
E14.0-E16.5	26 (17.0%)	$107^{2*,2\ddagger}$ (70.0%)	$20^{2\dagger,6*,1\ddagger}$ (13.0%)
E17.0-E18.5	44 (25.6%)	$112^{1\dagger}$ (65.1%)	$16^{4\dagger,1*,13\ddagger}$ (9.3%)
P1-4	54 (39.1%)	79 (57.3%)	$5^{5\ddagger}$ (3.6%)
>P20	51 (41.1%)	72 (58.1%)	$1^{1\ddagger}$ (0.8%)

n^*, the number of embryos showing abnormalities, including open or kinked neural tube, reduced branchial arches or abnormal head shape and eccentric limbs.

Dead or reabsorbed embryos were not included in the table but are given as $n^\dagger$. ‡Number of shorter/thinner embryos.

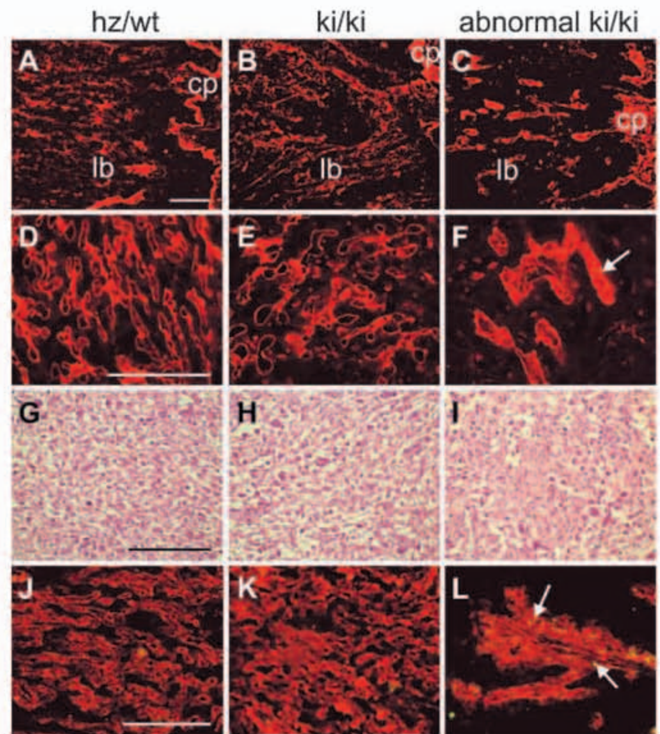

Fig. 2. At E14.5, abnormal *β1D ki/ki* embryos showed defective vascularisation and increased apoptosis of endothelial cells in the placental labyrinth. (A,D,G) Heterozygous, (J) wild-type (B,E,H,K) normal *β1D ki/ki* and (C,F,I,L) abnormal *β1D ki/ki* embryos. (A-C) Laminin immunostaining, in transverse sections of E14.5 placentae, showing the basal lamina of foetal blood vessels. (D-F) Higher magnification of the labyrinth in A-C showing reduced branching and obstructed blood vessels in abnormal *β1D ki/ki* embryos (arrow). (G-I) Haematoxylin and Eosin staining. (J-L) Transverse sections showing MECA32- (red) and TUNEL- (green) positive cells (arrows in L). cp, chorionic plate; lb, labyrinth. Scale bar: 200 μm.

distribution until E13.5, after which the *β1D ki/ki* genotype frequency decreased from about 24% to 9% of the total embryos recovered. The embryos that survived this first period of lethality developed further and were alive at E18.5. This contrasts with our previous results on a mixed background (50% 129Ola/50% FVB), where only two of a total of 35 embryos, which were collected at E16.5 and genotyped as *β1D ki/ki,* were dead and highly abnormal (Baudoin et al., 1998). Since the gross morphology of the *β1D ki/ki* embryos at E18.5 on a mainly FVB genetic background was relatively normal, we allowed subsequent litters to develop to term. Of the 138 pups on P1, five were *β1D ki/ki*; three were dead and one survived for 1 day. The fifth (male) *β1D ki/ki* pup survived for 5 months and was fertile. Of his offspring (two litters), nine individuals were heterozygous and six *β1D ki/ki*. These *β1D ki/ki* pups died soon after birth. The relatively normal gross morphology of *β1D ki/ki* embryos at E18.5 was therefore only exceptionally reflected in viable offspring and survival to adulthood. Thus, the FVB background resulted in reduced penetration of the phenotype and revealed a second period of lethality, occurring at, or soon after, birth.

Gross morphology of *β1D ki/ki* embryos

Between E8.0-E13.5, we observed similar external defects as described previously (Baudoin et al., 1998). These included abnormal head or branchial arches, open or kinked neural tube, and retarded or eccentric limbs (Table 1). However, the frequency was considerably lower (7.4% compared with 27.5% between E10.0 and E13.5), showing that on the present genetic background, replacement of β1A by β1D had less severe morphological consequences. Furthermore, *β1D ki/ki* embryos showed no signs of haemorrhage. Thus, from the external morphology alone, no defects severe enough to cause the previously observed early lethality were detected.

Migratory cells behave normally in *β1D ki/ki* embryos

One of the most prominent defects in E11.5 *β1D ki/ki* embryos on a mixed background was a reduction in the size of the first branchial arch (Baudoin et al., 1998), raising the possibility that the migration of *β1D ki/ki* cranial neural crest cells into the branchial arches could be impaired. Although on a mainly FVB background, only 7.4% of the *β1D ki/ki* embryos had a phenotype suggesting problems with cell migration, we determined whether a general failure in cell migration contributed to the first period of lethality. The behaviour of three migratory cell populations (cranial neural crest cells, limb muscle precursor cells and primordial germ cells; PGCs) was analysed in *β1D ki/ki* embryos and their littermates.

In E9.5 embryos there were no differences between *β1D ki/ki* embryos and their littermates in the expression of *snail* (Fig. 1A,D), which encodes a transcription factor in cranial neural crest cells (Nieto et al., 1992) and *pax3* (Fig. 1B,E), a marker for muscle precursor cells (Goulding et al., 1994).

Since wild-type PGCs only express integrin β1A (Fig. 1I), any delay in the rate of PGC migration in *β1D ki/ki* embryos would be due to the exclusive expression of the β1D splice variant. Comparison of PGC distribution in both E10.5 *β1D ki/ki* and wild-type embryos revealed no significant differences (Fig. 1C,F,G). Furthermore, the absolute number of PGCs in each of the genotypes was similar (Fig. 1H).

Although we cannot exclude defective cell migration in the few *β1D ki/ki* embryos showing external defects, our data show that three cell populations that undergo long-range cell

migration behave normally in the *β1D ki/ki* embryos analysed. These results suggest that on a predominantly FVB genetic background, β1D is able to support cell migration in vivo as efficiently as β1A.

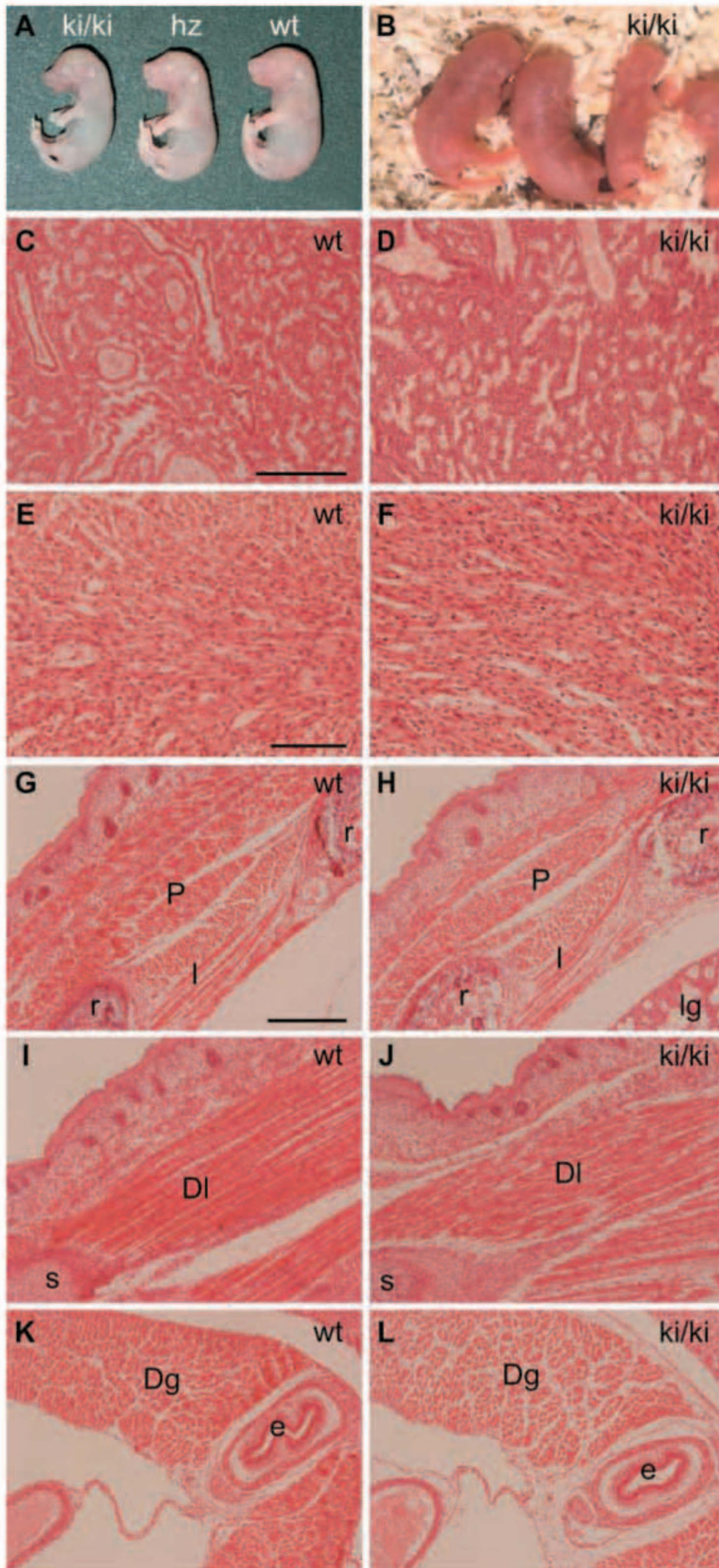

Fig. 3. Late gestation *β1D ki/ki* embryos and newborn pups are thinner/shorter than their littermates and exhibit a reduction in muscle mass. (A,B) Gross morphology of E18.5 embryos (A) and newborns (B). Note the curved posture of the E18.5 *β1D ki/ki* embryo. (C-L) Transverse Haematoxylin and Eosin-stained sections of E18.5 wild-type (C,E,G,I,K) and *β1D ki/ki* (D,F,H,J,L) embryos showing lung (C,D), heart (E,F), thoracic body wall (G,H), shoulder (I,J) and diaphragm (K,L) muscles. Dg, diaphragm; Dl, deltoideus; e, esophagus; I, intercostal; lg, lung; P, pectoral; r, rib; s, scapula. Scale bar: 200 µm.

Abnormal vascularisation and increased apoptosis in the placental labyrinth causes early death of *β1D ki/ki* embryos

The *β1D ki/ki* embryos that ceased development between E13.5-E14.5 were exceptionally pale (not shown), raising the possibility of placental malfunction. We examined the distribution of laminin, a marker for foetal blood vessels (Harbers et al., 1996), in placentae of E14.5 embryos. The results showed that embryonic blood vessels present in the labyrinth of both heterozygous and morphologically normal, living *β1D ki/ki* embryos were uniform in diameter and regularly spaced (Fig. 2A,B,D,E,G,H). In contrast, the labyrinth of the pale *β1D ki/ki* embryos contained a reduced number of large calibre blood vessels, the lumen of the vessels was irregular and many were obstructed. Furthermore, there was less branching, although strong laminin immunoreactivity was maintained (Fig. 2C,F,I). TUNEL assay and immunostaining with MECA32 antibody (recognising endothelial cells) revealed increased apoptosis in labyrinthine endothelial cells in two of three pale *β1D ki/ki* placentae, compared to wild-type or morphologically normal *β1D ki/ki* placentae (Fig. 2J-L). However, phospho-histone H3 immunostaining showed no difference in the number of proliferating cells (not shown). Interestingly, the one *β1D ki/ki* placenta that did not show increased apoptosis, did show a marked increase in proliferation (not shown), suggesting placental recovery (Plum et al., 2001).

These results suggested that some *β1D ki/ki* embryos have placentae with a reduced network of foetal blood vessels, and that placental endothelial cells undergo apoptosis. This could compromise maternal-foetal nutrition and be the cause of early lethality. However, the fact that the 36% *β1D ki/ki* embryos, surviving this period, developed normally until birth indicates that this defect is of variable severity on the FVB genetic background.

Reduction in muscle mass in late gestation *β1D ki/ki* embryos

The *β1D ki/ki* embryos recovered alive between E14.0 and E18.5 were as advanced in development as their wild-type littermates. However, a large proportion was thinner and a few were also shorter than heterozygous and wild-type embryos (Fig. 3A). This was particularly evident between E17.0-E18.5 where 13 of 16 *β1D ki/ki* embryos exhibited this phenotype.

