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Hematopoietic stem cell activity in the aorta-gonad-mesonephros region enhances after mid-day 11 of mouse development

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ABSTRACT The E11.5 aorta-gonad-mesonephros (AGM) region is a site of hematopoietic stem cell (HSC) development prior to colonisation of the embryonic liver. The generation of HSCs in the embryo starting from E11 is very rapid. Here, we have assessed hematopoietic development in the AGM region during E11 at precise somitic ages. Although the numbers of committed hematopoietic precursors fluctuate throughout the day, the repopulation activity in the AGM region noticeably increases from mid (44 s.p.) to end (48 s.p.) day 11 of gestation. While prior to mid day 11 two thirds of AGM regions contain no definitive HSCs, shortly prior to liver colonisation, all older day 11 embryos contain definitive HSC. Nevertheless, all E11 AGM regions even at early somitic stages have the capacity to expand numbers of definitive HSCs *ex vivo*. Quantitative anatomical analysis confirmed preferential localization of intra-aortic clusters (IACs) to the ventral domain of the dorsal aorta during entire day 11 of development. No clear correlation was established between IAC numbers and the presence of definitive HSCs.

KEY WORDS: *AGM region, HSC, mouse embryo, long-term repopulation*

Introduction

The AGM region is an important source of definitive hematopoietic activity during vertebrate embryogenesis. During mouse development definitive HSCs in the AGM region are generated during a narrow temporal window between E10.5 and E12.0 (Chen *et al.*, 2009; de Bruijn *et al.*, 2002; Dzierzak and Speck, 2008; Godin and Cumano, 2002; Kumaravelu *et al.*, 2002; Medvinsky and Dzierzak, 1996; Muller *et al.*, 1994) in the ventral domain of the dorsal aorta, AoV (Taoudi and Medvinsky, 2007).

It is broadly thought that intra-aortic cell clusters (IAC) are a morphological manifestation of progenitor and HSC formation from the endothelium of the dorsal aorta. The link between the endothelial and hematopoietic lineages is highlighted in a series of publications using *in vitro* and *in vivo* models (Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Chen *et al.*, 2009; Choi *et al.*, 1998; Eilken *et al.*, 2009; Kissa and Herbomel, 2010; Lancrin *et al.*, 2009; Nishikawa *et al.*, 1998; Zovein *et al.*, 2008). Some evidence suggests that definitive HSCs mature within intra-aortic clusters (Taoudi *et al.*, 2008). In addition to IACs supposedly forming by intra-aortic cell migration, hematopoietic cells and progenitors/HSCs, as has been shown in non-mammalian vertebrates, may migrate underneath the aortic endothelial lining (Bertrand *et al.*, 2010; Jaffredo *et al.*, 1998; Kissa and Herbomel, 2010). The relationship between intra-aortic clusters and HSC formation needs to be elucidated.

Here we studied and quantified the location of small and large intra-aortic clusters in the dorsal aorta and their numbers in E11 embryos at various somitic ages. Although intra-aortic clusters can be found around entire luminal surface of the dorsal aorta, they are located preferentially in the ventral domain of the dorsal aorta. We observed a trend of increasing numbers of small clusters and decreasing of numbers of large clusters after mid day 11.

Over the course of many years we have observed quantitative variations between experiments in the generation of definitive HSCs. Although variability in live systems under experimental

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; CFU-C, colony forming unit in cluture; HSC, hematopoietic stem cell; IAC, intra-aortic cluster.

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conditions is expected, we decided to investigate whether the age variability between embryos can play a role in that. Indeed, the hematopoietic system undergoes dramatic transformations over the course of several days. If rapid changes in hematopoietic development occur during one day, this may contribute into experimental variability. Here we studied E11 embryos staged accurately in accord with somite pair counts and morphological criteria. We also sought to correlate HSC formation with other characteristics, such as development of CFU-Cs and formation of intra-aortic clusters.

We observed variability in numbers of CFU-Cs throughout E11 even among embryos of equal somitic age. We also found that prior to mid E11 (up to 44 s.p.) some AGM regions did not contain definitive HSCs. However, from mid E11 HSCs were found in all AGM regions. In explant culture conditions AGM regions from each stage at day 11 were able to generate significant numbers of definitive HSCs.

