

Ventral embryonic tissues and Hedgehog proteins induce early AGM hematopoietic stem cell development

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Hematopoiesis is initiated in several distinct tissues in the mouse conceptus. The aorta-gonad-mesonephros (AGM) region is of particular interest, as it autonomously generates the first adult type hematopoietic stem cells (HSCs). The ventral position of hematopoietic clusters closely associated with the aorta of most vertebrate embryos suggests a polarity in the specification of AGM HSCs. Since positional information plays an important role in the embryonic development of several tissue systems, we tested whether AGM HSC induction is influenced by the surrounding dorsal and ventral tissues. Our explant culture results at early and late embryonic day 10 show that ventral tissues induce and increase AGM HSC activity, whereas dorsal tissues decrease it. Chimeric explant cultures with genetically distinguishable AGM and ventral tissues show that the increase in HSC activity is not from ventral tissue-derived HSCs, precursors or primordial germ cells (as was previously suggested). Rather, it is due to instructive signaling from ventral tissues. Furthermore, we identify Hedgehog protein(s) as an HSC inducing signal.

KEY WORDS: AGM, Hematopoiesis, Hedgehog, Gut, Mouse embryo, Positional information

INTRODUCTION

Patterning of the vertebrate embryo occurs through the complex interactions between concomitantly developing adjacent tissues (Rossant and Tam, 2004; Tam and Zhou, 1996). The hematopoietic system, although not generally thought of as a tissue that undergoes patterning, does develop in several distinct anatomical positions (Dzierzak and Speck, 2008), and is likely to be under the influences of the different surrounding tissues (Baron, 2005). The early hematopoietic tissues, the yolk sac, aorta-gonad-mesonephros (AGM) and chorioallantoic placenta, differ in their position in the conceptus and in morphologic complexity (Dzierzak and Speck, 2008). The yolk sac, consisting of extra-embryonic mesoderm and visceral endoderm, is the first hematopoietic tissue of the conceptus (Moore and Metcalf, 1970). Rapidly after gastrulation, this simple bilaminar tissue gives rise to the primitive hematopoietic system. Slightly later, definitive hematopoiesis and the generation of the first adult type hematopoietic stem cells (HSCs) takes place in the para-aortic splanchnopleura (PAS)/AGM region (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). This intra-embryonic tissue is more complex in its origins, being formed from the splanchnopleural mesoderm in close association with endoderm that will form the mid- and hindgut and the epithelium of the coelomic cavity. Dorsally, the splanchnopleural mesoderm of the developing PAS/AGM region faces the somitic mesoderm, and dorsal to the aorta are the axial structures, the notochord and neural tube. In the chick embryo, all the endothelial cells lining the walls of the paired aortae initially develop from splanchnopleural mesoderm (Pardanaud et al., 1996). As the paired aortae fuse, somitic mesoderm contributes to the dorsal endothelial wall of the aorta

(Pouget et al., 2006). In the mouse it is as yet unknown whether there is a contribution of somitic mesoderm to the mouse aorta/AGM but recent clonal marking experiments suggest some late contribution, at least to the mesenchymal cell populations ventral to the aorta (Esner et al., 2006). The chorioallantoic placenta may be the most complex of the early hematopoietic tissues (Alvarez-Silva et al., 2003; Corbel et al., 2007; Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006), being a chimeric tissue consisting of both fetal elements and maternally derived elements.

Intriguingly, within the aorta of the chick embryo there is a distinctive ventral polarity in the development of hematopoietic cell clusters along the luminal wall of the aorta (Dieterlen-Lievre and Martin, 1981). Clusters of hematopoietic cells are closely associated with the ventral aortic endothelium, and marking studies show that hematopoietic cells arise from this endothelium (Jaffredo et al., 1998). Aortic hematopoietic clusters have been described in many species, including mouse and human (Jaffredo et al., 2005). In the mouse, hematopoietic clusters appear at embryonic day 10 (E10) and the first adult-repopulating HSCs are autonomously generated in the aorta at E10.5 (>34 somite pairs) (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Although clusters are found both dorsally and ventrally, HSC activity has been localized exclusively to the ventral aspect of the mouse mid-gestation aorta, suggesting a strong positive ventral positional influence in the development of AGM hematopoiesis (Taoudi and Medvinsky, 2007). Indeed, required hematopoietic transcription factors are expressed in cells of the ventral clusters (Minegishi et al., 1999; North et al., 1999; North et al., 2002) and several developmental factors are expressed in the mesenchymal region underlying the ventral aorta (Durand et al., 2007; Marshall et al., 2000).

Accumulating evidence in several animal and cell models suggests that, for mesoderm to undergo hematopoietic development, the inductive influences of endoderm are required. For example, the formation of primitive erythroblasts in the chick yolk sac requires endodermal cells (Wilt, 1965). Avian somatopleural mesoderm was re-specified when briefly exposed to endoderm, exhibiting hematopoietic and vascular potential, much like splanchnopleural

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mesoderm that is normally in contact with endoderm (Pardanaud and Dieterlen-Lievre, 1999). In mouse embryo explant grafting studies, it was found that visceral endoderm can induce erythroid development in prospective neuroectoderm (Belaoussoff et al., 1998). Hedgehog proteins were shown to mimic the effect of endoderm in both experimental models and thus it was suggested that Hedgehog proteins act as the short-range, diffusible endodermal signal to induced endothelial and hematopoietic potential early in gastrulation (Dyer et al., 2001). Studies in zebrafish embryos show that Hedgehog is involved at three distinct stages in dorsal aorta and hematopoietic development (Gering and Patient, 2005). In avian studies several other factors, VEGF, bFGF and TGF β 1, were able to re-specify non-hematopoietic mesoderm to blood fate (Pardanaud and Dieterlen-Lievre, 1999). Interestingly, contact of splanchnopleural mesoderm with ectodermal tissue abolished its hematopoietic potential, and EGF and TGF α were found to mimic the negative effect of the ectoderm. These studies suggest an important role for dorsal and ventral positional information in hematopoietic induction. However, no inducing factor for HSCs has been identified in mammalian embryos.

In this study we test the hypothesis that tissues positioned dorsal or ventral to the AGM affect HSC induction. The HSC content of AGMs dissected with dorsal or ventral tissues was measured by the in vivo transplantation assay after organ culture. We show that ventral tissues have a positive influence on AGM HSC induction, whereas dorsal tissues are suppressive. Our data implicate Hedgehog protein(s) as one of the positive effectors in the induction and increase in AGM HSCs.

MATERIALS AND METHODS

Embryo generation

Animals were maintained and bred at the Erasmus Medical Center according to the institutional guidelines. Animal procedures were carried out in compliance with the Standards for Humane Care and Use of Laboratory Animals. Embryos were generated through timed matings between C57Bl/6 females and *CAG-EGFP* (Okabe et al., 1997) males; (C57Bl/10 \times CBA)F1 females and *Ln72* [hu β -globin transgenic (Strouboulis et al., 1992)] males; (C57Bl/10 \times CBA)F1 females and *Ly6a-GFP* (de Bruijn et al., 2002) males; (C57Bl/10 \times CBA)F1 females and *Ly6a-lacZ* (Miles et al., 1997) males; *Gli1-CreER^{T2}* (Ahn and Joyner, 2004) \times *R26R-lacZ* (Soriano, 1999) females and males; and (C57Bl/10 \times CBA)F1 females and males. The day of vaginal plug discovery was embryonic day 0. Pregnant dams were sacrificed and the developmental stage of embryos was determined by counting the number of somite pairs. Embryos with 31-34 somite pairs were grouped as early E10, and embryos with 35-39 somite pairs as late E10.