All *β1D ki/ki* pups alive on P1 were both thinner and shorter than their littermates (Fig. 3B). Two of the five

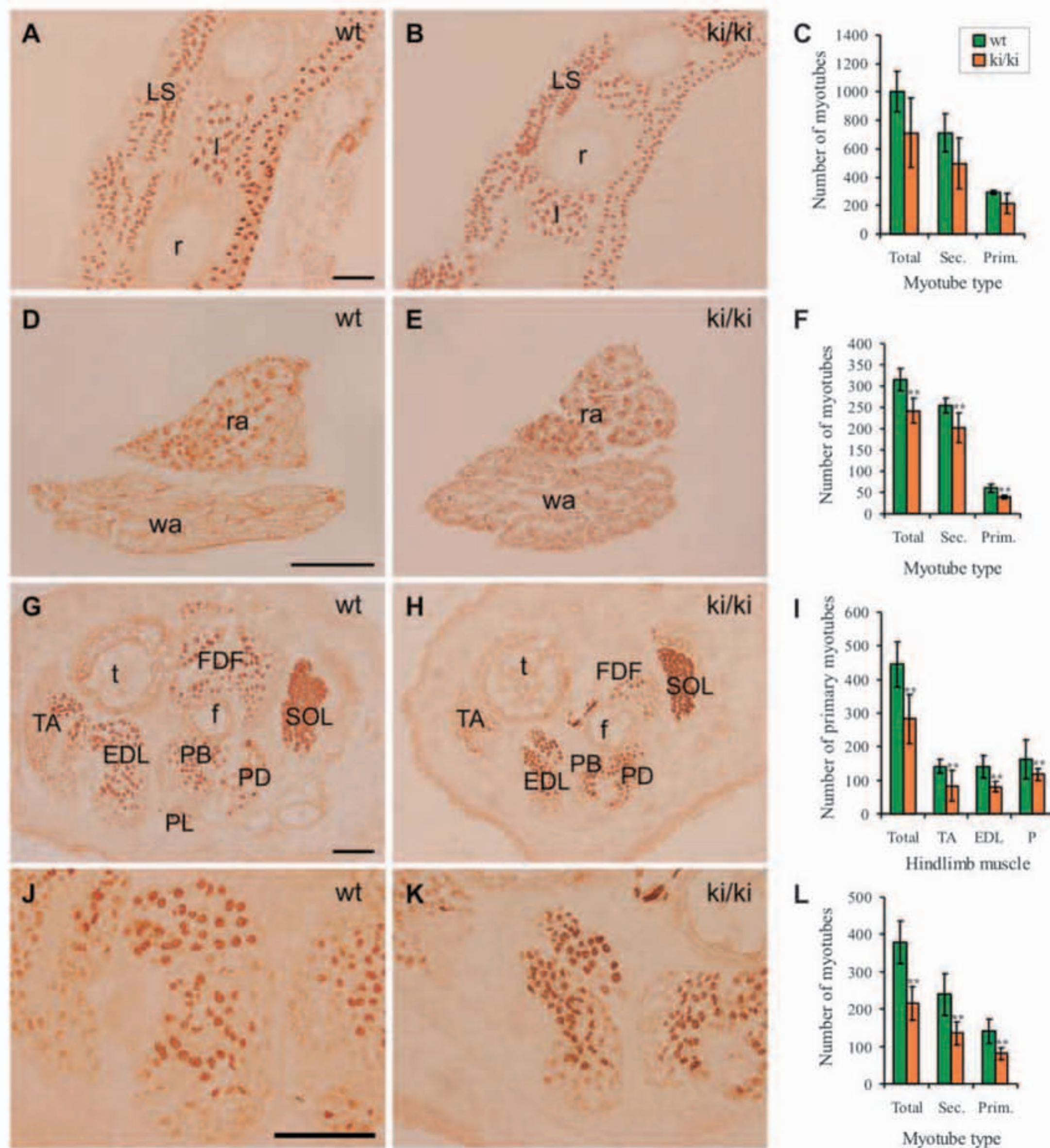

Fig. 4. Different muscle types of E18.5 embryos stained with the anti-slow MHC antibody show a reduction in primary and secondary myotubes in *β1D ki/ki* embryos (B,E,H,K) compared to wild-type littermates (A,D,G,J). Transverse sections of body wall (A,B), cleidomastoideus (D,E) and lower hindlimb (G,H) muscles. J and K are higher magnifications of EDL muscles in G and H. (C,F,I,L) Graphical representation of the differences in myotube number between wild-type and *β1D ki/ki* E18.5 muscles. (C) Total, secondary and primary myotube numbers/mm^2 in intercostal/serratus dorsalis muscles. (F) Total, secondary and primary myotube numbers in red area of cleidomastoideus muscle. (I) Total number of primary myotubes in lower hindlimb represents the sum of myotubes in TA, EDL, and P group. (L) Total, secondary and primary myotubes in EDL muscle. Bars represent means ± s.d.; $^{**}P\leq0.01$. EDL, extensor digitorum longus; f, fibula; FDF, flexor digitorum fibularis; I, intercostal; LS, latissimus dorsi/serratus dorsalis; P, peroneus group (including: PB, peroneus brevis; PD, peroneus digiti; PL, peroneus longus); r, rib; ra, red area of cleidomastoideus; SOL, soleus; t, tibia; TA, tibialis anterior; wa, white area of cleidomastoideus. Scale bars: 200 µm.

pups alive on P1 were also purple (suggesting respiratory distress) and unable to feed. The *β1D ki/ki* male that survived to adulthood behaved normally, but remained smaller than all littermates (weight on P36 was 15 g compared with an average of 22 g for 4 male littermates).

Histological analysis of E18.5 *β1D ki/ki* embryos did not reveal obvious gross morphological abnormalities, and all major organs, e.g. lung (Fig. 3C,D), heart (Fig. 3E,F) and kidneys (not shown), appeared normal. However, there was a marked reduction in skeletal muscle mass (Fig. 3G-L), probably causing the overall size reduction. Furthermore, while wild-type and heterozygous embryos/neonates had a straight spine and upright head, the posture of *β1D ki/ki* embryos was characterised by pronounced spinal curvature and downward facing head (Fig. 3A). Interestingly, myogenin-deficient mice (Hasty et al., 1993; Nabeshima et al., 1993) displayed a similar posture and gross phenotype, and also died perinatally due to a severe reduction in skeletal muscle mass.

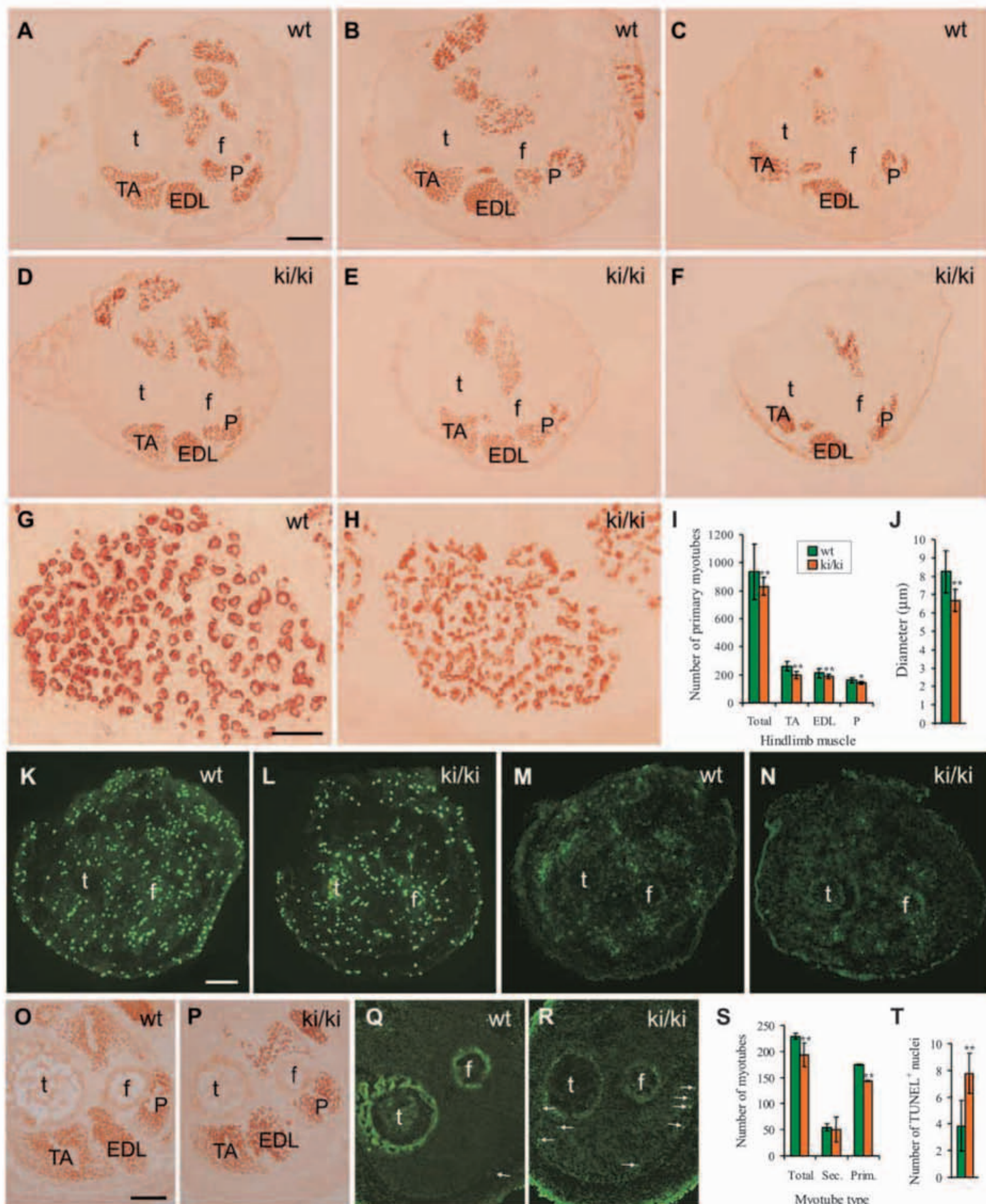

Fig. 5. Hindlimb muscles of *β1D ki/ki* E14.5 embryos (D,E,F,H) have reduced numbers of primary myotubes compared to wild-type embryos (A,B,C,G). Transverse sections of lower hindlimb: (A,D) upper (B,E) middle and (C,F) lower region, stained with anti-slow MHC, showing that muscle size is always reduced in *β1D ki/ki* embryos. (G,H) Higher magnification of EDL in (B,E) showing a clear difference in morphology of the primary myotubes. (I) Graphical representation of the differences in primary myotube numbers in TA, EDL, P muscles and their totals in E14.5 wild-type and *β1D ki/ki* embryos. (J) Primary myotube diameter in EDL, showing a difference in size between wild-type and *β1D ki/ki*. (K-N) Transverse sections of E14.5 hindlimbs (middle region), stained for phospho-histone-H3 (K,L) and subjected to TUNEL assay (M,N) show no differences in proliferation or apoptosis in muscle regions between wild-type (K,M) and *β1D ki/ki* (L,N) embryos. (O-R) Adjacent transverse sections of E17.5 lower hindlimbs stained for slow MHC (O,P) and exposed to the TUNEL assay (Q,R) show a reduction in primary myotubes and increased apoptosis (arrows) in *β1D ki/ki* (P,R) compared with wild type (O,Q). (S-T) Graphical representation of primary, secondary and total myotube numbers in E17.5 EDL (S) and TUNEL-positive nuclei in E17.5 TA, EDL and P per section, in wild type and *β1D ki/ki* (T). For abbreviations see Fig. 4. Legend. Bars represent means ± s.d. **P*≤0.05, ***P*≤0.01. Scale bars: 200 μm (A-F,K-R), 50 μm (G,H).