In summary, during E11 rapid changes occur in development of HSCs, which can contribute into variability of experimental results. In contrast to CFU-C, which vary rather chaotically independently of precise somitic age, the HSC numbers change in a more predictable monotonous manner. Thus, selecting specific embryo ages for experiments may provide more stable experimental outcome.

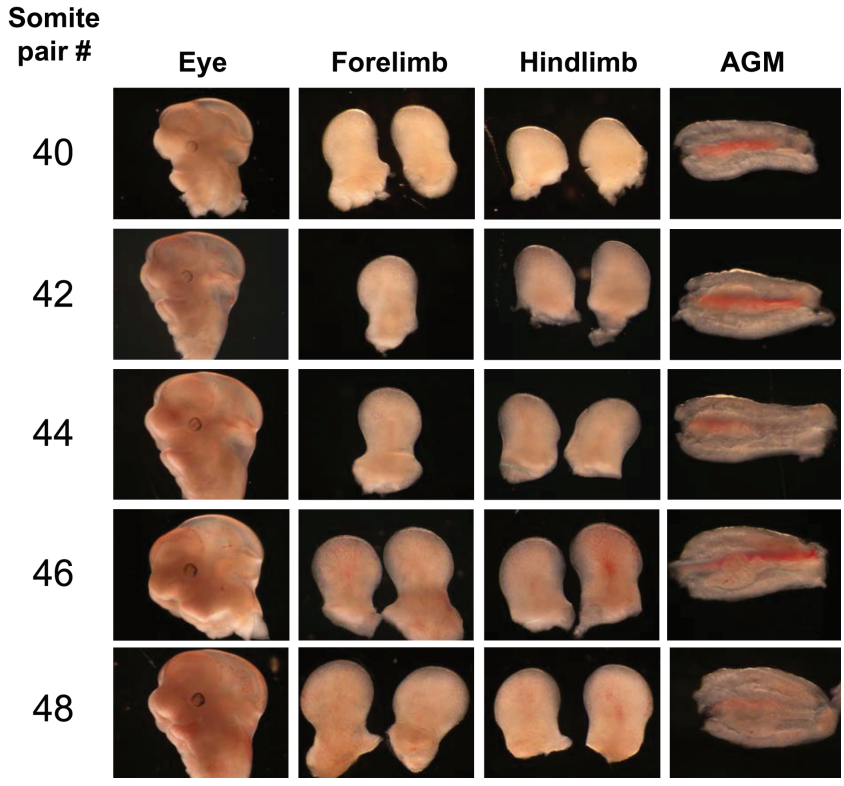


Fig. 1. Morphological characteristics of day 11 embryos staged according to somitic age. *Note changes in pigmentation of eyes, shape of forelimbs and hindlimbs and the aorta-gonad-mesonephros (AGM) region with increase of somitic age (see details in the main text).*

Results

Embryo staging

At mid-gestation, the development of 10 s.p. occurs in approximately 24 hours (Medvinsky *et al.,* Blood 1996; Medvinsky and Dzierzak, 1996). E11 encompasses a 40-48 somite pair range (Fig. 1). To facilitate staging we assessed pigmentation of eyes and limb development. At early E11 (40-44 s.p.) the outer layer of cuboidal cells, forming the optic cup, gives rise to the pigment layer of the retina (Fig. 1). By late E11 (45-48 s.p.) the retinal pigmentation spreads around the eye, becomes significantly darker and almost entirely encompasses the eye. The forelimbs develop earlier than hindlimbs and at 40 to 42 s.p. the forelimb bud is dividing into proximal and distal parts where the proximal region will develop into the arm and pectoral girdle and the distal region will become the hand plate (Fig. 1). The marginal vein can be seen adjacent to the apical ectodermal ridge of the forelimb bud but not of the hindlimb (not shown). The hindlimb does not catch up until around 44 s.p. when distal and proximal parts become apparent. At 46 s.p. the marginal vein can be seen in the hindlimb (not shown). The morphology of the AGM region changes throughout E11, characterized by an expansion of the indifferent gonads and the entire organ becomes bulkier (Fig. 1).