Tissue dissection, explant culture and cell preparation

The following tissues were dissected: AGM; gut (midgut, including the part of the vitelline artery, which is within the loop of the midgut); AGM:gut (AGM with midgut); and AGM:NT (AGM with neural tube, notochord and somite remnants just lateral to the neural tube).

Tissues were processed directly or after an explant culture of 3 days on permeable membrane filters in myeloid long-term medium (StemCell Technologies) with hydrocortisone (10^{-6} M, Sigma) (Dzierzak and de Bruijn, 2002). Medium was supplemented with 0, 2, 20 or 200 ng/ml Ihh or Shh (R&D Systems) or Hedgehog blocking antibody 5E1 (50 ng/ml) or IgG control. Tissues were treated with collagenase [0.12% w/v, type I (Sigma) in PBS/10% FCS] for 1 hour at 37°C and single cell-suspensions were prepared.

Chimeric reaggregate cultures were performed as follows: AGMs and guts of genetically marked embryos were dissected. Each AGM was then combined with a gut of a genetically differently marked embryo and a cell suspension was prepared. Each cell suspension (1 AGM and 1 gut) was

transferred as an individual droplet on a filter for culture. After 3 days the reaggregates were collected, and cell suspensions were made by collagenase treatment, pooled and used for in vivo transplantation assays.

In vivo transplantation assays

Progenitor assay (CFU-S₁₁)

After a 3-day explant culture, tissues (AGM, gut, AGM:gut and AGM:NT) were collected and cell suspensions were prepared. For AGM+gut_{mix}, separately cultured AGM and gut were combined and prepared as one cell-suspension. Cells were injected into lethally irradiated [10 Gy of γ -irradiation (¹³⁷Cs source)] recipients at a dose of 1-2 embryo equivalents (ee) per recipient. Each experiment contained one to three noninjected irradiation controls. Spleens were collected 11 days post-transplantation, fixed using Tellesniczky's fixative, and macroscopically visible colonies were counted (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). The number of colonies per ee was determined for each spleen. Spleens of recipients injected with a cell suspension of chimeric reaggregates were collected, and colonies were counted and checked with a fluorescent microscope for GFP expression. Colonies were excised, and DNA was extracted and checked for the presence of the GFP gene by PCR analysis.

HSC transplantation assay

After a 3-day explant culture of AGM, gut, AGM:gut and AGM:NT of *Ln72* \times (C57Bl/10 \times CBA)F1, *Ly6a-lacZ* \times (C57Bl/10 \times CBA)F1 or *Ly6a-GFP* \times (C57Bl/10 \times CBA)F1 embryos, tissues were collected and single cell-suspensions were prepared. Cells were injected into sublethally irradiated (9 Gy of γ -irradiation) (C57Bl/10 \times CBA)F1 recipients. Cells were injected at a dose of 2-2.5ee per recipient for early E10 and 1.2-1.8ee per recipient for late E10, and were coinjected with 2×10^5 spleen cells (recipient background). Repopulation was assayed at 4 months post-transplantation by donor-specific semi-quantitative PCR (*Ln72*, *GFP* or *lacZ*) on peripheral blood DNA. Similarly, recipients of the chimeric reaggregate cultures were analyzed for both donor markers. The percentage of donor chimerism was determined from ethidium bromide fluorescence intensity (ImageQuant) and calculated from a standard curve of DNA control dilutions (0, 1, 3, 6, 10, 30, 60 and 100% donor marker) (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Only recipients with >10% donor chimerism were considered repopulated. For multi-lineage repopulation analysis, DNA was isolated from peripheral blood, lymph node, spleen, thymus, bone marrow, or from FACS-sorted cells, and assayed for donor contribution by PCR. Secondary recipients received an irradiation dose of 9 Gy and were injected with 2×10^6 bone marrow cells from a primary recipient. Blood DNA was analyzed for donor contribution by PCR at 4 months post-transplantation.

Flow cytometric analysis

Single cell-suspensions were stained with labeled monoclonal antibodies anti-CD34 (PE-conjugated), anti-CD45 (PE-conjugated) and/or anti-c-Kit (APC-conjugated) (BD Pharmingen) in PBS with 10% FCS and 1% PenStrep on ice for 30 minutes. Cells were washed twice and resuspended in PBS/10%FCS/1%PenStrep, supplemented with 7AAD (Molecular Probes) or Hoechst 33258 (1 μ g/ml, Molecular Probes). Analysis was performed on FACScan or FACS Aria (Becton Dickinson) with Cell Quest software.

Expression analyses

RT-PCR

Total RNA was extracted with TRIzol (Gibco/Life Technologies) and treated with RNase-free DNase (Promega, RQ1). After phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, cDNA synthesis was performed using SuperScript II (Invitrogen/Life Technologies). Primer sequences and PCR fragment sizes are available upon request.

X-Gal staining

Explant cultures of AGM and AGM:gut of *Gli1CreER^{T2}:R26R-lacZ* embryos were supplemented with 4-hydroxy-Tamoxifen (4-OHT; 1 μ M, Sigma) and maintained for 3 days. Tissues were collected and washed in PBS, followed by fixation with 4% paraformaldehyde for 1 hour at room temperature. Tissues were rinsed in washing solution (0.02% NP-40 in PBS), incubated for 3 hours at 37°C in β -gal staining solution and washed.

Following X-gal staining, tissues were refixed, cryoprotected with 20% sucrose, embedded in gelatin, snap frozen in OCT compound (Tissue-Tek), sectioned (10 μ m) and counterstained with Eosin.

Whole embryo culture of E9 *Gli1-CreER^{T2}:R26R-lacZ* and C57Bl/6 conceptuses was performed. Conceptuses were dissected in HBSS/FCS/PenStrep and only embryos surrounded by an intact yolk sac with an ectoplacental cone were processed further. Embryos were cultured (Cockroft, 1990) at 38°C in rolling bottles with rat serum with glucose levels adjusted to 10 mM, with the addition of 0, 0.1 or 1 μ M 4-OHT. After 15 hours of culture, embryos were checked for viability (heart beat and yolk sac circulation) and the trunk region was processed for X-Gal staining as described above.