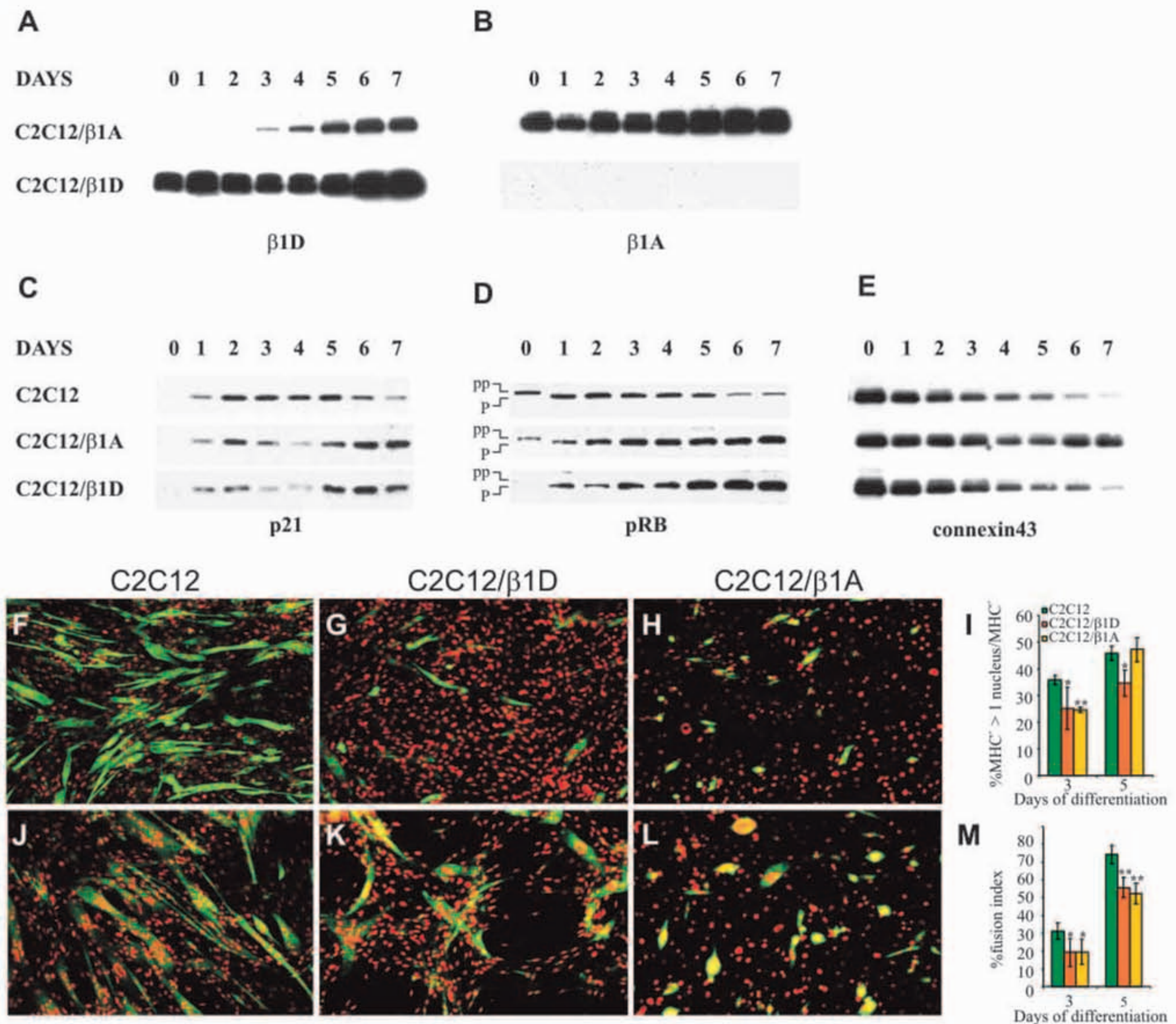

Fig. 6. Myogenic differentiation of C2C12/β1D and C2C12/β1A cells. (A,B) β1D and β1A expression during differentiation of C2C12/β1D and C2C12/β1A cells. (C-E) Expression of p21 (C), pRB (D) and connexin43 (E) analysed by western blotting. During differentiation, p21 is upregulated and pRB is dephosphorylated in all cell groups analysed. Connexin43 was downregulated in C2C12 and C2C12/β1D, but not in C2C12/β1A. (F-H,J-L) Immunostaining for MHC (green) and nuclear To-Pro3 staining (red). C2C12/β1D and C2C12/β1A had fewer MHC^+ cells than C2C12 at day 3 (F-H) and day 5 (J-L) of differentiation and C2C12/β1A myotube morphology was abnormal (H,L). (I) Percentage of MHC^+ cells with >1 nucleus. After 3 days of differentiation, the number of C2C12/β1D and C2C12/β1A myotubes was less than the C2C12 control. After 5 days, the number of C2C12/β1A myotubes is similar to the C2C12 control, in contrast to the number of C2C12/β1D myotubes, which was still lower. (M) Fusion index (percent), being the ratio of number of nuclei in myotubes (cells with >3 nuclei) to the total number of nuclei in MHC^+ cells. After 3 and 5 days of differentiation, both C2C12/β1D and C2C12/β1A myotubes have fewer nuclei than C2C12 myotubes. Bars represent means ± s.d. $^*P\leq 0.05$; $^{**}P\leq 0.01$.

Reduced primary and secondary myotube numbers in E18.5 *β1D ki/ki* embryos

The next question to be considered was whether the reduction in muscle mass resulted from selective loss of either primary or secondary myotubes, or whether these were affected equally. This is important since maternal malnutrition has been shown to affect the development of secondary, but not primary myotubes (Wilson et al., 1988). Thus, a selective reduction in secondary myotubes could indicate an indirect effect on skeletal muscle development due to placental insufficiency, rather than a direct effect of the *β1D ki/ki* genotype in skeletal muscle cells.

The number of primary and secondary myotubes were compared in three different muscle groups in E18.5 wild-type and *β1D ki/ki* embryos, namely cleidomastoideus (neck), intercostal/serratus dorsalis (trunk) and lower hindlimb muscles. A comparable reduction in the number of both primary (27%) and secondary (30%) myotubes per mm^2 in crosssections of trunk muscles of *β1D ki/ki* embryos was observed but this was not statistically significant (Fig. 4A-C).

However, there was a significant reduction in both primary (35%) and secondary (20%) myotube numbers within the red part of cleidomastoideus muscle (Fig. 4D-F). Interestingly, the ratio of secondary/primary myotubes was slightly higher in *β1D ki/ki* (5.20) than in wild-type (4.23) embryos.

Furthermore, *β1D ki/ki* lower hindlimb muscles showed a significant reduction (37%) in primary myotubes as did the individual muscles assayed (tibialis anterior, TA; extensor digitorum longus, EDL; and the peroneus group, P) (Fig. 4G-I). Secondary myotubes were counted only in EDL muscles (Fig. 4J-L); the results showed a significant decrease (44%) in *β1D ki/ki* embryos. The ratio of secondary/primary myotubes in EDL muscles was similar in wild-type (1.71) and *β1D ki/ki* (1.67) embryos at this developmental stage. These results demonstrate that the reduction in muscle mass observed in E18.5 *β1D ki/ki* embryos is due to a reduction in the number of both primary and secondary myotubes. However, since primary myotubes serve as a scaffold for the formation of secondary myotubes (Duxson et al., 1989), a reduction in primary myotubes will cause a proportional reduction in secondary myotubes, evident from E18.5 onwards (Ashby et al., 1993; Kegley et al., 2001). Together these results suggested that the reduction in muscle mass of the *β1D ki/ki* embryos was not due to placental insufficiency, but was rather a direct effect of defective primary myogenesis.

Primary myotube formation and survival are affected in *β1D ki/ki* embryos

The reduction in the number of primary myotubes in E18.5 *β1D ki/ki* embryos may result from the formation of fewer primary myoblasts, their reduced proliferation or subsequent loss by, for example, apoptosis. Primary myogenesis starts around E12.0 in the mouse, with the number of primary myotubes reaching a maximum at around E14.5 in hindlimb muscles, without secondary myotubes being present (Ashby et al., 1993; Kegley et al., 2001).

Serial sections of the lower hindlimb of E14.5 *β1D ki/ki* and wild-type embryos were stained with anti-slow MHC antibody. *β1D ki/ki* hindlimb muscles were smaller than wild type at all levels (Fig. 5A-F), and showed a significant average reduction in the number of primary myotubes (16%; Fig. 5I). Fewer serial sections of each muscle were obtained (not shown), suggesting that these muscles were also shorter. In addition, myotubes of *β1D ki/ki* embryos were smaller (Fig. 5G,H), EDL myotube diameter being 81% of those of wild type (Fig. 5J). This suggested that the exclusive presence of β1D resulted in a reduced capacity to form primary myotubes, which was reflected both in the lower number formed and their smaller size.

To determine whether this could be due to reduced proliferation of primary myoblasts, E14.5 hindlimbs sections were stained for phospho-histone H3, but no differences were observed between the number of proliferating cells per section of wild-type and *β1D ki/ki* embryos at this developmental stage (Fig. 5K,L).

At E14.5, apoptosis is a normal feature of developing muscle (Ashby et al., 1993). TUNEL assay carried out on sections of E14.5 hindlimbs revealed no differences in apoptosis between wild-type and *β1D ki/ki* embryos (Fig. 5M,N), suggesting that the presence of β1D does not lead to increased cell death in primary myoblasts or early myotubes.

It is, however, clear from the results above (see Fig. 4I and Fig. 5I) that the reduction in the number of primary myotubes in *β1D ki/ki* hindlimbs is much greater at E18.5 (37%) than at E14.5 (16%). Thus about 21% of *β1D ki/ki* primary myotubes are apparently lost during this period. To pinpoint the exact period of myotube loss, we exposed alternate cryosections of wild-type and *β1D ki/ki* E16.5 and E17.5 hindlimbs to anti-slow MHC antibody and TUNEL assay. Quantification of primary myotubes in E16.5 EDL showed a similar difference between wild type and *β1D ki/ki* as observed in E14.5 (not shown), suggesting that myotube loss had not yet started. However, at E17.5, primary myotube numbers in *β1D ki/ki* EDL (Fig. 5O,P) were reduced by 18% as compared to the wild type (Fig. 5S), an intermediate value between the reduction observed at E14.5 (11%) and E18.5 (40%) (Fig. 4I and Fig. 5I). The number of secondary myotubes was, however, identical both at E16.5 (not shown) and E17.5 (Fig. 5S) between genotypes, confirming that reduced number of secondary myotubes as a result of defective primary myogenesis becomes evident only by E18.5 (Ashby et al., 1993). The TUNEL assay revealed a twofold increase in apoptosis in *β1D ki/ki* hindlimb muscles compared with those of wild type both at E16.5 (not shown) and E17.5 (Fig. 5Q,R,T). Together these data show that there is an increase in apoptosis in *β1D ki/ki* muscles at E16.5, but myotube loss is only evident by E17.5.

Denervation has been shown to result in reduction of primary myotube numbers, although the myotube loss was observed before E15.5 (Condon et al., 1990; Ashby et al., 1993). Neurites differentiate and migrate normally on a permissive substrate in *β1D ki/ki* embryoid bodies (Gimond et al., 2000). No evidence for abnormal innervation was observed in E14.5 *β1D ki/ki* hindlimb sections stained for PGP9.5, a nerve marker (not shown). Clusters of acetylcholine receptors were also present in normal numbers in hindlimb muscles of E18.5 *β1D ki/ki* embryos (not shown), suggesting normal development of neuromuscular junctions.

Thus, these results show a significant impairment in primary myogenesis in E14.5 *β1D ki/ki* embryos, not caused by reduced myoblast proliferation, increased apoptosis or absence of nerves. In addition, our data indicate that long-term primary myotube survival is affected when β1A is replaced by β1D.

Differential effects of β1A and β1D overexpression during myogenic differentiation of C2C12 cells

To gain insight in the putative roles of β1A and β1D during myogenic differentiation, C2C12 cells were stably infected with either β1D or β1A constructs and differentiation and fusion parameters analysed. β1A is expressed by proliferating C2C12 cells and is downregulated during myogenic differentiation, while β1D becomes upregulated (Belkin et al., 1996). Infection of C2C12 cells with either splice variant caused constant and high expression of that splice variant (Fig. 6A,B) and infection with β1D totally inhibited the expression of β1A (Fig. 6B).

First, we analysed the effects of β1A and β1D on cell cycle parameters by western blotting for cyclin-dependent kinase inhibitor p21 and tumour suppressor retinoblastoma protein (pRB), markers for irreversible cell cycle arrest (Gu et al., 1993; Andrés and Walsh, 1996; Walsh and Perlman, 1997). No

differences were observed between wild-type and infected cells in the onset of p21 and dephosphorylation of pRB (Fig. 6C,D).

MHC, a marker for myoblast differentiation, was upregulated 2 days after induction of differentiation in C2C12 cells, as expected, and the levels of expression steadily increased until day 7 (Andrés and Walsh, 1996; Dedieu et al., 2002). Although both C2C12/β1A and C2C12/β1D cells upregulated MHC at a similar rate (not shown), immunostaining for MHC showed clear differences between infected and control cultures (Fig. 6F-H,J-L).

After 3 days of differentiation, both C2C12/β1A and C2C12/β1D cells showed a similar reduction in number of multinucleated MHC^+ myotubes/MHC^+ cells (30% reduction compared to C2C12 control) and in fusion index (Fig. 6L,M). After 5 days of differentiation, the number of multinucleated MHC^+ myotubes/MHC^+ cells formed by C2C12/β1A cells was similar to the C2C12 control, but the number of nuclei present per myotube was significantly lower. In contrast, both the number of multinucleated MHC^+ cells and the number of nuclei per myotube were reduced in C2C12/β1D cells (Fig. 6L,M).

During differentiation, C2C12/β1D myotubes elongated normally, but the elongation of C2C12/β1A myotubes was blocked and myotubes were rounded and had little cytoplasm (Fig. 6H,L). Finally, we analysed the expression of connexin43, a gap junction protein downregulated in C2C12 myotubes (Reinecke et al., 2000). C2C12/β1A cells did not downregulate this protein, in contrast to C2C12/β1D cells (Fig. 6E), despite a similar fusion index. Thus, we propose that the failure to downregulate connexin43 correlates with the failure in myotube elongation rather than fusion as such.

These data thus show that overexpression of β1A and β1D in C2C12 cells differentially affects myogenic differentiation.

DISCUSSION

β1D ki/ki embryos exhibit two periods of lethality on a FVB background

Phenotypes resulting from genetic modifications in mice are often highly dependent on the genetic background of the mice carrying the mutation. Here, we crossed *β1D ki/ki* mice generated on a mixed genetic background [50% 129Ola/50% FVB (Baudoin et al., 1998)], into a predominantly FVB background. We observed that the defects previously associated with the genotype became milder and less frequent and were unlikely to be responsible for the early lethality described.

We demonstrated that the great majority of *β1D ki/ki* embryos lost during midgestation, died not because of abnormal cell migration or haemorrhage as previously, but from defective placentation. Furthermore, the remaining *β1D ki/ki* embryos were lost at birth, most likely as a consequence of reduced muscle mass, which affected, among other muscles, the diaphragm and therefore their ability to breath.

By studying the phenotype of the β1D knock-in mice in a predominantly FVB background, we revealed the importance of β1 during placental labyrinth development and also demonstrated that precocious and exclusive expression of β1D in skeletal muscle leads to a reduction in muscle mass.