Intra-aortic cluster distribution at E11

IACs identified from serial sections were scored as either small 2-5 cell clusters protruding slightly into the lumen, or large 6-25 cell clusters protruding significantly into the lumen (Fig. 2). Frequently, the endothelial layer underlying large clusters can be seen distorting the circumference of the lumen (Taoudi *et al.*, 2008). IACs occupied all orientations (ventral, lateral and dorsal) along the luminal surface of the dorsal aorta, as was

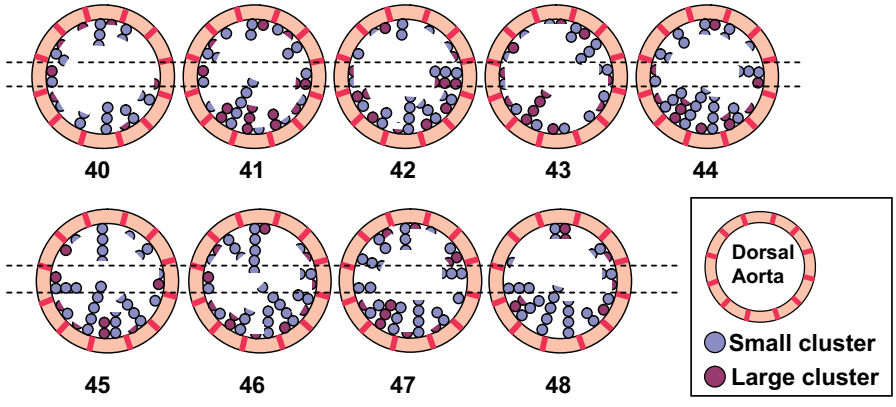


Fig. 2. Orientation of intra-aortic clusters (IACs). *Note that the majority of both large and small IACs are located in the ventral aspect. For each stage, two embryos were analysed. Half circles is a result of averaging of numbers of IACs in two different embryos.*

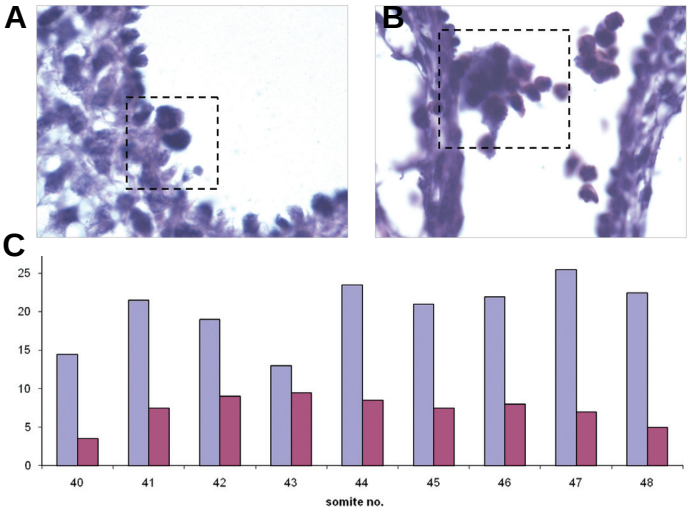
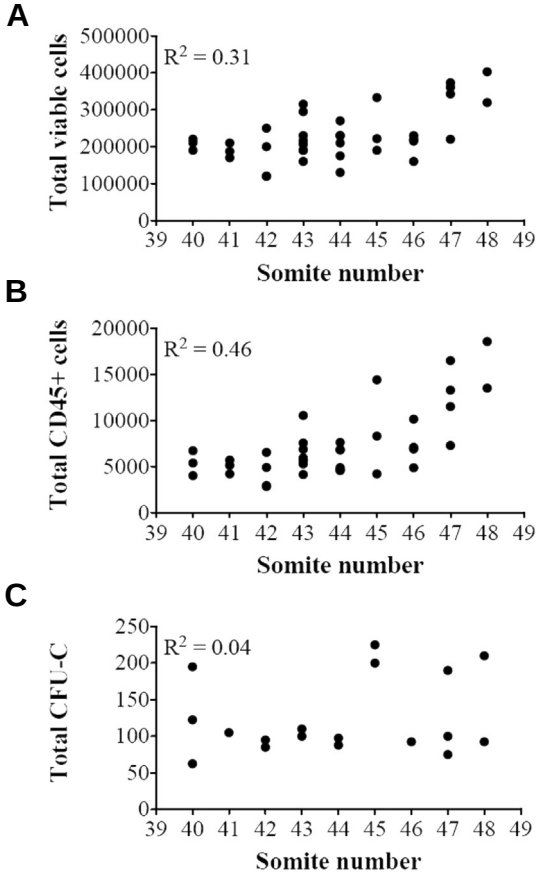