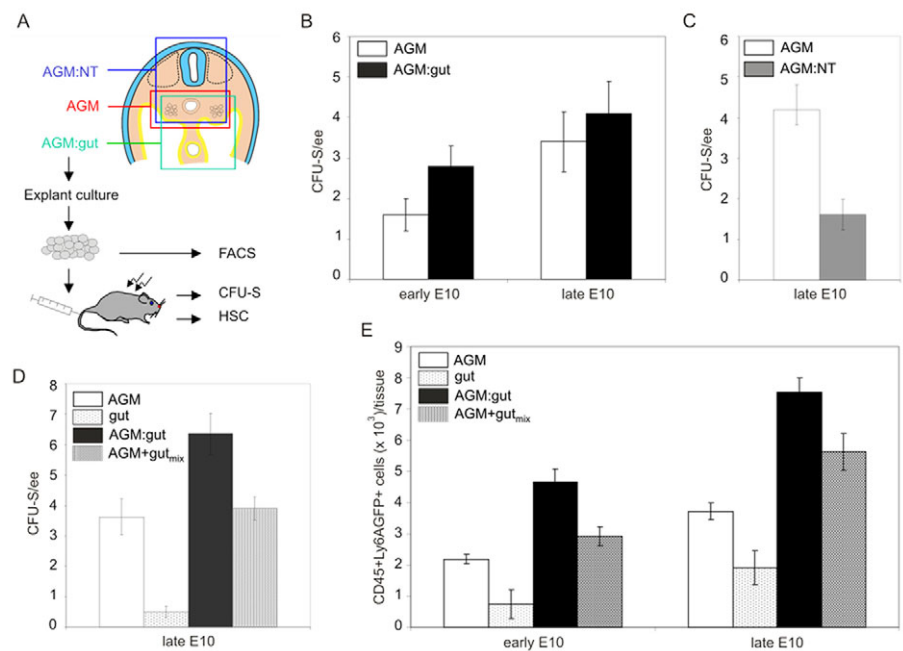
RESULTS

AGM hematopoietic progenitor numbers are affected by surrounding tissues

Previously, *ex vivo* studies examining the hematopoietic potential of the AGM have been performed on explants containing exclusively this tissue (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). In *vivo*, AGM hematopoiesis occurs in the context of the surrounding dorsal and ventral tissues. To examine whether surrounding tissues affect AGM hematopoiesis, we performed explant cultures in which the dorsal or ventral tissues were retained with the AGM. E10 tissues were dissected as AGM alone, AGM with dorsal tissues (AGM:NT) or AGM with ventral tissues (AGM:gut) and explants were cultured for 3 days (Fig. 1A). Tissues were harvested and single cell-suspensions were injected into irradiated recipient mice. The number of colonies for each spleen (colony forming unit-spleen; CFU-S) was counted on day 11 post-transplantation (CFU-S₁₁), as an indicator of the number of immature hematopoietic progenitors.

Fig. 1. Dissections and hematopoietic analyses of AGM explants.

(A) Schematic cross-section through the trunk region of an E10 embryo. The boxes indicate the tissues that were dissected: AGM, aorta-gonads-mesonephros; AGM:gut, AGM with midgut, including the part of the vitelline artery within the loop of the midgut; AGM:NT, AGM with neural tube, notochord and somite remnants just lateral to the neural tube. Tissues were processed directly or after a 3-day explant culture and treated with collagenase, single cell-suspensions were prepared and cells were analyzed by FACS or injected into irradiated recipients for the *in vivo* transplantation assays for progenitors (CFU-S₁₁) or HSCs. (B) Number of CFU-S₁₁ after explant culture of early and late E10 AGM and AGM:gut tissues. The difference in CFU-S₁₁ numbers obtained from early E10 AGM:gut as compared with AGM after explant was statistically significant (Student's *t*-test, $P < 0.05$). (C) Number of CFU-S₁₁ after explant culture of late E10 AGM and AGM:NT tissues. One to two embryo tissue equivalents (ee) of cells (wild-type or *Ln72* transgenic) were injected per recipient. Results are based on seven experiments (early E10 AGM and AGM:gut), four experiments (late E10 AGM and AGM:gut) and six experiments (late E10 AGM and AGM:NT). The total number of mice injected with cells in B and C was 138, with a total of 506 CFU-S detected (average: 3.6 CFU-S/spleen). Twenty-eight irradiated (noninjected) control mice were used in these studies (one to three mice/experiment) with only two colonies detected (average: 0.06 CFU-S/spleen). (D) Number of CFU-S₁₁ per ee after explant culture of late E10 AGM, gut and AGM:gut. After explant culture some of the separately cultured AGMs and guts were pooled (ratio 1:1; referred to as 'AGM+gut_{mix}'). Each recipient received 2 ee of cells. Total number of recipients is 8 (AGM:gut), 12 (AGM), 12 (gut) and 12 (AGM+gut_{mix}). No colonies were detected in irradiated (noninjected) control mice. (E) Number of CD45 Ly6a-GFP double-positive cells per ee after explant culture of early and late E10 AGM, gut and AGM:gut as determined by flow cytometric analysis. The data are based on three (early E10) and four (late E10) experiments. s.e. is indicated. Differences in CFU-S/ee in C and D and in CD45⁺ Ly6a-GFP⁺ cells/tissue in E are statistically significant ($P < 0.05$).



Both at early E10 (31-34 somite pairs) and late E10 (35-39 somite pairs), the number of CFU-S₁₁/explant was increased when the ventrally located gut tissue was kept intact and cultured with the AGM (Fig. 1B). A 1.8-fold increase was found in the early E10 AGM:gut explants and a 1.2-fold increase was found in late AGM:gut explants as compared with the CFU-S₁₁ obtained from equivalent age AGM explants. E11 AGM:gut explants showed no increase in CFU-S₁₁ numbers as compared with E11 AGM explants (data not shown). The influence of dorsally located tissue on E10 AGM hematopoiesis was also examined. Neural tube and associated dorsal tissue was kept intact with the AGM in explant culture for 3 days and the number of CFU-S₁₁/explant was determined. In sharp contrast to the increased number of CFU-S₁₁ in AGM:gut explants, the number of CFU-S₁₁/AGM:NT explant was decreased by 2.6-fold as compared with AGM explants (Fig. 1C). Thus, ventral tissue increases hematopoietic progenitors, and dorsal tissue negatively affects hematopoietic progenitor numbers in AGM explants at early mid-gestation stages.

Ventral tissue increases AGM hematopoietic cell numbers

The increased numbers of CFU-S₁₁ in the E10 AGM ventral tissue explants could simply be due to the presence of hematopoietic progenitors in the gut tissue itself. Hence, the CFU-S₁₁ content of the E10 gut was examined. Gut and AGM tissues were dissected and cultured separately as explants. For comparison of CFU-S numbers, AGM:gut explants were also cultured. Irradiated mice were injected with cells from gut explants, AGM explants, a mixture of gut and AGM explant cells combined at the time of injection (AGM+gut_{mix}) or AGM:gut explant cells (Fig. 1D). Gut explants gave few or no

(0.5) CFU-S₁₁, whereas AGM explants gave 3.6 CFU-S₁₁/tissue. AGM+gut_{mix} yielded similar numbers of CFU-S₁₁ as AGM explants alone and gave 1.6-fold fewer CFU-S₁₁ than AGM:gut explants. These data suggest a synergy between physiologically intact AGM:gut tissues in the growth of immature hematopoietic progenitors.