Replacement of β1A with β1D supported cell migration in vivo

Cell migration on a predominantly FVB background was not perturbed by the substitution of β1A by β1D in any of the migratory cells analysed in vivo (neural crest cells, limb muscle precursor cells and PGCs), meaning that their migratory behaviour was unaffected by the exclusive presence of β1D. Independent experiments have shown that myoblasts and neural crest cells derived from mES cells deficient in β1 were able to migrate normally in vivo in wild-type/β1-null chimeric embryos (Fässler and Meyer, 1995; Hirsch et al., 1998). This suggested that migration in general was not dependent on β1 integrins. An alternative explanation, however, could be the low percentage of chimerism used in those experiments. Thus β1-null cells could have adhered to wild-type cells and been passively transported to their destination. However, PGC colonisation of the gonads has been shown to be dependent on β1 integrins, by analysis of wild-type/β1-null chimeric mice (Anderson et al., 1999) and therefore β1 is necessary for normal PGC migration even in the presence of a large number of wild-type cells. Our results clearly show that replacement of β1A with β1D had no effect on either the total number of PGCs or their migration towards the gonads. This strongly suggests that β1D is able to support normal cell migration in vivo on a predominantly FVB background.

Placental labyrinth defects are responsible for early lethality in *β1D ki/ki* embryos

The placental labyrinth starts forming immediately after chorioallantoic fusion, when foetal blood vessels growing from the allantois contact the chorionic plate, the precursor of the labyrinthine trophoblast. The chorionic trophoblast cells then proliferate, differentiate and fuse into multinucleated syncytiotrophoblast, a prerequisite for labyrinthine trophoblast branching morphogenesis and subsequent embryonic vascular invasion (Anson-Cartwright et al., 2000; Rossant and Cross, 2001).

In the mouse, β1 integrin is strongly expressed in both the labyrinthine trophoblast and foetal blood vessels throughout their development (Bowen and Hunt, 1999). α4β1 in the chorionic plate plays an important role in chorioallantoic fusion by binding to VCAM1 expressed by the allantois (Yang et al., 1995; Kwee et al., 1995; Gurtner et al., 1995). This early event does not appear to be affected in *β1D ki/ki* embryos. However, labyrinthine branching defects were observed in the minority of VCAM1-null embryos that underwent chorioallantoic fusion (Gurtner et al., 1995), suggesting that VCAM1-α4β1 interactions are also important during later stages. αvβ1 and αvβ3 heterodimers are expressed in the labyrinth (Bowen and Hunt, 1999), which is poorly developed in 80% of αv-null embryos (Bader et al., 1998). Furthermore, α7β1 is strongly expressed in the chorionic plate, but is downregulated during labyrinthine branching (Klaffky et al., 2001). Although placental defects have not been described in α7-null embryos, about half of these embryos are lost at midgestation (Mayer et al., 1997), raising the question of whether α7 is important in trophoblast development as suggested by in vitro assays (Klaffky et al., 2001). Although α6β1 is present in labyrinthine blood vessels, α6-null embryos

develop to term (Georges-Labouesse et al., 1996), indicating normal placental development. Laminin receptors are likely to play a role in labyrinthine development since inactivation of the laminin α5 chain (present in laminin 10/11) causes a reduction in labyrinthine branching and leads to embryonic lethality between E13.5 and E16.5 (Miner et al., 1998).

β1A is the only β1 splice variant present in the early placenta and trophoblast cell lines (Klaffky et al., 2001). Based on the phenotype observed in two out of three *β1D ki/ki* embryos, we hypothesise that the replacement of β1A by β1D might impair chorionic trophoblast morphogenesis, leading to abnormal vascularisation of the labyrinth (Rossant and Cross, 2001). Alternatively, β1D expression on invading embryonic blood vessels might impair their morphogenesis directly.

The placenta was not examined in *β1D ki/ki* embryos on a mixed background (Baudoin et al., 1998). We cannot rule out placentation defects, at least in part, causing the observed embryonic lethality, since 5 of the 13 *β1D ki/ki* embryos analysed at E12.5 were severely anaemic. It was suggested that the anaemia was the result of the extravasation of red blood cells through weak-walled vessels throughout the bodies of *β1D ki/ki* embryos. However, in the predominantly FVB background described here, extravascular blood cells were not observed and we believe that the anaemia resulted largely or entirely from abnormal placentation.

Precocious expression of β1D inhibits primary myogenesis

From its expression pattern it is clear that β1D is not involved in primary myogenesis. β1D is detected on the surface of myotubes from E17.5 and then becomes enriched in myotendinous junctions and at costameres, where it might be important for the formation of strong adhesion sites (van der Flier et al., 1997).

We show here that precocious and exclusive expression of β1D in skeletal muscle affects primary myogenesis. Not only were there fewer primary myotubes at E14.5, but they were also smaller in diameter and shorter.

Fewer primary myotubes could be the result of: (1) a reduction in the number of primary myoblast precursors, (2) a reduction in the proliferation rate of primary myoblasts, (3) their increased apoptosis and (4) an inhibition in myoblast differentiation and subsequent fusion.

Our results suggest that myogenic precursor cells delaminate and migrate normally towards the limb buds in *β1D ki/ki* embryos and that proliferation and apoptosis is not altered at E14.5. However, we cannot exclude an earlier reduction in the numbers of myoblast precursors. The fact that primary myotubes are smaller in *β1D ki/ki* embryos does, however, strongly suggest a defect in myoblast differentiation and/or fusion. A significant reduction in primary myotubes was also found in embryos lacking the transcription factor NFATC3 (Kegley et al., 2001). However, there, the E15.0 EDL primary myotubes were normal in size and morphology, leading the authors to exclude a defect in differentiation and/or fusion and favour a reduction in the pool of primary myoblasts.

Several studies have suggested a role for β1 integrins in the differentiation and fusion of myoblasts in vitro (Sastry et al., 1996; Gullberg et al., 1998), but because β1-null cells participate in the formation of skeletal muscle in β1-null/wild type chimeras (Fässler and Meyer, 1995) the role of β1 in this process in vivo has been questioned. However, Hirsch et al. (Hirsch et al., 1998) have shown that while β1-null myoblasts isolated from E16.0 embryos (i.e. secondary myoblasts) fuse and form normal myotubes, myoblasts derived from β1-null mES cells (i.e. predominantly primary myoblasts) show a significant inhibition in myotube formation. This study together with our data strongly suggest that β1A plays an important role in the formation of primary myotubes and that substitution of β1A by β1D significantly impedes this process.

Long-term myotube survival is clearly affected in *β1D ki/ki* embryos. This could be due to inhibition of myotube growth caused by a progressive impairment in the addition of myoblast nuclei to the early myotubes (Zhang and McLennan, 1995). Alternatively, the precocious presence of β1D might interfere with the establishment of myotube connections to the surrounding basement membrane (Vachon et al., 1996) or the myotendinous junction (Miosge et al., 1999), compromising myotube survival by forming strong adhesion sites too early.

Overexpression of β1D and β1A produces different effects on C2C12 differentiation

C2C12 cells overexpressing β1A or β1D exhibit inhibition of myotube formation, suggesting that excessive amounts of either β1 splice variant interferes with this process. However, the more long-term effect of β1D overexpression shows that this splice variant causes a stronger inhibition. Strikingly, myotube morphology was severely affected in C2C12/β1A cells and downregulation of connexin43 did not occur, suggesting that β1A inhibits myotube maturation. This is probably due to overexpression since inhibition in myotube maturation is not observed in β1D knock-out mice, where β1A expression persists in mature muscle fibres (Baudoin et al., 1998). Overall, these results suggest that C2C12 myotube formation and maturation is dependent on a quantitative balance of different integrins, an idea supported by studies on the effect of integrin α-subunit ratios on β1A signalling during myoblast differentiation (Sastry et al., 1996; Sastry et al., 1999).

Secondary myogenesis is unaffected in *β1D ki/ki* embryos, suggesting different roles for β1 integrins in primary versus secondary myogenesis

At E16.5 and E17.5, the number of secondary myotubes was similar between *β1D ki/ki* and wild-type embryos, while at E18.5, it was significantly lower in *β1D ki/ki* embryos. The ratio of secondary to primary myotubes, however, was similar in the muscles studied at E18.5. Thus the number of secondary myotubes is only reduced when the reduced number of primary myotubes in *β1D ki/ki* embryos becomes a limiting factor for the formation of more secondary myotubes (Ashby et al., 1993). These results suggest that secondary myogenesis is unaffected in *β1D ki/ki* embryos. It is becoming evident that different regulatory pathways control these two waves of myogenesis in vivo. For example, in myogenin null embryos secondary myogenesis is much more affected than primary myogenesis (Venuti et al., 1995), while inactivation of NFATC3 causes a selective reduction in primary myotubes (Kegley et al., 2001). Our data show that primary myogenesis is more sensitive to the precocious presence of β1D than secondary myogenesis. This not only demonstrates different roles for β1A and β1D, but also strongly suggests that β1

integrins play distinct roles in primary versus secondary myogenesis in vivo.

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chapter 6

CTGF expression and Smad signaling during mouse heart development and myocardial infarction

submitted

CTGF expression and Smad signaling during mouse heart development and myocardial infarction

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Abstract

Connective tissue growth factor (CTGF) has been reported to be a target gene of transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) *in vitro*. Its physiological role in angiogenesis and skeletogenesis during mouse development has recently been described. Here, we have mapped expression of CTGF mRNA during mouse heart development, postnatal adult life and following experimental myocardial infarction. In contrast to observations *in vitro*, CTGF and TGFβ/BMP show little crosstalk during development *in vivo*, but we report that CTGF is upregulated independently of TGFβ or BMP in the developing heart under hypoxic conditions.

Strikingly one week after myocardial infarction, when myocytes have disappeared from the infarct zone, CTGF and TGFβ expression as well as activated forms of TGFβ but not BMP, Smad effector proteins are co-localized exclusively in the fibroblasts of the scar tissue, suggesting possible cooperation between CTGF and TGFβ during the pathological fibrotic response.

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Introduction

Connective tissue growth factor (CTGF), also known as FISP12 in the mouse, was first isolated from human umbilical vein endothelial cells [1]. This secreted growth factor belongs to the CCN family, which includes CYR61 and NOV and has been identified in mammals as well as amphibians. Mouse and human CTGF are both 38kDa proteins and share 90% of their amino-acid sequence, but differ in that human CTGF can be glycosylated, whilst mouse cannot. CTGF contains 4 conserved structural domains: an insulin-like growth factor binding domain, a von Willebrand type C domain, a thrombospondin type I repeat and a C-terminal cystine knot module and further 38 conserved cysteine residues [2,3].

CTGF binds to extracellular matrix proteins (ECM) and integrins [3], but also to transforming growth factor-β1 (TGFβ1) and bone morphogenetic protein (BMP4) modulating their activity [4]. CTGF has also been shown, at least *in vitro*, to promote ECM production in different types of fibroblasts [5,6,7]. Its physiological role during mouse development has recently been described by Ivkovic and colleagues [8]. *Ctgf* deficient mice have defects in skeletal development leading to perinatal death. Pathologically, CTGF is known to play important roles during wound repair, tumorigenesis, artherosclerosis and fibrotic disorders of the skin, kidney, lung, liver and pancreas [reviewed in 3,9].

TGFβ1 and BMP4 are members of the TGFβ superfamily of secreted growth factors. TGFβ signaling involves the activation of type I receptor serine-threonine kinases and subsequent phosphorylation of receptor-regulated (R-)Smads, which then form complexes with Smad4 and translocate from the cytoplasm to the nucleus and regulate transcription of target genes [reviewed by 10].

To what extent CTGF interacts with TGFβ/BMP signaling *in vivo* during development and disease is still unclear. *In vitro*, TGFβ(1) has been shown to increase mRNA and protein synthesis of CTGF in various fibroblastic cell types [3]. A TGFβ-response element identified in the *Ctgf* promoter suggests that this may be a direct effect [11,12] although possibly independently of Smads [13]. In dermal fibroblasts, the basal activity of the *Ctgf* promoter appears TGFβ Smad-dependent, but the constitutive increase in activity associated with sclerodoma is Smad independent [14]. The relation between BMPs and CTGF is less clear, although they are co-expressed in developing bone [8,15] and BMP2 upregulates CTGF mRNA in chondrocytes [16].

Here, the distribution of CTGF mRNA was studied in the heart during embryonic development and in adult mice. In addition, possible interactions between CTGF and TGFβ/BMP *in vivo* during development and disease were investigated. Analysis of *Alk5* and *Bmp4* deficient embryos at embryonic day (E)9.5 revealed similar changes in the expression of CTGF mRNA in the heart, despite signaling through different Smads, suggesting that CTGF may be upregulated there as a consequence of common defects in yolk sac formation. Analysis of *Ctgf* deficient mouse embryos at E16.5 showed no alterations in either the activation of TGFβ or BMP Smad-dependent signaling in the heart. Interestingly, CTGF expression was essentially restricted to the atria in adult hearts, but after experimental induction of myocardial infarction (MI) both CTGF and TGFβ mRNA were upregulated specifically in the scar tissue of the ventricle 1 and 4 weeks post-MI, where they may interact. We show for the first time increased TGFβ Smad-dependent signaling in fibroblasts of the heart combined with enhanced CTGF expression in the same cells and show that the myocytes are only marginally involved.

Material and Methods

Mouse strains, genotyping and isolation of embryos and hearts

Embryos and mice were BL6/CBA. *Bmp4*^{tm1} mice [17] were maintained on a BL6/CBA background, *TgfbrI*$^{+/-}$ (or *Alk5*$^{+/-}$) mice [18] on a BL6 background and *Ctgf*$^{+/-}$ mice [8] on a 25% Balbc/75% 129 background. All embryos and hearts were isolated/kept in cold Dulbecco's minimal essential medium (DMEM, Invitrogen) supplemented with 7.5% fetal calf serum (FCS) and 10 mM HEPES, fixed overnight (o/n) in 4% paraformaldehyde in PBS (PFA), washed twice 30 minutes in 0.83% NaCl, kept 30 minutes in 0.42% NaCl in 50% EtOH and washed twice 30 minutes in 70% EtOH, all steps performed at 4^0C. Embryos and hearts were embedded in paraffin wax, sectioned (6 µm) onto coated slides (Klinipath) and stored at 4^0C. Genotyping was performed as described [8,17,18].