Fig. 3 (above). Numbers of intra-aortic cell clusters (IACs) in the dorsal aorta of day 11 embryos. *Numbers of small IACs* **(A)** *have slight tendency to grow with age (C). Numbers of large IACs* **(B)** *have a slight tendency to decrease with age (C).* **(C)** *The number of IACs per AGM region. Blue, small; red, large. These changes are not statistically significant. Values indicated are means calculated for two embryos analysed for each stage.*

reported previously (Taoudi and Medvinsky, 2007). However, the majority of both large and small IACs were located in the ventral aspect (Fig. 2). This was true whether the boundary for the ventral aspect includes the lateral 9 and 3 o'clock positions or not. At 43, 44 and 47 s.p. large clusters were found frequently at the 7 o'clock position sometimes associated with inter-somitic vessels.

Through day 11 the number of small clusters per AGM region fluctuated between 15 and 25 (Fig. 3). Slightly more small IACs per AGM region were found after mid day 11 of development. Large IACs were also observed at all somitic ages through day 11 of development (ranging from 3-9 per AGM region) with a modest decrease in number from mid day 11 (Fig. 3).

The overall cellularity of the AGM region increases during E11, from $2.1x10^5 \pm 1.6x10^4$ cells at 40 s.p to $3.6x10^5 \pm 5.9x10^4$ cells at 48 s.p. Significant positive correlation was observed (R^2=0.31) (Fig. 4). The number of $CD45^+$ hematopoietic cells significantly correlates with increasing somite numbers, ranging from $5.4x10^3 \pm 1.3x10^3$ cells at 40 s.p to $1.6x10^4 \pm 3.6x10^3$ cells at 48 s.p (Fig. 4). Interestingly, the number of CFU-Cs within individual AGM regions varied greatly (from 50 to 250 colonies per AGM) at the same somitic age and did not significantly correlate with increasing somite number (R^2=0.04)(Fig. 4).



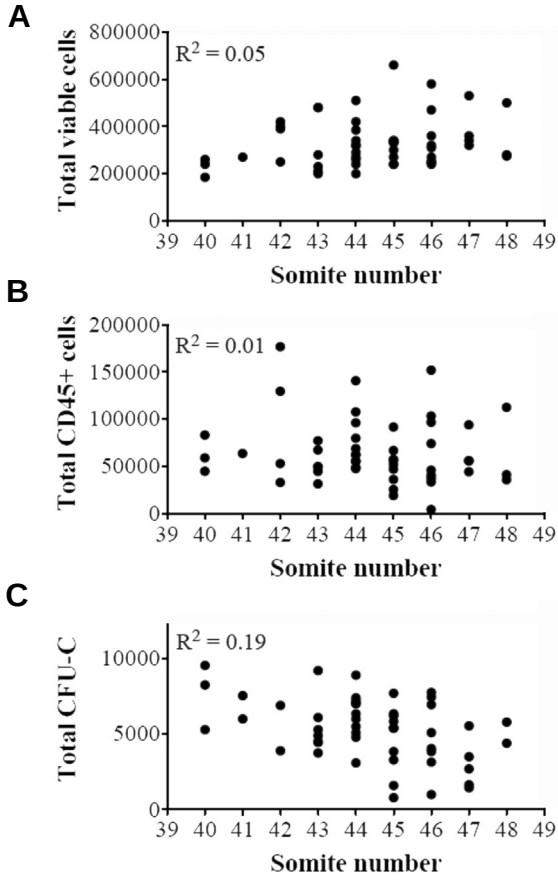
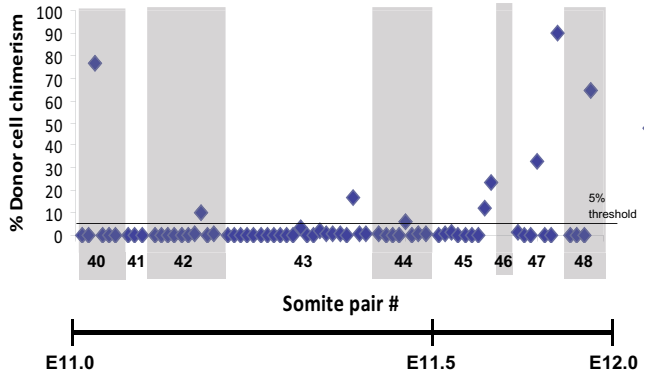