To further examine the effects of ventral tissue on AGM hematopoiesis, we performed flow cytometric analysis for cells expressing hematopoietic markers after culture of E10 AGM, gut and AGM:gut. We reported previously that all AGM HSCs in *Ly6a-GFP* transgenic embryos are GFP positive (de Bruijn et al., 2002) and most AGM HSCs are positive for CD45 (Ptprc – Mouse Genome Informatics) (North et al., 2002). Hence, HSCs are within the pool of *Ly6a-GFP* CD45 double-positive cells. As shown in Fig. 1E, early and late E10 AGM explants contained ~2200 and 3700 GFP⁺ CD45⁺ cells/tissue, respectively. Gut explants contained many fewer GFP⁺ CD45⁺ cells/tissue; 700 at early E10 and 1900 at late E10. Interestingly, AGM:gut explants contained more than double the number of GFP⁺ CD45⁺ cells/tissue compared with the AGM cultured alone, and were more than the simple sum of GFP⁺ CD45⁺ cells from the AGM and gut cultured separately. Thus, the phenotypically defined population containing HSCs is synergistically increased in AGM:gut explants.

Ventral tissue induces hematopoietic stem cells in the early E10 AGM

To examine whether functionally defined AGM HSCs are affected by dorsal and ventral tissues we performed *in vivo* transplantation assays after explant culture of early and late E10 tissues. Previous studies show that HSCs cannot be detected in early E10 (<35 somite pair) AGM explants (Medvinsky and Dzierzak, 1996). Similarly, in this study early E10 AGM explants gave no repopulation after *in vivo* transplantation (Fig. 2A). Also, no recipients receiving early E10 AGM:NT or gut explant cells were repopulated. Surprisingly, transplantations of early E10 AGM:gut explant cells did result in 4 repopulated recipients out of 14. Peripheral blood donor engraftment levels of the four recipients were 86%, 93%, 60% and 98% at greater than 4 months postinjection. Two early E10 AGM:gut recipients were further examined for multi-lineage engraftment. High levels of donor-derived cells were found in all hematopoietic tissues and cell lineages analyzed (Fig. 2C). Moreover, bone marrow recovered from these primary recipients contained HSCs, as high level, long-term engraftment was found in the peripheral blood of secondary recipients (Fig. 2D). Thus, the tissue ventral to the AGM region induces the early E10 production of HSCs. By late E10 the differences in repopulation potential of AGM:gut cells were less pronounced. AGM explant cells resulted in 54% of recipients repopulated with donor cells, whereas late E10 AGM:gut resulted in 62% of recipients repopulated (Fig. 2B). Late E10 AGM:NT explants resulted in 40% of recipients repopulated. Thus, as observed for hematopoietic progenitors, dorsal tissues inhibit AGM HSC activity, whereas ventral tissues increase HSC activity, and this activity is most pronounced at the early E10 stage.

CFU-S₁₁ and HSCs are AGM-derived and not ventral tissue-derived

Whereas the decrease in AGM explant hematopoietic activity by the dorsal tissue suggests the presence of inhibitory signals, the increase in hematopoietic activity by the ventral tissue could be because precursors to hematopoietic progenitors/stem cells are harbored in this tissue. It has been suggested previously that primordial germ cells (PGCs) are the precursors to the hematopoietic cells detected

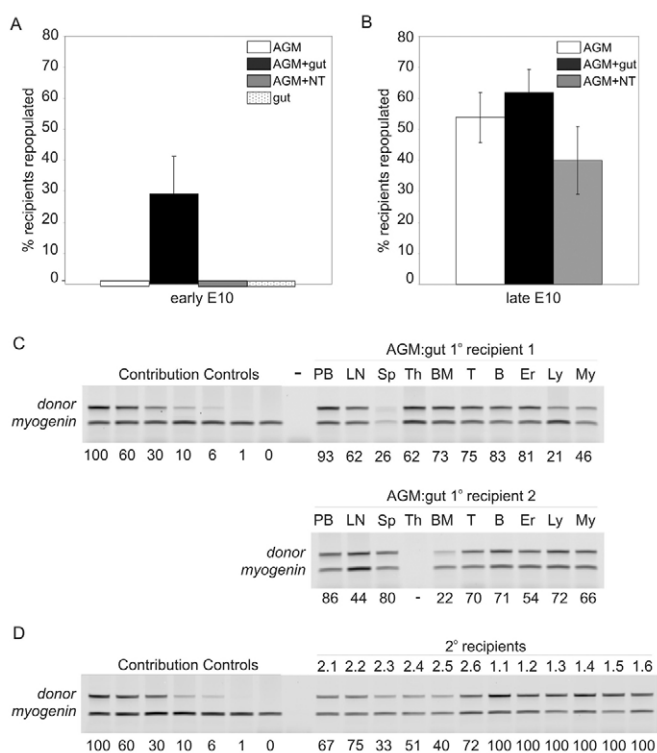


Fig. 2. HSC analysis after explant culture of AGM and AGM with surrounding tissues. Percentage of adult recipient mice repopulated with HSCs from explants of early E10 (A) and late E10 (B) AGM, gut, AGM:gut and AGM:NT tissues (from *Ln72*, *Ly6a-lacZ* or *Ly6a-GFP* transgenic embryos). Cells were injected into irradiated mice (1.2–1.8 ee per recipient for late E10 and 2–2.5 ee per recipient for early E10). Each column represents the number of mice repopulated per number of recipients transplanted (late E10: 21/39 for AGM, 26/42 for AGM:gut, 6/15 AGM:NT; early E10: 0/17 for AGM, 4/14 for AGM:gut, 0/5 for AGM:NT, 0/15 for gut). Error bars indicate s.d. for the combined results of 12 separate experiments. Percentages of donor cells in peripheral blood of the four recipients repopulated with early E10 AGM:gut are 86%, 93%, 60% and 98%. (C) Representative semi-quantitative PCR analysis for multi-lineage hematopoietic repopulation of primary (1°) recipient 1 and 2) injected with cells from early E10 AGM:gut. Donor marker PCR was performed on DNA from hematopoietic cells and tissues. PB, peripheral blood; LN, lymph node; Sp, spleen; Th, thymus; BM, bone marrow cells; T, sorted spleen T lymphocytes; B, sorted spleen B lymphocytes; Er, sorted BM erythroid cells; Ly, sorted BM lymphoid cells; My, sorted BM myeloid cells. (D) PCR analysis performed on DNA of peripheral blood of secondary recipients transplanted with 2×10^6 BM cells from the primary recipients (shown in C). Percentage donor marker contributions (*Ln72*) are indicated below each lane. Controls include the myogenin gene for DNA normalization and quantitation standards containing 100, 60, 30, 10, 6, 1 and 0% donor (*Ln72*) DNA.

in the mid-gestation AGM (Rich, 1995), as at this time point PGCs are migrating up the hindgut to the genital ridges/gonads. Alternatively, the ventral tissue might provide factors that enhance AGM-derived hematopoiesis. To examine whether ventral tissue-derived cells are responsible for the increase in hematopoietic activity, we performed a variation of the whole tissue explant culture. We developed a reaggregate culture system for AGM cells. Briefly, AGM tissue is collagenase-treated, made into a single cell suspension and centrifuged. The cells are suspended at high cell

density and transferred as a droplet onto a filter. A tightly adherent cell reaggregate tissue is formed rapidly. As for the AGM explant culture, the reaggregate is cultured on the filter at the air-medium interface. Such AGM reaggregates cultured for several days contain CFU-S and HSC activity (see Table S1 in the supplementary material). Although hematopoietic activity might be slightly reduced as compared with whole AGM explants, this culture system is useful for cell tracing experiments.