In situ hybridization

To generate the CTGF probe, the vector pGEM4Z containing a 0.65kb *Pst*I fragment of *hCtgf* cDNA (235-825bp) [19] was linearized with *BamH*I. The anti-sense probe was made using [α^{35}S]-UTP (Amersham) and the T7 promoter. The TGFβ1 probe was generated from a *Kpn*I-*Apa*I fragment (553-1152bp) from the mouse full length *Tgfβ1* [20] cloned into Bluescript and linearized with *EcoR*I. The anti-sense probe was made using [α^{35}S]-UTP (Amersham) and the T3 promotor. *In situ* hybridization was performed essentially as described [21]. Consecutive adult heart sections were used for Azan –Mallory staining.

Cell culture and northern blot

C2C12 cells were cultured to subconfluence in DMEM supplemented with 10% FCS at 37^0C and 5% CO_2, kept for 16 hours in serum-free medium, then treated with 50 ng/ml BMP4 (R&D systems) in fresh serum-free medium for 1 or 2 hours. Total RNA was isolated using TRIzol Reagent Kit (Invitrogen). The CTGF probe (described above) was labeled with [α^{32}P]dCTP using a Rediprime kit (Amersham-Pharmacia). Prehybridization and hybridization was carried out as described [22].

Coronary artery ligation

MI was experimentally induced by coronary artery ligation as described [23]. Male mice (Swiss) weighing 30-45 g (10-12 weeks old) were used for surgery. Sham-operated mice were subjected to similar surgery, except that no ligature was placed. The hearts were dissected 1 week and 4 weeks post-MI and processed as above.

Immunohistochemistry

Immunohistochemistry using the primary antibodies rabbit anti-PSmad2 [24], anti-Troponin I (Chemicon), anti-PSmad1/5/8 (Cell Signalling Technology) and anti-fibronectin (Sigma) was performed as described [25,26].

Results

Expression pattern of CTGF mRNA during early mouse development (E7.5 – E10.5)

Although well-studied biochemically and to some extent in disease, CTGF expression through early mouse development has not been fully documented. At E7.5, high levels of CTGF mRNA were detected by *in situ* hybridization in the parietal endoderm and deciduum, particularly in crypt cells (Fig. 1A,B). Later in development but before the embryo undergoes "turning" (E8.0-E8.5), transcripts were detected in the notochord, cephalic mesenchymal cells, first branchial pouch, foregut diverticulum and faintly in roof and floor plate of the neural tube (Fig. 1C,D). CTGF

was upregulated throughout the heart myocardium, but was not detected in the endocardium (Fig. 1C). At E8.5-E9.0, CTGF mRNA became more prominent, particularly in the heart myocardium, where CTGF-positive cells were clustered randomly in patches (Fig. 1E,F).

At E9.5-E10.5, most cells of the common atrial chamber, the sinus venosus, the truncus arteriosus, the aortic sac and myocardial cells of the bulbus cordis and ventricle contained CTGF mRNA, again in a characteristic patched/punctated pattern (Fig. 1G, 2C,D). Furthermore, the common cardinal vein, branchial arch arteries and the dorsal aorta all contained CTGF mRNA, as well as the notochord, cephalic mesenchyme, the outer-lateral parts of both the first and second branchial arches, the foregut and some tissues in the nervous system (Fig.1G-I).

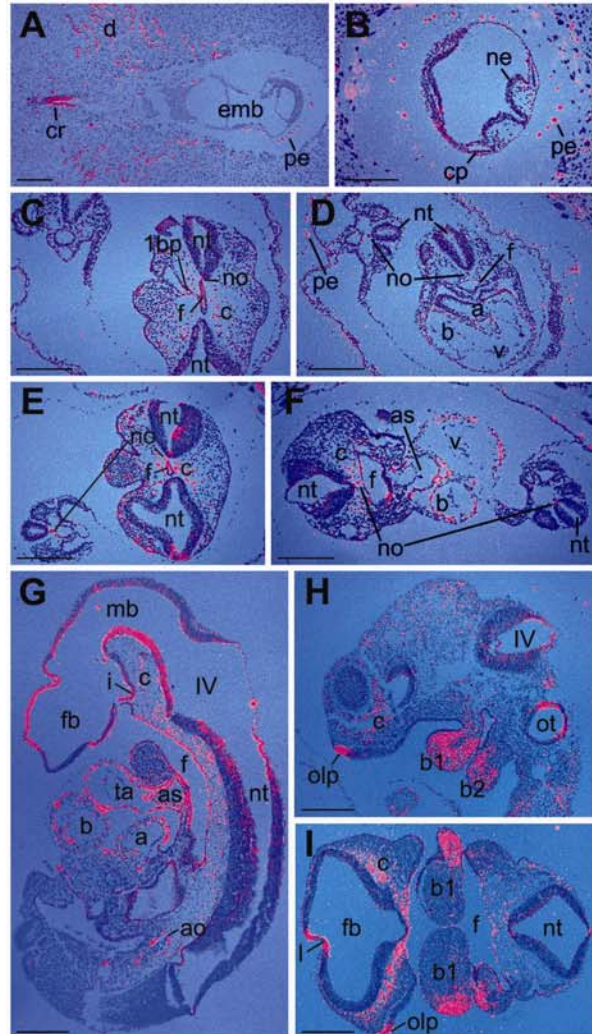

Fig. 1. Expression of CTGF mRNA during early development (E7.5-E10.5). Sections of E7.5 embryos in deciduum (A, sagittal; B, transverse), E8.0-E8.5 embryos (C, tranverse; level of head; D, transverse; level of heart), E8.5-E9.0 embryos (E, tranverse; level of head; F, transverse; level of heart) and E9.5-E10.5 embryos (G, sagittal; through midline; H, sagittal; through branchial arches; I, transverse; level of head). Abbreviations: 1bp, first brachial pouch; IV, fourth ventricle of the brain; a, common atrial chamber; ao, aorta; as, aortic sac; b, bulbus cordis; b1, first branchial arch; b2, second branchial arch; c, cephalic mesenchyme; cp, cardiogenic plate; cr, deciduum cript; d, deciduum; emb, embryo; f, foregut; fb, forebrain; i, infundibulum; l, lamina terminalis; mb, midbrain; ne, neural ectoderm; no, notochord; nt, neural tube; olp, olfactory placode; ot, otocyst; pe, parietal endoderm; ta, truncus arteriosus; v, common ventriclar chamber of the heart.
Scale bar: 0.25 mm (A-F) and 0.5 mm (G-I).

Abnormal CTGF expression in the heart at E9.5 in the absence of TGFβ/BMP signaling

In cardiac fibroblasts, CTGF has been described as an early TGFβ target gene [27]. To determine whether CTGF is also an early target gene of BMP, C2C12 cells were treated with BMP4 and analyzed by Northern blotting. CTGF mRNA was upregulated transiently 8 fold within one hour, decreasing after two hours, suggesting that *in vitro*, CTGF is indeed an early target gene of BMP4 (Fig. 2A).

CTGF mRNA expression was then analyzed in *Bmp4* deficient mouse embryos. On a mixed genetic background, *Bmp4* deficient embryos die around E9.5, showing a reduced number of blood islands in the visceral yolk sac [28]. The most retarded *Bmp4* embryo mutants analyzed at E9.5 were very disorganized and CTGF mRNA was absent from the heart (data not shown). In E9.5 *Bmp4* deficient embryos that did not undergo "turning", CTGF mRNA expression was similar to that in wildtype littermates, except that expression in the myocardium of the common atrial chamber was greatly increased (Fig. 2E,F). By contrast, in E9.5 *Bmp4*

deficient embryos that developed further and "turned", CTGF mRNA was highly upregulated in the aortic sac and bulbus cordis, while the rest of the heart, including the common atrial chamber showed normal levels of CTGF transcripts (Fig. 2G,H).

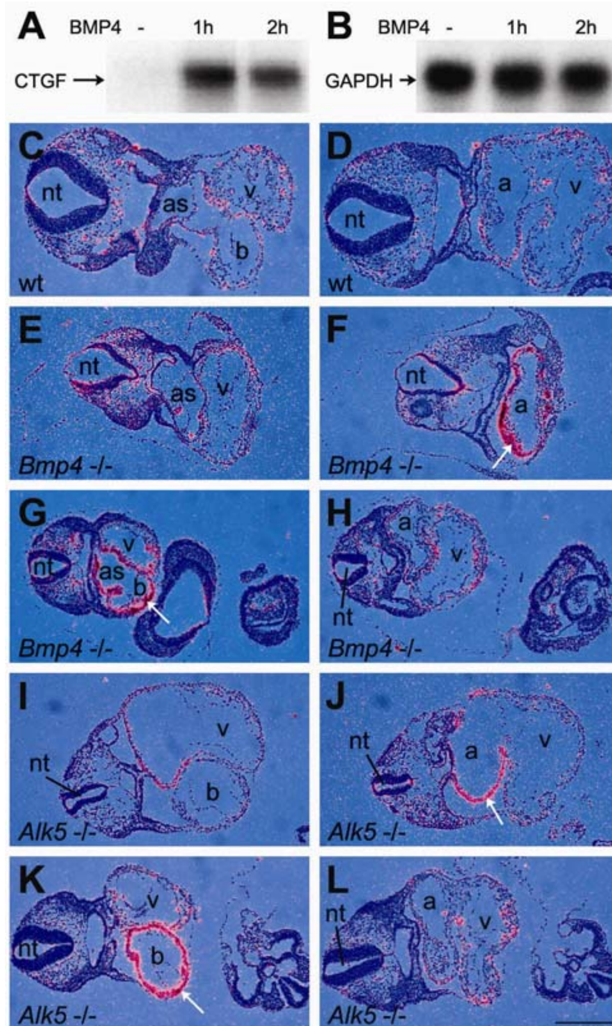

Fig. 2. CTGF expression in the heart of Bmp4 and Alk5 deficient embryos at E9.5. In C2C12 cells, CTGF mRNA was transiently upregulated after one hour of exposure to BMP4 (A). As A, reprobed with GAPDH as loading control (B). CTGF mRNA expression in transverse sections through the hearts of E9.5 embryos wildtype control (C, aortic sac level; D, atrium level), Bmp4 deficient E9.5 embryos not turned (E, aortic sac level; F, atrium level) and turned (G, aortic sac level; H, atrium level) and Alk5 deficient E9.5 embryos not turned (I, aortic sac level; J, atrium level) and turned (K, aortic sac level; L, atrium level). White arrow i ndicates aberrant CTGF mRNA expression in the heart in F,G,J,K. Abbreviations: as legend to Fig.1. Scale bar: 0.25 mm.

To identify tissues where CTGF might be regulated by the TGFβ signaling *in vivo*, we analyzed E9.5 embryos lacking *Alk5* (or *Tgfbrl*$^{-/-}$), a TGFβ type I receptor that does not bind BMPs. *Alk5* deficient embryos die around E10.5 with pronounced defects in vascularization of the visceral yolk sac [18]. Curiously, CTGF expression in *Alk5* deficient embryos resembled that in *Bmp4* deficient embryos: in E9.5 *Alk5* mutant embryos that did not "turn", CTGF mRNA was also upregulated in the common atrial chamber (Fig. 2I,J), while in morphologically normal E9.5 *Alk5* mutants, CTGF mRNA was upregulated in the aortic sac and bulbus cordis (Fig. 2K,L). CTGF mRNA expression throughout the rest of the embryo, including the other chambers of the heart was similar to wildtype littermates. Together, these data suggest that neither BMP4 nor TGFβ signaling pathways regulate CTGF expression (differentially or directly) during early mouse development, but the altered CTGF expression observed in the heart may indirectly result from the abnormal development of the mutants visceral yolk sac.

CTGF expression in the heart becomes atrium restricted in adult mice

After septation (E12.5), the heart structure remains essentially unchanged, although it undergoes a substantial increase in size and the valves acquire their final shape and position. CTGF mRNA was highly expressed in the heart at E12.5-E13.0 (Fig. 3A,B) and E14.5 (Fig. 3C,D), particularly in the myocardial trabeculation and cushion tissue, where it showed the characteristic patched pattern observed at earlier stages. Furthermore, it was detected in the aorta, pulmonary artery and in particular in the ductus arteriosus and pulmonary trunk (Fig. 3A-D), as well as in all large blood vessels. The leaflets of both

the pulmonary and aortic valves showed no/low CTGF mRNA, but the non-trabeculated region of the ventricles underneath the leaflets contained particularly high levels of transcripts (Fig. 3A,C). By E16.5, the domains of CTGF expression were unchanged (results not shown), but in addition CTGF mRNA was observed in the leaflets of both the aortic and pulmonary valve (insert in Fig. 4C).