Fig. 4 (Left). Total cellularity, contents of CD45+ hematopoietic cells and CFU-Cs significantly vary within and between precise somitic stages.

Fig. 5 (Right). Total cellularity, contents of CD45+ hematopoietic cells and CFU-Cs in aorta-gonad-mesonephros (AGM) regions in cultured precisely-staged AGM regions. *Variability for these parameters enhances after the culture (compare with Fig. 6).*



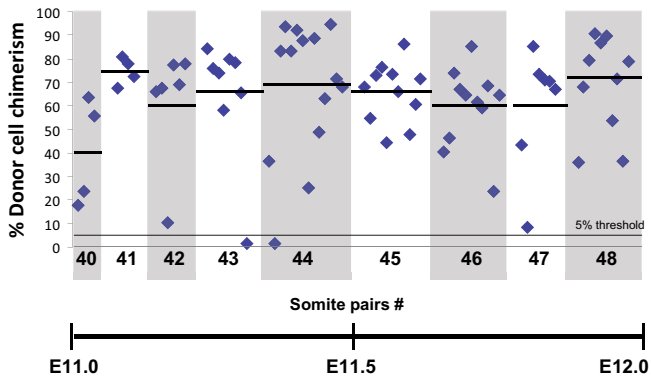


Fig. 6 (Left). Contents of definitive hematopoietic stem cells (HSCs) in the aorta-gonad-mesonephros (AGM(region increase after mid day 11. *Each irradiated recipient mouse received 0.25 e.e. of individual, uncultured, precisely-staged AGM region. Note that 12 AGM regions prior to mid day 11 (40-44sp embryos) repopulated in total only 4 out of 48 recipients. Thus only 4 out of 12 embryos contained definitive HSCs. In contrast, all 5 AGM regions older than 45 s.p. contained definitive HSCs (5 out of 20 mice were reconstituted). Each symbol represents one recipient mouse.*

Fig. 7 (Right). Production of definitive HSCs by cultured AGM regions. *Each irradiated recipient received 0.05 e.e. of individually cultured, precisely- staged AGM region. Note that despite low contents of definitive HSCs in AGM regions isolated from younger embryos, they are capable of efficient generation of HSCs in culture. Each symbol represents one recipient mouse. Horizontal bars represent means.*

Culture of the AGM region increases the inter- and intra-stage variations in CFU-Cs

AGM regions were dissected from individual embryos and cultured as whole organ explants. After 4 days the range of cellularity of individual AGMs varied more noticeably than before culture (between $2x10^5$ to $6x10^5$ cells); no significant correlation was observed between somite number and explant cellularity (R^2=0.05)(Fig. 5). $CD45^+$ numbers in individual AGM regions also fluctuated greatly after culture, ranging from $1x10^4$ to $1.7x10^5$ cells; no significant correlation with somitic age was observed (R^2=0.01)(Fig. 5). Interestingly, the capacity of AGM regions to generate CFU-Cs demonstrated a significant negative correlation with increasing developmental stage (R^2=0.19) (Fig. 5).

Long-term reconstitution by uncultured AGM regions

AGM regions isolated from individually staged embryos were dissociated and cell suspensions prepared separately. Each suspension was split into 4 aliquots and each was transplanted into an individual recipient mouse. Thus, each recipient received a 0.25e.e. dose of individually staged AGM region (Fig. 6). A total of 12 embryos between 40 to 44 s.p. were transplanted into 48 recipients. Only 4 recipients were reconstituted at high level, indicating that only 4 out of 12 embryos prior to mid day 11 contained one definitive HSC.