The tissue origins of the increased CFU-S and HSC activity found in the AGM:gut whole tissue explants were examined in chimeric reaggregates. E10 gut tissues and AGM tissues were dissected from embryos containing a *GFP* transgene, as well as from nontransgenic embryos (Fig. 3A). Single cell-suspensions of AGM and gut cells, each with distinct genetic markers, were mixed in a 1:1 ratio (one embryo tissue equivalent of each) and cultured as a reaggregate. After 3 days, cells were harvested, tested *in vivo* for CFU-S₁₁ and colonies were analyzed by fluorescence microscopy for GFP expression (Fig. 3B) and/or by PCR for the *GFP* transgene (Fig. 3C). When cultures of *GFP* gut cells were reagggregated with wild-type (WT) AGM cells, no *GFP*-positive CFU-S₁₁ were found. However, in reaggregate cultures of WT gut cells with *GFP* AGM cells, all CFU-S₁₁ were *GFP* positive. These cumulative results showing that all CFU-S₁₁ are AGM-derived are shown in Table 1.

Similarly, chimeric reaggregates of genetically distinguishable gut and AGM cells were transplanted and recipient mice were examined for long-term multi-lineage hematopoietic engraftment at >4 months post-transplantation. As shown in Table 2, when

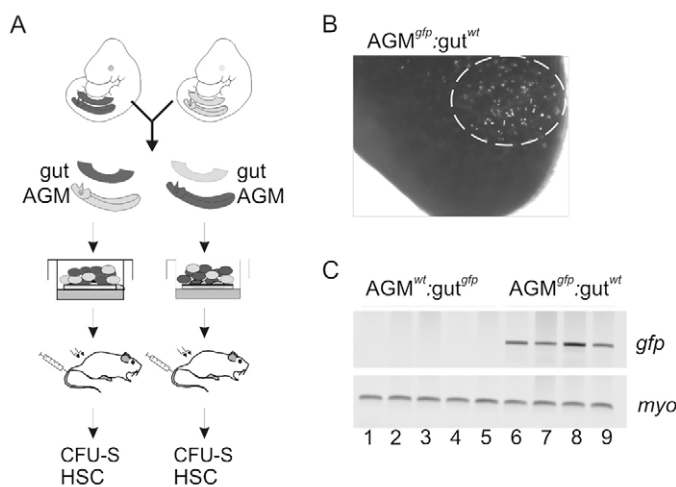


Fig. 3. Chimeric tissue reaggregates indicate the origin of hematopoietic progenitors/HSCs after AGM explant culture with ventral tissues. (A) Schematic diagram of reaggregate protocol.

Genetically distinct AGMs and guts were combined in a cell suspension and reaggregates deposited on a filter for culture. After 3 days the reaggregate cell suspensions were injected into recipients. CFU-S were examined at 11 days post-transplantation, and HSC engraftment at 4 months post-transplantation. (B) Part of a spleen containing one CFU-S₁₁ from a recipient injected with cells from an AGM^{GFP}:gut^{WT} reaggregate. Within the circled area is one representative colony of GFP-expressing cells (white spotted area). (C) Representative PCR analysis of DNA from individual CFU-S₁₁ from recipients injected with AGM^{WT}:gut^{GFP} reaggregate cells (lanes 1-5) or AGM^{GFP}:gut^{WT} reaggregate cells (lanes 6-9). Each lane contains the PCR product of DNA isolated from one CFU-S₁₁. The myogenin (*myo*) normalization control indicates that all CFU-S yielded DNA (lanes 1-9). See Table 1 for complete results.

early E10 chimeric reaggregates were tested 4 out of 12 recipients were repopulated. The hematopoietic system of all four repopulated mice was from AGM-derived cells and not from gut cells. Transplantation of late E10 chimeric reaggregates resulted in the repopulation of four out of eight recipients and, similar to early E10 chimeric reaggregates, all repopulated recipients were engrafted by donor AGM cells. All hematopoietic tissues of these primary recipients showed high level engraftment by AGM-derived cells. To confirm that the AGM-derived engrafting cells are indeed HSCs (that self renew) and also to test whether the gut contains precursors that need extra time to mature to HSCs, secondary transplantations were performed. Bone marrow from two recipients transplanted with late E10 chimeric reaggregates was harvested at >4 months post-transplantation and injected into secondary recipients. In each case, five out of five recipients were high level and were long-term repopulated with cells bearing the AGM genetic marker (Table 2). No gut-derived hematopoietic cells were found. Thus, the increase in hematopoietic progenitors and HSCs, as well as the induction of early E10 AGM HSCs in the AGM:gut explants is not due to gut-derived cells, but instead is most likely due to the influence of ventral-derived soluble factors (morphogens) or cell-cell interactions of the ventral-derived cells with the AGM-derived cells.

Hedgehog signaling pathway molecules are expressed in the E10 AGM region and in the AGM with associated tissues

To gain insight into soluble factors or morphogens that may regulate AGM HSC induction, we focused on the Hedgehog-related genes, as this signaling pathway is one of the earliest effectors of hematopoiesis in zebrafish embryos, mouse ES cell hematopoietic differentiation cultures and mouse neuroectoderm re-specification (Byrd et al., 2002; Dyer et al., 2001; Gering and Patient, 2005; Maye et al., 2000). Moreover, *Ihh* and *Shh* are expressed in gut endoderm and *Dhh* in the endothelium at mid-gestation (Bitgood and McMahon, 1995).

RT-PCR analysis of AGM, AGM:gut and AGM:NT shows the expression of all three Hedgehog ligands (*Dhh*, *Ihh*, *Shh*) and their membrane associated receptors (*Ptch1*, *Ptch2*), signal transducer (*Smo*) and intracellular effectors (*Gli1*, *Gli2*, but not *Gli3*) in freshly dissected E10 AGM tissue (Fig. 4A). AGM:gut and AGM:NT tissues also express these genes, as well as *Gli3*. There appears to be no obvious dorsal-ventral polarity difference in the expression of Hedgehog signaling pathway molecules.

The expression of Hedgehog signaling molecules was next tested in E10 AGM subpopulations. AGM cells were enriched for hematopoietic progenitors/stem cells (c-Kit⁺ CD34⁺), mesenchymal cells (c-Kit⁻ CD34⁻) and endothelial cells (c-Kit⁻ CD34⁺) by flow cytometric sorting. RT-PCR analysis (Fig. 4A) shows that mesenchymal cells express all three receptor/transducer genes, whereas hematopoietic progenitor/stem cells and endothelial cells express *Ptch1* and *Smo*, but no/low levels of *Ptch2*. Downstream targets *Gli1*, *Gli2* and *Gli3* are highly expressed in mesenchymal cells, whereas endothelial cells express only low levels of *Gli1*, *Gli2* and *Gli3*. Hematopoietic progenitor/stem cells express low levels of *Gli1* and *Gli3*, but not *Gli2*. Thus, relevant Hedgehog signaling pathway genes are expressed most highly in the mesenchymal subset of cells within the E10 AGM region.