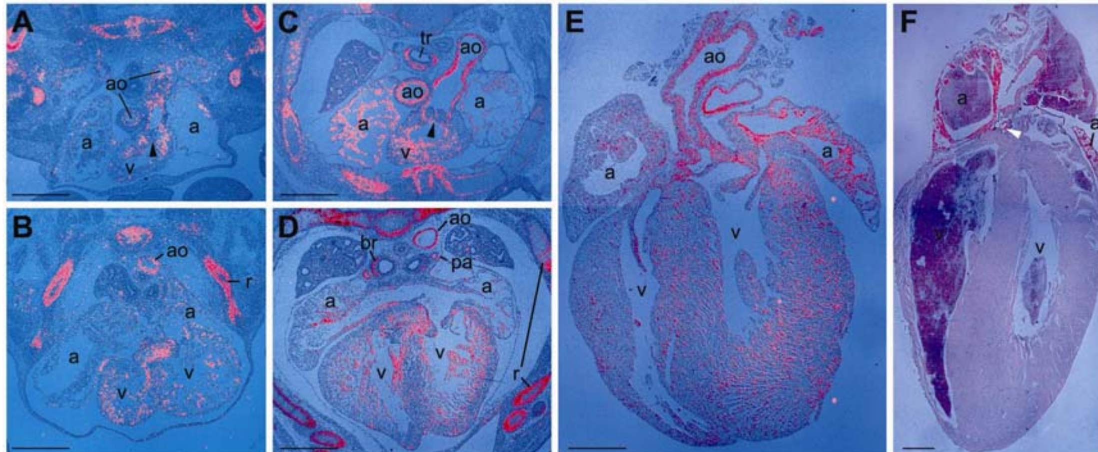

Fig. 3. CTGF mRNA expression during late heart development (E12.5-adult). Transverse sections through E12.5 embryos at the level of the atria (A) and ventricles (B), E14.5 embryos at the level of the atria (C) and ventricles (D) and frontal sections through an isolated newborn (E) and adult (F) heart. Black arrowhead in A and C indicates the pulmonary valve and white arrowhead in F indicates a large blood vessel in the adult heart. Abbreviations: a, atrium; ao, aorta; br, main branchus; pa, pulmonary artery; r, precartilage condensation of the ribs; tr, trachea; v, ventricle. Scale bar: 0.5 mm.

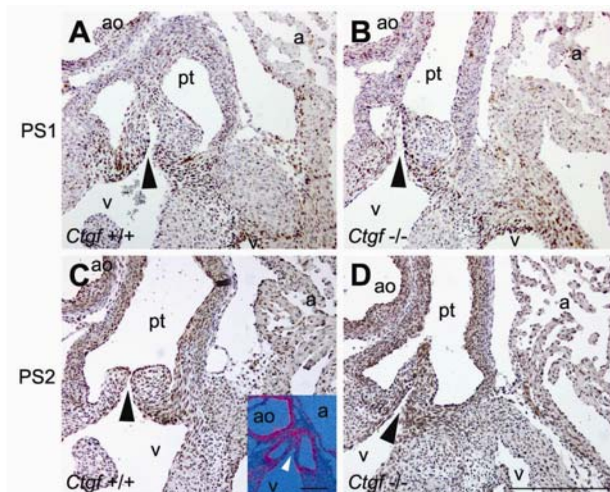

Fig. 4. CTGF does not regulate TGFβ or BMP signaling in the heart at E16.5. Detail of hearts isolated from E16.5 Ctgf deficient embryos (B,D) and wildtype littermates (A,B) immunostained for anti-PSmad1/5/8 (PS1, A-B) and PSmad2 (PS2, C-D), showing the pulmonary valve (black arrowhead) and pulmonary trunk. Insert in C shows expression of CTGF mRNA in the same area at E16.5 and white arrowhead indicates the pulmonary valve. Abbreviations: a, left atrium; ao, aorta; pt, pulmonary trunk; v, ventricle. Scale bar: 0.25 mm.

Interestingly, analysis postnatally revealed that although expression in newborn hearts was similar to that in embryos, *i.e.* patchy but ubiquitous (Fig. 3E), in adult hearts CTGF mRNA was restricted to the atria and had been entirely excluded from both myocytes and fibroblasts in the ventricles (Fig. 3F). However, CTGF expression remained in the large blood vessels of the ventricles.

No effect of *Ctgf* deficiency on BMP/TGFβ signaling in the embryonic heart

BMP/TGFβ signaling is known to regulate CTGF expression *in vitro* but the converse has also been reported: CTGF modulates both BMP(4) and TGFβ(1) signaling [4]. BMP and TGFβ signaling play important roles in heart development after septation, in particular in the development of the heart valves [29,30,31,32]. We observed strikingly high levels of CTGF mRNA in all heart valves at E16.5 (insert in Fig. 4C and results not shown), and therefore investigated whether CTGF regulates BMP/TGFβ signaling there after septation. Hearts of E16.5 *Ctgf* deficient embryos

were analyzed for the expression of the activated form (C-terminal phosphorylated) of BMP and TGFβ R-Smads, using anti-PSmad1/5/8 (Fig. 4A,B) and anti-PSmad2 (Fig. 4C,D), respectively. We detected neither morphological defects nor alterations in (nuclear) PSmad staining compared with wildtype littermates. Moderate BMP signaling and TGFβ signaling were detected throughout the heart both in the endocardium and myocardium. As expected, there were many PSmad1/5/8-positive and PSmad2-positive cells in all heart valves. Furthermore, PSmad2 staining was prominent in the aortic smooth muscle and endothelial cells and in the blood vessels embedded in the myocardium.

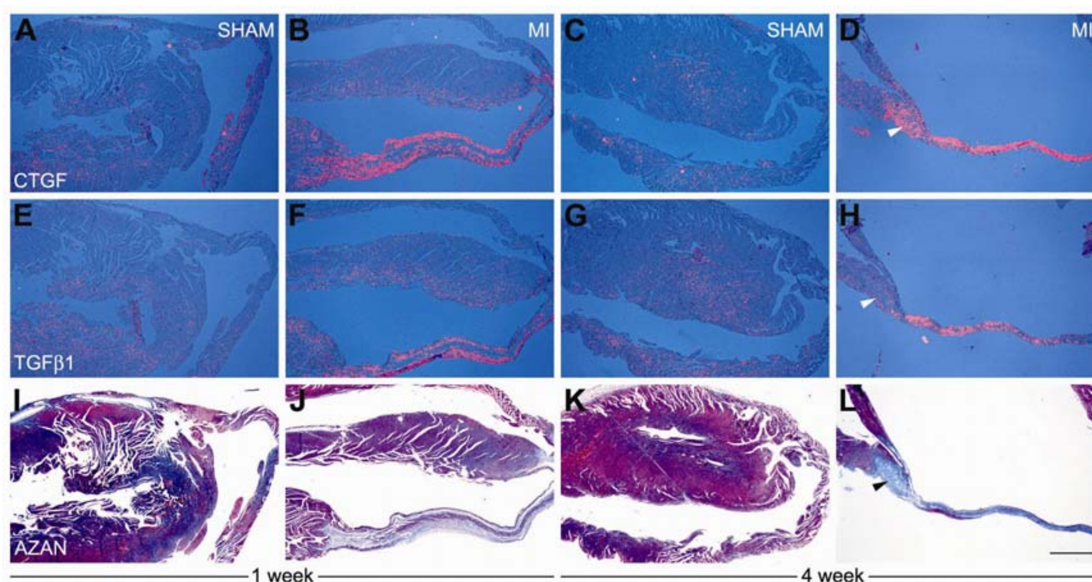

Fig. 5. Upregulation of CTGF and TGFβ in the scar tissue of the heart after experimentally induced MI. Expression of CTGF mRNA was low throughout sham-operated hearts after 1 (A) and 4 weeks (B), post- MI, CTGF mRNA expression was highly upregulated in the scar tissue after 1 (C) and 4 weeks (D). TGFβ 1 mRNA expression was also low or absent throughout sham-operated hearts after 1 (E) and 4 weeks (F), but highly upregulated in the scar tissue both 1 (C) or 4 weeks (D) post-MI. The borders of the scar after 4 weeks post-MI (white and black arrowhead) exhibited strong CTGF expression, but low TGF 1 levels. Consecutive heart sections were used for Azan-Mallory staining (I-L), showing the infarcted area (blue) and viable tissue (red/magenta) in hearts after 1 and 4 weeks post-MI and sham-operated. Scale bar: 1 mm.

CTGF and TGFβ are upregulated in the scar tissue post-MI in adult mice

Both CTGF and TGFβ1 are associated with fibrosis in several pathological conditions. However, the relationship between CTGF and TGFβ1 during the onset and progression of fibrosis in the heart post-MI is still unclear. To clarify this, we examined their mRNA expression 1 week and 4 weeks post-MI. Both CTGF and TGFβ1 mRNA were absent or expressed at low levels in the ventricles of sham-operated hearts after 1 and 4 weeks of recovery (Fig. 5A,C,E,G), but were strikingly upregulated 1 week post-MI in the scar tissue (Fig. 5B,F). Furthermore, whereas TGFβ1 mRNA was only detected in the fibroblasts of the scar tissue, CTGF mRNA was present both in fibroblasts and in the thin layer of surviving myocytes that contacted the lumen of the left ventricle. Cells of the myocardial septum close to the lumen of the left ventricle also contained low levels of both transcripts. 4 weeks post-MI, high levels of CTGF and TGFβ1 mRNA were still observed in the scar, whereas CTGF mRNA only was detected in fibroblasts in the border of the scar tissue (Fig. 5D,H). Together, these data indicate that CTGF mRNA is slightly more widespread than TGFβ1 mRNA and more importantly that both transcripts are produced by the scar post-MI largely by fibroblasts.

We tested whether TGFβ signaled to fibroblasts or myocytes using an anti-PSmad2 antibody and observed that 1 week post-MI TGFβ signaling took place in the majority of the fibroblasts of the scar (fibronectin positive, Troponin I negative),

but not in the few remaining myocytes (fibronectin negative, Troponin I positive) that directly contacted the lumen of the left ventricle (Fig. 6). PSmad2-positive cells were also observed at moderate levels scattered throughout the viable myocardium (Fig. 6) even in sham-operated hearts (data not shown), indicating that low levels of TGFβ signaling are present continuously in the myocardium, but 1 week after infarction robust increases in both production of TGFβ(1) and TGFβ signaling are observed exclusively in the fibroblasts of the scar tissue. Analysis of TGFβ Smad-dependent signaling 4 weeks post-MI surprisingly showed that PSmad2-staining was no longer in the fibroblasts of the scar tissue (Fig. 6). This suggests a dynamic and transient role for TGFβ Smad-dependent signaling during heart remodeling following MI.

We also studied BMP signaling by means of PSmad1/5/8 expression 1 and 4 weeks post-MI (Fig 6), but only low numbers of PSmad1/5/8-positive cells were detected both in the scar tissue and viable myocardium, suggesting that BMP signaling does not play a role remodeling the heart after MI.

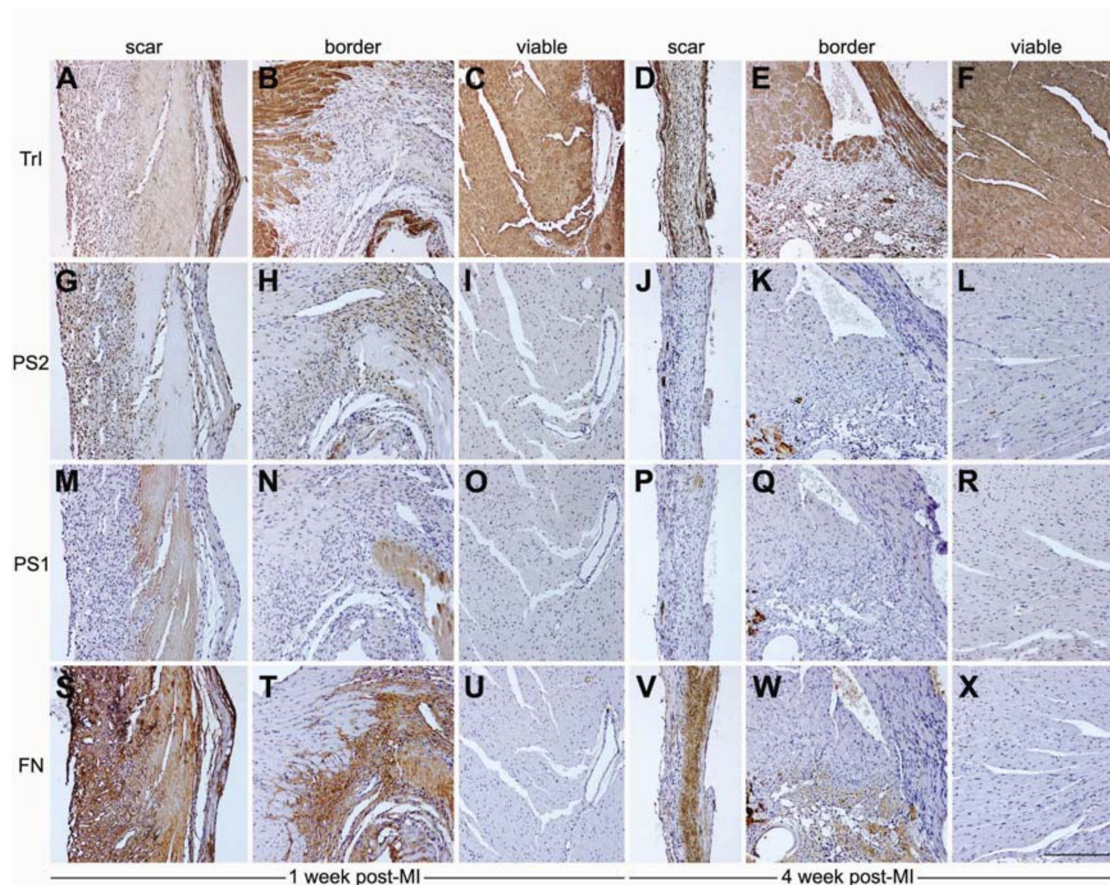

Fig. 6. TGFβ and BMP signaling in the heart, 1 and 4 weeks after experimentally induced MI. Immunostaining for Troponin I (TrI) showed the viable cardiomyocytes present in the scar (A,D), border (B,E) and viable (C,F) part of the heart, 1 week and 4 weeks post-MI. Immunostaining for PSmad2 (PS2) showed TGFβ (Smad-dependent) signaling in the majority of the fibroblasts in the scar and border 1 week post-MI (G,H). However, PS2 positive cells were also scattered through the population of cardiomyocytes in the viable part of the heart (H,I). 4 weeks post-MI, only few cardiomyocytes showed PS2 staining and the majority of the fibroblasts in the scar were negative for PS2. Immunostaining for PSmad1/5/8 (PS1) showed BMP (Smad-dependent) signaling in both cardiomyocytes and fibroblasts throughout the heart, with no preferential localization either 1 or 4 weeks post-MI. Immunostaining for fibronectin (FN) indicated fibrotic regions (scar and border) of the heart, 1 and 4 weeks post-MI (S-X). Scale bar: 0.1 mm.