In similar experiments transplantation of total 5 AGM regions from older embryos (45-48 s.p.) resulted in long-term hematopoietic repopulation of 5 out of total 20 recipients transplanted (Fig. 6), indicating that each region contained one definitive HSC.

Long-term reconstitution by cultured AGM regions

Although only 4 out of 12 uncultured early pre-mid day 11 AGM regions contained definitive HSCs, after the culture all individual AGM regions developed strong HSC activity (Fig. 7). Upon transplantation of 0.05 e.e. per recipient all except two mice of entire cohort of 74 recipients were repopulated at high level. It is not possible to establish whether these two recipients were not repopulated because of technical reasons or because of intrinsic incapacity of two embryos to develop HSCs efficiently. Apart from these two non-repopulated outliers, the system is saturated with HSCs, which means that no less than 20 HSCs have been generated by each E11 AGM region in culture.

Discussion

This study was initiated as a result of our observations that quantitative outcome of experiments with AGM-derived HSCs can vary. We previously found that the number of definitive HSCs per one day 11 AGM region in (C57Bl/6 X CBA)F1 embryos is on average about 1 (Kumaravelu *et al.*, 2002). Since transferring our research to a pure C57BL/6 background we have observed a slightly reduced frequency in definitive HSC number in the E11 AGM region.

Here we staged E11 C57Bl6 embryos according to the precise somite number age and functionally assessed the hematopoietic characteristics of individual AGM regions. We have: (i) quantitatively described the positional distribution of intra-aortic clusters on the luminal surface of the dorsal aorta; (ii) determined CFU-C numbers in individual AGMs; (iii) semi-quantitatively characterized HSC activity in the AGM region. To assess the hematopoietic potential of individual embryos the analysis was further extended to cultured AGM regions.

Based on functional experiments, we have demonstrated that E11 can be split into two major stages: early day 11 (40-44 s.p.) and late day 11 (45-48 s.p.). While only 1/3 of embryos prior to mid day 11 p.c. contained definitive HSCs, starting from mid day 11 p.c. ($\geq$ 45 s.p) all embryos contained HSCs. Since each embryo contained no more than 1 HSC, the average frequency of HSCs in E11 C57Bl/6 AGM regions is less than 1 per AGM region. Thus, depending on the precise age range of embryos in a litter taken for experiment, HSC numbers can vary. Our results demonstrate that only shortly prior to colonisation of the liver at E12, HSCs in the AGM can be consistently detected.

Although some early E11 embryos contain no definitive HSCs, all cultured AGM regions were able to generate definitive HSCs.

Although two out of 48 recipients which received AGM transplants were not reconstituted, it remains unclear whether this is because of a technical reason, or because some of the embryos intrinsically had lower potential to expand HSCs.

Interestingly, direct measurement of CFU-C activity in uncultured embryos revealed significant variations, even within the same somitic age. This is despite clear difference in HSC activity prior to and after mid day 11. Thus, CFU-C activity cannot be used as a reference for age-related changes in hematopoietic activity. The yolk sac is a major source of CFU-Cs in the body of the embryo (Ghiaur *et al.*, 2008; Lux *et al.*, 2008) and we presume that cell colonisation via circulation is an inherently variable process.

Intra-aortic clusters (IACs) are considered to be a morphological manifestation of HSC activity (Godin and Cumano, 2002). Definitive HSCs originate from the ventral niche of the dorsal aorta and our recent data suggest that maturation of definitive HSCs occurs in large clusters (Taoudi *et al.*, 2008). Our current data provide quantitative analysis for dorso- ventral distribution of IACs and confirms that although IACs can be readily identified along the entire luminal surface of the dorsal aorta, their majority are localised to the ventral domain of the dorsal aorta (this is the case for both small and large IACs).

In conclusion, age variability between embryos throughout day 11 may be a contributing factor to the outcome of experiments analysing HSC development. Meanwhile, CFU-C activity is a poor indicator of HSC activity in the AGM region. This study indicates that significant changes in hematopoietic development may occur during one day.