Localization of the cells activated by Hedgehog signaling in the AGM region was performed by inducible Cre-*lox* recombination marking. *Gli1-Cre^{T2}* mice were crossed with *R26R-lacZ* transgenic mice. Whole embryos were isolated at E9 and cultured overnight

Table 1. CFU-S₁₁ derived from chimeric reaggregates containing AGM and gut cells

E10 stage	AGM marker	Gut marker	Number of reaggregates	Number of recipients	Number of CFU-S
Early	–	<i>GFP</i>	11	4	8 GFP [–] 0 GFP ⁺
Early	<i>GFP</i>	–	3	2	4 GFP ⁺ 0 GFP [–]
Late	–	<i>GFP</i>	4	2	4 GFP [–] 0 GFP ⁺

Cells from E10 tissues from *CAG-EGFP* transgenic or nontransgenic embryos were made into chimeric reaggregates and cultured for 3 days. Harvested reaggregate cells were transplanted at a dose of 1.5–3 reaggregates (early E10) and 2 reaggregates (late E10) per adult irradiated recipient. Spleens were harvested at 11 days after injection and CFU-S₁₁ (colonies forming unit-spleen) were macroscopically counted under visible and fluorescent light. No CFU-S were found in noninjected irradiated control mice. DNA PCR verified the transgenic or nontransgenic genotype of the CFU-S₁₁. *n*=3.

in the presence of tamoxifen. Embryos exhibiting normal growth (E10 equivalent, ~32 somite pair stage) were stained for β-galactosidase activity and sectioned. Gli1 (β-Gal)-positive cells were found mainly in the mesenchymal regions (Fig. 4B, arrow), as determined by their scattered position around the dorsal aorta. Whereas many Gli1-positive cells were positioned distal to the dorsal aorta, some Gli1-positive cells were directly underlying the aortic endothelium and aortic endothelium with an associated hematopoietic cluster (Fig. 4C). These results were verified by AGM explant culture. E10 *Gli1-Cre^{T2};R26R-lacZ* compound transgenic embryos were dissected and AGM explants cultured in tamoxifen for 3 days. Following staining and sectioning, Gli1 expression was again found in the mesenchymal areas surrounding the aorta (Fig. 4D). These data confirm the high level of *Gli1* expression found in the RT-PCR analysis of sorted AGM (c-Kit[–]CD34[–]) mesenchymal cells.

Hedgehog proteins affect AGM HSCs

To examine whether the Hedgehog proteins do indeed play a functional role in HSC development, E10 and E11 AGM explants were cultured in the presence or absence of Hedgehog proteins for 3 days. Cells were harvested and injected into irradiated adult recipients. The recipient hematopoietic population was assayed 4 months following injection. As shown in Table 3, both *Ihh* and *Shh* protein at a concentration of 20 ng/ml induced HSC activity in early E10 AGM explants. These HSCs yielded long-term, high level (33–100% donor chimerism) engraftment of 40% of the

adult irradiated mice that were transplanted. Since culture of E10 AGM explants with 2 and 200 ng/ml Hedgehog proteins yielded no HSC activity, their effect on HSC induction is dose dependent.

The specific nature of the signal emanating from the gut was tested by culturing E10 and E11 AGM:gut explants with anti-Hedgehog specific activity-blocking antibody, 5E1 (or with control IgG). Cells were harvested and injected into irradiated adult recipients, and engraftment was measured at 4 months post-transplantation. As shown in Table 3, HSC repopulating activity was completely blocked by anti-Hh antibody in early E10 AGM:gut explants, as compared with the IgG controls. Slightly less blocking by anti-Hh antibody was observed with late E10 AGM:gut explants, whereas blocking was negligible in E11 AGM:gut explants, as compared with the IgG controls. These results demonstrate that endogenous Hedgehog protein has a positive influence on the induction and growth of early E10 AGM HSCs, that this influence is time-dependent and that the ventral tissue might be a source of the signal.

DISCUSSION

We have shown here that positional information is important for the development of functional HSCs and progenitors in the mid-gestation AGM region. Ventral tissues enhance the growth of AGM HSCs and progenitors, whereas the dorsal tissues inhibit this growth (Fig. 4E). Hedgehog proteins appear to be major effectors of early HSC induction and the gut is known to be a local ventral source of Hedgehog proteins.

Table 2. HSC repopulation by chimeric reaggregates containing AGM and gut cells

E10 stage	AGM marker	Gut marker	Number of reaggregates	Number of recipients	Number of repopulated recipients (% chimerism)	
					AGM marker	Gut marker
Early	<i>lacZ</i>	<i>Ln72</i>	15	5	1 (43%)	0
Early	<i>GFP</i>	<i>Ln72</i>	6	2	0	0
Early	<i>Ln72</i>	<i>GFP</i>	6	2	0	0
Early	<i>Ln72</i>	<i>lacZ</i>	9	3	3 (45%, 47%, 4%)	0
Late	<i>lacZ</i>	<i>Ln72</i>	6	3	0	0
Late	<i>GFP</i>	<i>Ln72</i>	2	1	0	0
Late	<i>Ln72</i>	<i>GFP</i>	2	1	1 (6%)	0
Late	<i>Ln72</i>	<i>lacZ</i>	6	3	3 (98%, 99%*, 100%†)	0

Secondary transplantation	AGM marker	Gut marker	Number of BM cells/recipient	Number of recipients	Number of repopulated recipients (% chimerism)	
					AGM marker	Gut marker
*BM	<i>Ln72</i>	<i>lacZ</i>	2×10 ⁶	5	5 (99%, 100%, 100%, 29%, 100%)	0
†BM	<i>Ln72</i>	<i>lacZ</i>	2×10 ⁶	5	5 (68%, 53%, 83%, 58%, 60%)	0

Cells from E10 tissues from *lacZ*, *Ln72* and *GFP* transgenic embryos were made into a chimeric reaggregate and cultured for 3 days. Harvested reaggregate cells were transplanted at a dose of 3 reaggregates (early E10) and 2 reaggregates (late E10) per adult irradiated recipient. At >4 months after transplantation, peripheral blood DNA PCR was performed with *lacZ*-, *Ln72*- and/or *GFP*-specific primers. Percentage donor contribution was determined by comparison of the specific signal that was normalized for DNA content (*myogenin* control) with serial dilutions (100, 60, 30, 10, 6, 1 and 0%) of control transgenic DNA. *n*=3.

*†Individual primary recipient mice repopulated with cells derived from late E10 AGM (*Ln72*)/gut (*lacZ*) reaggregates. At the time of sacrifice, hematopoietic tissues were collected and checked by PCR for *Ln72* and *lacZ* markers. Only *Ln72* contribution was detected. Bone marrow (BM) of these two primary recipients was used for transplantation into five secondary recipients. At >4 months after transplantation, peripheral blood DNA PCR was performed with *lacZ*- and *Ln72*-specific primers.