Discussion

CTGF during heart development and adulthood

In agreement with high CTGF expression in developing bone and blood vessels, analysis of *Ctgf* mutant embryos revealed that CTGF is indeed important

during skeletogenesis and angiogenesis [8]. CTGF is also highly expressed in the developing heart, however we were not able to detect morphological defects in the hearts of *Ctgf* deficient embryos, probably due to functional redundancy there with other CCN family members. Although expression during heart valve development is dynamic and may be related to matrix deposition, cell migration or proliferation, CTGF expression seems not to be necessary for normal valvulogenesis in contrast to TGFβ and BMP signaling.

Postnatally, CTGF mRNA expression in the heart becomes restricted to the atria and large epicardial blood vessels. This adds CTGF to the list of chamber-specific genes [reviewed by 33] and implies a tight regulation of expression in the heart immediately after birth. Interestingly, levels of PSmad2 were low/absent throughout the adult heart (data not shown) and therefore basal CTGF levels in the atria are TGFβ Smad2-independent. CTGF mRNA was expressed in developing blood vessels and also in large blood vessels of the adult heart, suggesting that it may be involved in maintenance of blood vessel integrity during adulthood.

CTGF and TGFβ/BMP do not interact during (heart) development

Although *Ctgf* deficiency in mice is not embryonic lethal [8], it is interesting to note that this gene is highly expressed at very restricted sites, for example at E9.5 in the lamina terminalis, the olfactory placode or the outer layers of both branchial arches, suggesting that there is a tight gene regulation during development.

To address whether TGFβ/BMP signaling modulated CTGF expression *in vivo*, we examined CTGF mRNA expression in embryos lacking TGFβ signaling via ALK5 or BMP4 signaling, both embryonic lethal around E10, half way through gestation. CTGF mRNA expression in the heart was altered in both mutants but in a surprisingly similar manner that appeared to depend only on their degree of retardation. This suggested that it was a feature of the phenotype rather than the particular signaling pathway affected. I*n vitro*, hypoxia is known to stimulate CTGF production [34,35] and, indeed, yolk sac defects result in impaired development, including characteristic heart defects and eventual death under hypoxic conditions [18,28]. This would seem the most likely explanation of the effects observed. However, we cannot exclude that TGFβ/BMP signaling may modulate CTGF expression later during development (after E9.5), but this could not be addressed using *Alk5* or *Bmp4* conventional knockout.

CTGF mRNA expression was prominent throughout the heart at E16.5, however we were not able to detect differences in TGFβ or BMP signaling in E16.5 *Ctgf* deficient hearts. Therefore, this study provides no evidence for direct interaction between CTGF, on the one hand, and TGFβ or BMP Smad-dependent signaling on the other during development, in contrast to reports *in vitro* and a single study *in vivo* showing that CTGF induced anti-BMP phenotypes when injected in *Xenopus* embryos [3,4].

Possible interaction between CTGF and TGFβ, but not BMP, in scar fibroblasts after experimentally induced MI

Heart remodeling post-MI is a complex process involving multiple factors and pathways; but although extensively studied, particularly using the rat as an animal model, it is still incompletely understood [36,37]. In rats, CTGF and TGFβ have both been detected in the scar tissue, 1 and 4 weeks post-MI respectively but only in independent studies [38,39]. Furthermore, treating either fibroblasts or myocytes isolated from newborn rat hearts with TGFβ increased CTGF expression [27].

We examined for the first time co-expression of CTGF and TGFβ1 mRNA and activation of downstream Smads in mice 1 and 4 weeks post-MI and observed simultaneous upregulation and Smad2 phosphorylation in the scar tissue. There, the two ligands co-localized with fibronectin and are therefore clearly related to the development of fibrosis post-MI in mice. Interestingly, at least in rats, a short TGFβ1

mRNA transcript of 1.9kb rather than the usual 2.4kb transcript is selectively upregulated post-MI, although it encodes the same protein [40]. This shorter transcript is also detected in undifferentiated mouse embryonic stem cells, but is rapidly downregulated after differentiation [41], and is generally regarded as an embryonic isoform. It is thus one of several examples of so-called "embryonic genes" that are upregulated post-MI [42,43]. The combined studies described here on the expression of CTGF in development, adulthood and post-MI indicates that CTGF also falls into this category.

The restriction of PSmad2 staining to the fibroblasts of the scar suggests that CTGF mRNA in surviving myocytes contacting the lumen of the left ventricle (and in the atria of normal adult hearts) is not regulated by TGFβ Smad2-dependent signaling, but that CTGF expressed in the scar fibroblasts probably is. This is not unexpected given that it is known that CTGF expression may be regulated by TGFβ Smad-independent pathways and that there may be cell type specific factors that determine the cellular response [13,14,44]. On the other hand, CTGF could bind TGFβ1, positively modulating TGFβ Smad-dependent signaling in the scar fibroblasts.

Subcutaneous injection of both CTGF and TGFβ into newborn mice resulted in persistent fibrosis, while the effect of TGFβ alone was transient [45]. Moreover, mice overexpressing TGFβ1 specifically in cardiomyocytes developed atrial but not ventricular fibrosis, suggesting that TGFβ1 activity by itself is not sufficient to induce persistent ventricular fibrosis [46] and that co-expression of CTGF, upregulated by a TGFβ independent mechanism is also required. Such a mechanism could be activated by MI and as we show here, CTGF expression and the fibrotic response in fibroblasts are persistent whilst TGFβ-mediated signaling via Smads is only transient, returning to background levels 4 weeks post-MI. Whilst the molecular basis for this remains to be elucidated, it seems from the data in the mutant embryos, that a simple mechanism whereby CTGF is downstream of TGFβ (or BMP) is unlikely. Furthermore, co-expression of CTGF and TGFβ to induce persistent fibrosis post-MI may be required. We report upregulation of CTGF under hypoxic conditions either during abnormal development (defects in yolk sac development) or post-MI (left coronary artery ligation) and suggest that intervening at the level of CTGF may be sufficient to control situations of persistent but undesirable fibrosis.

Acknowledgments

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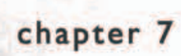

discussion

The purpose of this thesis was to analyse several aspects of cell lineage allocation, differentiation, growth and migration during early mouse development in particular the roles of TGFβ/BMP signalling pathway in these processes. The primary focus was on the behaviour of primordial germ cells. However, as is inevitable when approaching the study of development from the point of view of a specific molecular signalling pathway, rather than a specific process, we also observed novel roles for TGFβ/BMP signalling and the target genes, CTGF and β1 integrin in skeletal and cardiac muscle development as well as during placental development.

BMP signalling and the formation of PGCs

The BMP signalling pathway has been associated with the formation of PGCs, since Lawson and colleagues (1999) observed that *Bmp4* deficient embryos formed no PGCs. To date, other members of the BMP ligand family have been shown to be involved in this process and this remains the only pathway associated with the instruction of epiblast cells to a PGC fate. In the model originally proposed, it was though that the BMPs signalled directly from the extraembryonic ectoderm, where both BMP4 and BMP8b are produced, to the most proximal cells of the epiblast. These cells would later, during gastrulation, give rise to both extraembryonic mesoderm and PGCs. We demonstrated not only that ALK2, a BMP type I receptor is necessary for the formation of PGCs, but that it functions in the visceral endoderm. Therefore, in addition to a possible direct BMP signal to the proximal epiblast, there is clearly an indirect BMP signal via de visceral endoderm (and ALK2) to instruct the proximal layer of epiblast cells to a PGC (and possibly extraembryonic mesodermal) fate. Although, BMP2 is produced in the visceral endoderm, this tissue was until now not regarded as being required for PGC formation. This resulted mainly from the fact that most groups investigating PGC development cultured embryonic explants on a layer of feeder cells, such as STO cells. Under this experimental condition, E6.5 proximal epiblast cells are able to generate PGCs in culture in the absence of both extraembryonic ectoderm and visceral endoderm. However, STO cells produce survival (Dolci et al., 1991; Godin et al., 1991) and inducing factors and are therefore in co-culture not a good system to investigate signals important for the development of PGCs.

In a feeder-free system, it is clear that the presence of the visceral endoderm is necessary, in addition to the extraembryonic ectoderm, to instruct epiblast cells to a PGC fate. Moreover, we were able to demonstrate that only the proximal visceral endoderm adjacent to the extraembryonic ectoderm and proximal epiblast (and not the distal visceral endoderm) has the ability to induce epiblast cells to a PGC fate, if provided with an external source of BMP4 (exogenous or produced by the extraembryonic ectoderm). These results strongly suggest that BMP8b is in fact not necessary for the formation of PGCs, in contrast to reports suggesting that BMP4 and BMP8b, both segregated by the extraembryonic ectoderm were needed to induce PGC formation in distal epiblast cultured on feeder cells (Ying et al., 2001). Although we were able to demonstrate that signalling via ALK2 is necessary for the formation of PGCs in the visceral endoderm, we cannot exclude that ALK2 may function in parallel in the proximal epiblast cells. To test this possibility, the development of PGCs should be analysed in chimeric embryos generated from *Alk2* deficient recipient blastocysts and wildtype embryonic stem cells. The resulting (100% chimeric) embryos would be formed by *Alk2* deficient visceral endoderm and extraembryonic ectoderm, but wildtype epiblast. Absence of PGCs in those chimeric embryos would indicate that ALK2 acted exclusively on the visceral endoderm.

TGFβ signalling, β1 integrin and the migration of PGCs

Two mechanisms have been proposed to regulate PGC migration to the gonadal ridges: chemotaxis and an ECM gradient. From *in vitro* studies, it has been suggested that TGFβ signalling regulates the migration of PGCs towards the gonadal

ridges and that TGFβ1 produced by the gonadal ridges would function as a chemoattractent (Godin and Wylie, 1991). However, we found no evidence that TGFβ signalling regulates the migration of PGCs to the genital ridges or proliferation of PGCs during this period *in vivo*, although on the basis of morphology, PGCs (as well as surrounding somatic cells) did exhibit active TGFβ Smad-dependent signalling. To show direct evidence that these cells were indeed PGCs, CAGA:*gfp* reporter embryos were analyzed. However, the levels of the reporter were probably below detection limit and therefore either there is no TGFβ signal present in the embryo (and visceral yolk sac) during the first half of gestation or the reporter is not reflecting TGFβ signalling faithfully. Ablation of TGFβ1, TβRII and ALK5 clearly indicate a function before E10.5, at least in the vascularization of the visceral yolk sac (Dickson et al., 1995; Oshima et al., 1999; Larsson et al., 2001) and nuclear PSmad2 was detected by us in many tissues at E10.5, including the visceral yolk sac, confirming the lack of sensitivity of the CAGA:*gfp* transgenic mice in early development. New CAGA reporter transgenic lines have been generated in our group, this time using the more sensitive β-galactosidase as a read-out (Bouwman et al., unpublished results). We also analysed embryos with a conditional deletion of TβRII which should have been exclusively in the PGCs. However, the promoter of the tissue non-specific alkaline phosphatase (TNAP) used for the Cre-recombinase was not as specific as described (Lomelí et al., 2000) and Cre expression was rather mosaic and ubiquitous. Although we found no evidence that TGFβ Smad-independent signalling controls cell shape changes occurring as the PGCs arrive to the gonadal ridges, the putative role of TGFβ Smad-dependent signalling controlling mitotic arrest of the PGCs in the gonadal ridges is worth investigating further,

β1 integrin has two isoforms in the mouse, β1A and β1D. PGCs express β1A, but not β1D. Therefore, the failure of *β1 integrin* deficient PGCs to colonize the gonadal ridges of chimeric wildtype/β1 integrin embryos efficiently (Anderson et al., 1999) is probably exclusively due to the lack of the β1A isoform. Replacement of β1A by β1D resulted in neural crest migration abnormalities on a mixed Ola/FVB genetic background (Baudoin et al., 1998). We tested whether replacement of β1A by β1D would have an effect on the migration of PGCs. However, unfortunately the β1D knock-in mice with a mixed Ola/FVB genetic background were no longer available and the β1D knock-in mice analysed had a mainly FVB genetic background. These mice showed a less penetrant abnormal phenotype with normal neural crest and PGC migration and some mice in fact survived to birth. This illustrates the importance of the genetic background for the characterization of an abnormal phenotype associated with the ablation of a specific gene. However, the reduced penetration of the phenotype on the FVB background revealed a novel role of the β1D isoform in skeletal muscle and placental development (see below).

TGFβ signalling and β1 integrin can interact at several levels. Although we did not investigate a direct relationship between them, we can speculate that TGFβ, in addition to direct signalling to the migrating PGCs (with still unclear function), may also control ECM deposition in the somatic cells surrounding the developing gonadal ridges contributing to the formation of an ECM gradient, which would facilitate β1 integrin mediated homing to the gonadal ridges.