Materials and Methods

Mice

All experiments were performed using C57BL/6 mice. Mice were housed and bred in animal facilities at the University of Edinburgh in accordance with Home Office regulations. Pregnant females were culled 11 days later and embryos removed. At this stage the embryos have characteristic eye pigmentation and limb development (Fig. 1). To accurately determine developmental stage of individual embryos the numbers of somite pairs (s.p.) were counted.

Histology

For histological analysis two embryos of each somitic stage were selected for further processing. Embryo trunks were dissected immediately inferior to the forelimb buds and immediately superior to the hindlimb buds. Tissues were fixed in 4% PFA overnight, dehydrated using sequentially increasing concentrations of ethanol and after clearing with xylene embedded in paraffin wax. Transverse sections were obtained in a rostral to caudal order and serially sectioned. Every fifteenth section (average 11 μm thickness) was mounted on slides and stained with hematoxylin (Ehrlich) and eosin. Two categories of intra-aortic clusters, small (2-5 cells) and large (6 or more cells) were distinguished and their position within the aorta was recorded (Fig. 2,3). Where clusters were found in the same position over a series of sections these were classed as one cluster. Sections were viewed and imaged using an Olympus BX61 upright light microscope and Q imaging camera with Volocity software (Improvision).

Tissue culture

Staged whole AGM regions from C57BL/6 (Ly5.2/2) embryos were dissected and cultured on the top of 0.65μm Durapore filters (Millipore, Cat. No. DVPP02500) at the gas-liquid interface in IMDM$^+$ medium (IMDM (Invitrogen), 20% fetal calf serum, L-Glutamine (4mM), penicillin/streptomycin (50 units/ml), mercaptoethanol (0.1mM), IL-3 (100ng/ml), SCF (100ng/ml) and Flt3L (100ng/ml) in 5% CO_2 at 37ºC in a humidified incubator for 4 days (Taoudi *et al.*, 2008). All growth factors were purchased from Peprotech.

Phenotypic characterisation of cell populations

Cells were analysed in individual AGMs either directly after dissection or after 4 days explant culture. Cell suspensions were obtained from individual embryos and stained with anti-CD45 (ly-5) APC and anti-CD31 (PECAM-1) PE monoclonal antibodies. Cell viability was determined by 7-aminoactinomycin D (7-AAD) exclusion. All immunoreagents were purchased from either BD Biosciences or eBioscience (San Diego, CA, USA). All samples were analysed using a FACSCalibur (BD Bioscience, Oxford, UK) and data processed using FlowJo software (TreeStar, Ashland, OR, USA).

CFU-C assay

Colony-forming (CFU-C) assay was performed on individual AGM regions (without pooling) in accord with their somitic age using M3434 medium (StemCell Technologies). Colonies were scored after 7-9 days as described (Medvinsky *et al.*, 2008).

Long-term repopulation assay

Donor AGM regions were isolated, cultured and transplanted individually into irradiated C57Bl6 (Ly5.2/1) recipients along with 20,000 bone marrow (Ly5.1/1) carrier cells. The number of transplanted uncultured and cultured cells is expressed throughout the article in doses, defined as a unit of cells equivalent to their proportion present in one fresh or cultured organ. For example a 0.05 dose is equal to 5% of the total cells present in one cultured organ. Donor chimerism was determined after 16 weeks in the peripheral blood. To this end blood samples were collected into PBS/EDTA and red blood cells lysed using PharmLyse (BD Bioscience, Oxford, UK). Donor contribution was assessed by flow cytometry using anti-Ly5.2-PE and anti-Ly5.1-APC antibodies (BD Biosciences). Dead cells were excluded by 7-aminoactinomycin D (7-AAD) staining. Mice demonstrating ≥5% donor derived chimerism after a minimum of 16 weeks were considered to be reconstituted by definitive HSCs. All samples were analysed using a FACSCalibur and data processed using FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

GraphPad Prism software (CA, USA) was used to analyze the correlation between ell/CFU-C numbers and somite number. The statistical significance of correlations was assessed using Student's t-test, differences with $p<0.05$ were regarded as significant. Correlation coefficients (R^2) are denoted for analyses. Where appropriate, data are reported as mean ± standard deviation.

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