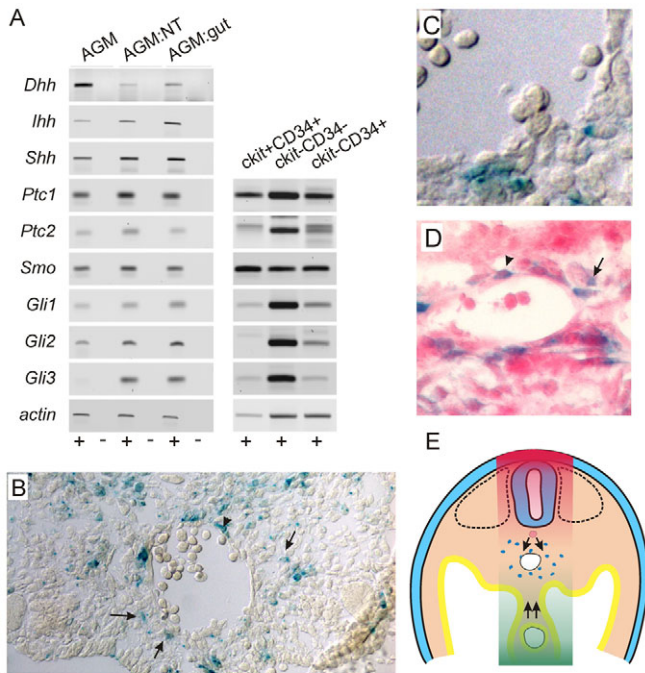


Fig. 4. Expression of Hedgehogs, receptors and targets in the AGM. (A) RT-PCR analysis of freshly dissected AGM, AGM:NT and AGM:gut tissues, and sorted HSCs/progenitor cells (c-Kit⁺ CD34⁺), endothelial cells (c-Kit⁻ CD34⁺) and mesenchymal cells (c-Kit⁻ CD34⁻) for the expression of Hedgehogs, receptors (*Ptch1*, *Ptch2*), signal transducer (*Smo*) and intracellular transcriptional effectors (*Gli1*, *Gli2*, *Gli3*). c-Kit⁺ CD34⁺, c-Kit⁻ CD34⁺ and c-Kit⁻ CD34⁻ represented 0.8%, 2.0% and 95.4%, respectively, of the total AGM population. Sorted cells were 83–96% pure. + indicates reaction with reverse transcriptase and – indicates no reverse transcriptase. (B) β -Galactosidase-stained (blue) transverse section through the trunk of an early E10 *Gli1-CreERT2;R26R-lacZ* embryo after overnight culture in tamoxifen. Arrows indicate *Gli1*⁺ mesenchymal cells and arrowhead indicates mesenchymal cells directly underlying aortic endothelial cells. Top is dorsal, bottom is ventral. (C) *Gli1*-expressing mesenchymal cells (blue) underlying the aortic endothelium (underlying a hematopoietic cluster) of an early E10 *Gli1-CreERT2;R26R-lacZ* embryo after overnight culture in tamoxifen. (D) Section through an early E10 AGM *Gli1-CreERT2;R26R-lacZ* explant cultured for 3 days in tamoxifen. *Gli1*⁺ mesenchymal cells are dispersed throughout the tissue (arrow) and under the endothelial cells of the vessel (arrowhead). (E) Positional information influences AGM HSC induction. Hedgehog protein production is dorsal and ventral to the aorta (arrows). The Hedgehog pathway exhibits no dorsal/ventral polarity in activation, as *Gli*-expressing cells (blue) are found surrounding the aorta. However, ventral tissue induces the production of HSCs through gut-expressed Hedgehog protein and perhaps other ventral factors (such as *Bmp4*), as shown by the green gradient. Dorsal tissue inhibits AGM HSC induction as shown by the red gradient, suggesting that unknown dorsal factors (or antagonists) act to inhibit HSC induction and effect a ventral polarized induction of HSCs.

AGM hematopoiesis is influenced by exogenous information

Much of our knowledge about the development of HSCs has come from the study of the AGM region *in vivo* and *ex vivo*. AGM tissue explants autonomously increase HSC numbers by 15-fold or more after 3 days in culture (Medvinsky and Dzierzak, 1996). As the first tissue to generate HSCs in the embryo at E10.5, the initial challenge

was to identify the local mediators of HSC induction within the AGM tissue itself. Indeed, we previously have identified *Bmp4* as one of the local factors effecting an increase in AGM HSCs (Durand et al., 2007). Also, *Il3* has been identified as a positive effector, but its source may not be within the AGM (circulating cells and/or gut cells) (Robin et al., 2006).

Although AGM explants can autonomously promote the growth of HSCs, the influence of surrounding tissues had not been previously examined. Within the normal physiology of the embryo, the AGM lies between the ventral tissue, which includes the endoderm-derived gut and the dorsal tissue, which includes the ectoderm-derived notochord and neural tube. In agreement with the results of avian embryo studies (Pardanaud and Dieterlen-Lievre, 1999), we found that dorsal tissues suppress and ventral tissues enhance hematopoietic potential. Both immature hematopoietic progenitors and HSCs are positively influenced in AGM:gut explants. The CFU-S studies show that the positive influence of the ventral gut tissue is time dependent. An increase in CFU-S is seen with early E10 AGM:gut explants, and CFU-S increases are less apparent at late E10 and are absent by E11. More strikingly, HSC activity is present in early E10 AGM:gut explants, whereas no HSCs are found in AGM or gut explants alone. A slight increase in HSCs in late E10 AGM:gut explants was also observed but a more quantitative (limiting dilution) analysis must be performed to verify the extent of this. Thus, the positive influence of ventral gut tissue affects early events leading to HSC induction, and later might also affect HSC maintenance/expansion, although this aspect is less certain.

HSC induction: trophic effect of ventral tissues, not ventral cell contribution

Despite many data supporting the development of HSCs from hemogenic endothelium or hemangioblasts (reviewed by Dzierzak and Speck, 2008), it has also been proposed that PGCs are the direct precursors to AGM HSCs (Rich, 1995). Studies on the mid-gestation mouse gonads show a clear migration of PGCs along the hindgut into the genital ridges of the AGM region (Ginsburg et al., 1990; Gomperts et al., 1994). Most PGCs enter the developing gonads, but some can be found in other areas of the AGM region, such as in the vicinity of the aortic endothelium. Upon arrival in this region, it was suggested that the PGCs mature to HSCs and hematopoietic progenitors (Rich, 1995). Until our studies, no lineage tracing had been performed to test this notion. Using the novel chimeric AGM reaggregate culture system, we demonstrated that HSCs and CFU-S in AGM:gut cultures are always AGM-derived, thus ruling out the possibility that PGCs or any other cells from the gut are the source of AGM hematopoietic cells. These data further support AGM HSC emergence from hemogenic endothelium or hemangioblasts.

The reaggregate culture system clearly disrupts the structure of the AGM. However, the rapid reaggregation of cells and the detection of relatively normal quantities of CFU-S and HSCs within the control reaggregates suggest that the microenvironment of the tissue is quickly restored (see Table S1 in the supplementary material). The chimeric reaggregate culture system will be instrumental in identifying various candidate hemogenic cells (Taoudi et al., 2008), in further dissecting the signaling network(s) that determine HSC fate in the AGM region and in segregating cell-autonomous from non-cell-autonomous effects when used with mutant donor tissue. Eventually, we should also be able to test the time dependence of the various component interactions that lead to HSC generation.