TGFβ/BMP signalling, β1 integrin, CTGF and development of the heart

BMP/TGFβ signalling is thought to be important in valve formation during heart development (Kim et al., 2001; Bartram et al., 2001; Gaussin et al., 2002; Jiao et al., 2003), while BMP signalling also plays a role during the induction of the heart fields during gastrulation (Chuva de Sousa Lopes et al., unpublished results). CTGF is strongly expressed in the developing heart, in particular underneath the heart aortic and pulmonary valve leaflets. Moreover, *in vitro* CTGF has been demonstrated to regulate the BMP/TGFβ signalling (Abreu et al., 2002). We analysed the activation of the BMP/TGFβ (Smad dependent) signalling pathway during valvulogenesis in

Ctgf deficient embryos, but found no alteration in signalling activation compared to that in wildtype littermates. Therefore, if CTGF functions as an accessory receptor *in vivo*, positively mediating TGFβ signalling and negatively regulating BMP signalling, it seems not to interact significantly with either pathway during heart development. Although CTGF may promote the production of ECM in the heart, its function there is still unclear. On the other hand, we and others have shown that CTGF is an early target of both BMPs and TGFβs at least *in vitro* (Moussad and Brigstock, 2000; Nakanishi et al., 1997). Interestingly, in the absence *Alk5*, a TGFβ type I receptor, we observed a specific alteration of the CTGF expression in the heart, suggesting initially that CTGF is a direct target of TGFβ *in vivo*. However, deficiency in *Bmp4* induced similar alterations in the CTGF expression pattern, although TGFβ (via ALK5) and BMP(4) use completely different signalling pathways. Both, *Alk5* and *Bmp4* deficient embryos exhibit prominent but clearly different defects in visceral yolk sac formation. We propose that the abnormal CTGF expression pattern observed in the mutant hearts is not the result of direct regulation by the TGFβ signalling pathway as initially thought, but the indirect result of hypoxia in the mutant embryos. At E8.5, the embryo does not produce its own "embryonic" blood, but is entirely dependent on "extraembryonic" blood (with primitive nucleated red blood cells) generated by the blood islands of the visceral yolk sac. The chorio-allantoic placenta has not yet been formed and therefore there is no close contact between the circulation of the mother and of the embryo self. The embryo depends on the so-called yolk sac placenta for nutrient and waste exchange which occurs by diffusion. It is therefore the development of a cardiovascular system around E8.5 that allows the embryo to growth in size and complexity. Abnormalities in the "extraembryonic" blood supply are usually related to abnormal heart development, one of the first organs to be functional during development, followed by embryonic lethality a couple of days later. In addition, we were surprised by the different patterns of CTGF expression observed in more retarded and less retarded mutants hearts and still have no explanation for it. We examined if the CTGF expression in the mutants was related to differences in cell proliferation, but observed no correlation (data not shown).

In adult mice, CTGF upregulation was observed independently of BMP/TGFβ (Smad dependent) signalling in the scar tissue of the left ventricle 4 weeks after experimentally induced myocardial infarction. This clearly suggests that although TGFβ/BMP signalling is able to induce CTGF, this is not the only mechanism that regulates CTGF transcription. We suggest that hypoxia, both during heart development and disease, also contributes to the regulation of CTGF transcription. Moreover, from our work and that of others it is becoming clear that TGFβ expression is not sufficient to cause persistent fibrosis in the heart, but that TGFβ-independent CTGF expression may also be required (Nakajima et al., 2000). Although, *Ctgf* deficient mice die perinatally from breathing problems caused by skeletal abnormalities (Ivkovic et al., 2003), it would be interesting to analyse heart remodelling and fibrosis development after experimentally induced myocardial infarction in *Ctgf* heterozygous adult mice.

β1 integrin is expressed during development in the heart: β1A is detected during early embryogenesis, but at E11 both β1D and β1A are colocalized in the heart (Brancaccio et al., 1998; van der Flier et al., 1997). Thereafter, expression of β1A, declines while β1D increases to become the predominant β1 isoform expressed in adult cardiac myocytes. Analysis of chimeric wildtype/β1 integrin embryos, showed that *β1 integrin* deficient cardiac cells exhibit abnormal sarcomeres, and progressively deteriorate and die during adult life as the heart has contracted for a extensive period of time (Fässler et al., 1996). Surprisingly, deletion of the β1D isoform resulted only in mild heart defects during adulthood (Baudoin et al., 1998). In addition, the replacement of β1A for β1D caused no gross morphological abnormalities in the heart throughout development, suggesting that the precocious and exclusive expression of β1D in fact supported normal heart development.

Nevertheless, the upregulation of the β1D isoform is associated with mitotic arrest and differentiation of myoblasts (Belkin and Retta, 1998), therefore it would have been interesting to look in more detail at the expression of functional and morphological molecular markers to confirm that indeed β1D knock-in embryos developed normal hearts.

β1 integrin and development of skeletal muscle and placenta

The replacement of β1A for β1D resulted in the formation of fewer, thinner and shorter primary myofibres. In addition, the number of secondary myofibres was also reduced, but proportionally reduced to the number of primary myofibres, suggesting that this is not the direct effect from the exclusive expression of β1D in the muscle cells, but results from the fact that there are less primary myofibres to use as a scaffold during secondary myogenesis. In chimeric wildtype/β1 integrin embryos, *β1D integrin* deficient myoblasts were able to withdraw from the cell cycle, differentiate and fuse with wildtype myoblasts (Fässler and Meyer, 1995) and more importantly *β1D integrin* deficient myoblasts differentiated normally (Baudoin et al., 1998), suggesting that β1D integrin is not required for myogenesis. However, our data indicates that myogenesis occurs less efficiently in the exclusive presence of the β1D isoform.

Defective myogenesis was the cause of death of about one third of the β1D knock-in embryos late during gestation, however two thirds of the mutants died during mid-gestation due to defects in the development of the placenta, in particular the vascularization of the labyrinth layer. The observed abnormalities could result from a direct defect in blood vessel formation or the differentiation of chorionic trophoblasts. Interestingly, the chorionic trophoblasts differentiate into the multinucleated syncytiotrophoblasts that function as blood circulation network for the maternal blood in the labyrinth layer of the placenta. We hypothesise that there may be a common developmental defect in both the development of myotubes and syncytiotrophoblasts in particular involving fusion, resulting from the replacement of β1A by β1D. During peri-implantation, β1A is the only isoform expressed in the tissues that will give rise to the placenta (Klaffky et al., 2001), however expression of the β1 isoforms was not analysed later during labyrinth development. It would be interesting to investigate whether β1D is upregulated and involved in the maturation (fusion) phase of syncytiotrophoblasts, similarly to its function during primary myogenesis.

Final remarks

Although we focused mainly on the role of TGFβ/BMP signalling, β1 integrin and CTGF during the development of the PGCs, heart, skeletal muscle and placenta in the mouse embryo, these are by no means the only pathways or the most important factors regulating the development of these organs/tissues. For a comprehensive analysis of the molecular components involved during embryonic development of each specific organ/tissue, we refer to the reviews mentioned in Chapter 1. Secondly, we would like to emphasise that not only does TGFβ interact with CTGF and β1 integrin, but CTGF and β1 integrin are also known to interact with each other (Brigstock, 2003 and references therein; Weston et al., 2003) and although beyond the scope of this thesis to cover the relevant literature, all three factors are angiogenic modulators, clearly involved in the development of fibrotic disorders and tumorigenesis (Moussad and Brigstock, 2000; Derynck et al., 2001; Wakefield and Roberts, 2002; Wayne and Noble, 1994; Planque and Perbal; 2003; Brigstock, 2003; Brakebusch et al., 1997). Further research on their interactions and the analysis of conditional mouse mutants, revealing tissue specific functions during development and disease may lead to new perspectives approaching several human diseases, including fibrotic disorders and cancer.

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We characterized the expression of **PSmad1/5/8 (BMP R-Smads)** and **PSmad2 (TGFβ R-Smad)** during peri-implantation and gastrulation in the mouse, and further characterized the expression of PSmad2 until E12.5, showing were the BMP and TGFβ Smad-dependent signalling pathways are active (Chapter 2, 3 and 4).

The role of **ALK2 (BMP signalling)** and **ALK5 (TGFβ signalling)** in the development of PGCs in the mouse was studied in detail. ALK2 is needed for the **formation of PGCs** before gastrulation occurs and it signals in the **visceral endoderm**, a tissue that was previously not associated with primordial germ cell formation (Chapter 3). In contrast to suggestions derived from studies *in vitro*, ALK5 is probably not involved in PGC migration through the hindgut and to the gonadal ridges and TGFβ1 is unlikely to be a chemoattractant for PGCs as proposed (Chapter 4). However, there may be a role for TGFβ signalling in PGC development in controlling mitotic arrest of PGCs in the gonadal ridges.

β1 integrin was also implicated in PGC migration to the genital ridges. β1 integrin has two isoforms in mice: a more common one β1A and a muscle specific β1D that is upregulated late during development. We studied whether the replacement of β1A by β1D influenced the **development of PGCs**, but this occurred normally and we found no evidence of an effect on migration on the genetic background used. Furthermore, β1 integrin has been implicated in **heart development**. We showed that the replacement of β1A by β1D did not cause heart abnormalities during development. However, we were able to show a clear role for β1A during **myogenesis**. The differentiation of primary myoblasts was abnormal when only β1D was expressed, while secondary myoblasts were formed normally. Furthermore, we showed a novel role of β1 integrin during **placentation**, in particular the development of the labyrinthine layer (Chapter 5).

CTGF is known to crosstalk with both TGFβ and BMP *in vitro*. We studied possible interactions between them, focusing on **heart development**, where BMP and TGFβ are known to play prominent roles. However, we were not able to detect direct crosstalk. Both CTGF and TGFβ are associated with fibrosis. We investigated whether CTGF and TGFβ were expressed following experimental induction of **myocardial infarction** in the mouse and showed that both genes are present in particular in the scar tissue and that TGFβ Smad-dependent signalling is transient 1 week post-infarction and largely restricted to cardiac fibroblasts (Chapter 6).

In dit proefschrift is de expressie van **PSmad1 (BMP R-Smads)** en **PSmad2 (TGFβ R-Smad)** gedurende de implantatie en gastrulatie gekarakteriseerd in de muis. Het expressiepatroon van PSmad2 gedurende de embryonale ontwikkeling is verder bestudeerd tot en met E12.5. Deze expressiestudies laten zien waar TGFβ en BMP Smad-afhankelijke signaaltransductiepaden actief zijn (Hoofdstuk 2,3 and 4).

De rol van **ALK2 (BMP signaaltransductie)** en **ALK5 (TGFβ signaaltransductie)** in de ontwikkeling van primordiale kiemcellen (PGC's) in muizenembryo's werd in detail bestudeerd. Voordat de gastrulatie optreedt is ALK2 noodzakelijk voor de **vorming van PGC's**. ALK2 is functioneel in het **viscerale endoderm**, een cellaag die voorheen niet geassocieerd was met de vorming van PGC's (Hoofdstuk 3). In tegenstelling tot resultaten die verkregen zijn uit *in vitro* experimenten vonden wij dat ALK5 waarschijnlijk niet betrokken is bij de migratie van PGC's door de achterdarm naar de voorlopers van de gonaden en dat TGFβ dus geen *chemo-attractor* is voor PGC's (Hoofdstuk 4). Een rol voor TGFβ in de aanzet van het mitotische arrest dat optreedt zodra de PGC's aangekomen zijn in de voorlopers van de gonaden kan echter niet uitgesloten worden.

β1 integrine werd ook geacht een rol te spelen in de migratie van de PGC's naar de voorlopers van de gonaden. β1 integrine heeft twee iso-vormen in de muis: een algemene 1A en een spierspecifieke 1D, waarvan de expressie laat in de ontwikkeling verhoogd is. In het proefschrift is bestudeerd of de vervanging van β1 integrine 1A door β1 integrine 1D de **ontwikkeling van PGC's** zou beïnvloeden, maar er werd gevonden dat in dat geval de PGC's normaal ontwikkelen. β1 integrine is ook geassocieerd met de **ontwikkeling van het hart**, maar wij toonden aan dat de vervanging van 1A door 1D geen hartafwijkingen veroorzaakte gedurende de ontwikkeling. Wèl werd een duidelijke rol voor 1D gedurende de **ontwikkeling van spieren** gevonden. De differentiatie van primaire myoblasten was abnormaal indien alleen 1D werd geëxpresseerd terwijl secundaire myoblasten normaal werden gevormd. Ook hebben we een tot nu toe onbekende rol van β1 integrine gedurende de **placentatie** ontdekt, in het bijzonder in de vorming van de labyrinthine laag (Hoofdstuk 5).

Van **CTGF** is bekend dat het betrokken is bij de signaaltransductiepaden van TGFβ's en BMP's. Wij bestudeerden mogelijk interactie tussen CTGF en deze signaaltransductiepaden, met daarbij een specifieke aandacht voor **hartontwikkeling**, omdat zowel TGFβ's als BMP's daar belangrijk voor zijn. Een directe interactie werd echter niet gevonden. Zowel CTGF als TGFβ zijn betrokken bij de ontwikkeling van fibrose en onderzocht werd of CTGF en TGFβ betrokken zijn bij de ontwikkeling van fibrose na de experimentele inductie van een **infarct van het myocard** in de muis. Er werd aangetoond dat expressie van beide genen aanwezig was, in het bijzonder in littekenweefsel. Ook werd aangetoond dat TGFβ Smad-afhankelijke signalering tijdelijk aanwezig is 1 week na het infarct (Hoofdstuk 6).

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