Table 3. Hedgehog signalling affects HSC activity in AGM explants

Embryonic day	Tissue	Factor or antibody	Concentration (ng/ml)	Number repopulated/ number transplanted	% Recipients repopulated	% Chimerism
Early E10	AGM	Ihh	2	0/9	0	–
			20	4/10	40	100, 89, 99, 91
			200	0/12	0	–
Early E10	AGM	Shh	2	0/3	0	–
			20	4/10	40	95, 89, 95, 33
			200	0/8	0	–
Early E10	AGM:gut	IgG	50	2/8	25	60, 99
		Anti-Hh	50	0/7	0	–
Late E10	AGM:gut	IgG	50	3/5	60	11, 47, 83
		Anti-Hh	50	1/6	17	99
E11	AGM	IgG	50	4/11	36	>10, >10, >10, >10
		Anti-Hh	50	3/12	25	>10, >10, >10

E10 AGM explants (*Ln72*) were cultured for 3 days and cells transplanted into irradiated adult recipients (B10 × CBAF1). Recipients received 2–2.5 embryo equivalents of AGM cells. At >4 months after transplantation, peripheral blood DNA PCR was performed for the *Ln72* transgene. Percentage donor contribution was determined by comparison of the specific signal that was normalized for DNA content (*myogenin* control) with serial dilutions (100, 60, 30, 10, 6, 1 and 0%) of control transgenic DNA. Total number of experiments=14. For each condition $n=2-4$.

Hedgehog signaling plays a role in AGM HSC induction and growth

Our observations that the ventral tissue in AGM:gut explants induced the early appearance of HSCs and increased the number of hematopoietic progenitors (as compared with AGM alone), and that all HSCs and CFU-S were AGM-derived, strongly suggested that the observed effect is through the provision of factors/morphogens by the early ventral tissue. Indeed, AGM explant culture experiments, in which Indian or sonic hedgehog protein was added, showed an early induction of HSCs. Moreover, this effect was dose dependent and induction of AGM HSCs by the gut could be inhibited with a Hedgehog activity-blocking antibody.

Transcriptional analyses of AGM tissue and sorted cell populations show that Hedgehog signaling pathway-related molecules are expressed at early E10. Our histologic analyses provide further evidence that this pathway is active in cells in the AGM region. As a general concept, *Gli1* is transcriptionally induced by Hedgehog signaling, and thus by detecting *Gli1* transcription it is possible to monitor target cells at any moment during development. Using *Gli1-CreER^{T2};R26R-lacZ* mice in two different culture systems, we observed *Gli1*-expressing cells (or cells that recently expressed *Gli1*) in the AGM region by tamoxifen-induced recombination. Similar results were obtained when tamoxifen was added to overnight cultures of whole E9 embryos or to 3-day cultures of early E10 AGM explants. Although AGM explants lose some morphologic features in culture, *Gli1*-positive cells were found in mesenchyme surrounding the aorta. A clearer localization of the *Gli1*-expressing cells was obtained from the histologic sections of cultured whole embryos. *Gli1*-expressing cells are found in a punctate distribution in the mesenchymal areas surrounding the aorta, both at a distance and just underlying the aortic endothelium (and a hematopoietic cluster). Preliminary immunostaining data of these sections with anti-CD31 antibody (endothelial and hematopoietic cell-specific; also known as *Pecam1*) support the mesenchymal nature of the *Gli1*-expressing cells. This expression pattern is also consistent with the high level of *Gli1* expression found by transcriptional analysis of mesenchymal cells sorted from the AGM, as compared with sorted AGM endothelial and hematopoietic cells. Thus, Hedgehogs might act indirectly (through mesenchymal cells) to effect AGM HSC induction. As the notochord is a known dorsal source of Hedgehog protein and the gut is a ventral source of Hedgehog, it is not surprising that *Gli1*-expressing cells are found circumferentially. Our results showing the opposing influences of

the dorsal and ventral tissues on AGM HSCs suggest that, although the Hedgehog signaling pathway is active in the whole AGM region, the dorsal tissue might override positive influences. We speculate that this is due to dorsal-derived negative regulators and/or the cell origin of aortic endothelium in the dorsal wall [which has been shown to be replaced in chick embryos (Pouget et al., 2006)] at this stage in development.

Implications of Hedgehog signaling in vertebrate hematopoietic development

Studies in zebrafish have shown a clear requirement for Hedgehog signaling in the generation of the definitive hematopoietic system (Gering and Patient, 2005). Using a morpholino knockdown approach and the Hedgehog inhibitor cyclopamine, it was found that Hedgehog acts at several distinct embryonic stages. First, Hedgehog is required during the gastrulation stage for the medial migration of dorsal aorta precursors (most likely *Flk1*⁺) from the lateral mesoderm. At early somitogenesis, Hedgehog influences the expression of a gene cascade, including the *Vegf*, *Notch*, *ephrinb2a* and *runx1* genes that are required for arterial specification, and as the production of HSCs is dependent upon the dorsal aorta, Hedgehog is essential.

In the mouse, deletion of *Ihh* leads to defective development of yolk sac vasculature (Byrd et al., 2002) and to mid-gestation lethality (St-Jacques et al., 1999). *Ihh*^{−/−} embryoid bodies do not form blood islands (Byrd et al., 2002). *Ihh* is expressed in the visceral endoderm and *Dhh* is expressed in the yolk sac vasculature. *Smo*^{−/−} embryos are indistinguishable from *Shh*^{−/−}; *Ihh*^{−/−} embryos (Zhang et al., 2001), with only rudimentary yolk sac vasculature. Recently, it has been found that *Foxf1* expression is lost in these mutants and is most likely the mesodermal target of Hedgehog signaling (Astorga and Carlsson, 2007). *Bmp4* appears to be a downstream target of *Foxf1*, as the addition of *Bmp4* to *Foxf1*^{−/−} explants leads to a well-developed plexus of endothelial tubes. Thus, Hedgehog induction of murine vasculogenesis acts through *Foxf1* and *Bmp4*.

Unfortunately, the functional redundancy of murine Hedgehogs and the early lethality of *Smo*^{−/−} mice prevent investigation of their role in dorsal aorta specification and hematopoiesis. Nonetheless, some aspects of the signaling cascade involved in zebrafish dorsal aorta specification are conserved in the mouse (Rossant and Hirashima, 2003). Considering the demonstration of a Hedgehog-*Foxf1*-*Bmp4* genetic cascade by Astorga and Carlson, and our

previous findings that Bmp4 affects the growth of AGM HSCs, future experiments will focus on the molecular interface between Hedgehog and Bmp4 signaling in the spatiotemporal events leading to HSC induction in the early AGM.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/15/2613/DC1>

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Table S1. E11 AGM reaggregates contain similar CFU-S and HSC activity as compared with whole AGM explants

Type of culture	CFU-S ₁₂ /AGM*	Number of recipients reconstituted/transplanted†	
		1 ee/recipient	2 ee/recipient
Normal tissue	3.9±0.8	3/3	3/3
Reaggregate	3.0±1.2	3/3	4/4

Tissues were dissected from wild-type or *Ln72* embryos for the CFU-S experiments and *Ln72* or *Ly6A-LacZ* embryos for long-term transplantation experiments and were cultured for 2 to 4 days.

*Data are the mean±s.d. of three independent experiments. A total of 12 AGMs for each culture condition were injected (2-2.5 ee per recipient, five recipients per culture condition). No CFU-S were found in the noninjected irradiation controls that were included in each experiment (one to two controls per experiment).

†Data are compiled from two independent experiments. Mice were bled at >4 months postinjection.

CFU-S, colony forming unit-spleen; ee, embryo equivalent.