The *SCL* gene specifies haemangioblast development from early mesoderm

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The *SCL* gene encodes a basic helix–loop–helix (bHLH) transcription factor that is essential for the development of all haematopoietic lineages. *SCL* is also expressed in endothelial cells, but its function is not essential for specification of endothelial progenitors and the role of *SCL* in endothelial development is obscure. We isolated the zebrafish *SCL* homologue and show that it was co-expressed in early mesoderm with markers of haematopoietic, endothelial and pronephric progenitors. Ectopic expression of *SCL* mRNA in zebrafish embryos resulted in overproduction of common haematopoietic and endothelial precursors, perturbation of vasculogenesis and concomitant loss of pronephric duct and somitic tissue. Notochord and neural tube formation were unaffected. These results provide the first evidence that *SCL* specifies formation of haemangioblasts, the proposed common precursor of blood and endothelial lineages. Our data also underline the striking similarities between the role of *SCL* in haematopoiesis/vasculogenesis and the function of other bHLH proteins in muscle and neural development.

Keywords: haemangioblast/haematopoiesis/*SCL*/vasculogenesis/zebrafish

Introduction

During mammalian embryogenesis, haematopoiesis occurs sequentially at several different sites. Early data suggested that haematopoietic stem cells first arose in the yolk sac and subsequently colonized the fetal liver and bone marrow. However, the yolk sac progenitors appeared to have limited potential (Moore and Metcalf, 1970; Wong *et al.*, 1986; Liu and Auerbach, 1991; Cumano *et al.*, 1993; Huang and Auerbach, 1993). More recently, an intra-embryonic source of multipotent haematopoietic stem cells has been identified in the posterior splanchnopleura (Godin *et al.*, 1993, 1995; Cumano *et al.*, 1996) and the region of the dorsal aorta, genital ridge and mesonephros (AGM) in mice (Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996). The relationship between haematopoietic stem cells in the yolk sac and those arising within the embryo remains unclear, but it has been suggested that the intra-embryonic haematopoietic stem cells colonize both yolk

sac and fetal liver (Dzierzak and Medvinsky, 1995). Haematopoietic development is also highly conserved in lower vertebrates. Teleost fish produce differentiated cells analogous to most of the mature blood lineages found in mammals (Rowley *et al.*, 1988). Embryonic haematopoiesis develops within the body of the embryo in many bony fish. Early haematopoietic stem cells first arise within the lateral mesoderm and subsequently migrate medially to form the intermediate cell mass (ICM), where they are closely associated with the dorsal aorta and axial vein (Al-Adhami and Kunz, 1977; Detrich *et al.*, 1995; Zon, 1995).

Several lines of evidence suggest a close relationship between the development of blood and endothelium in vertebrates. Both cell types emerge simultaneously during the formation of yolk sac blood islands (Sabin, 1920; Murray, 1932; Wagner, 1980), and early intra-embryonic sites of haematopoiesis are closely associated with the dorsal aorta and other vessels (Godin *et al.*, 1993, 1995; Medvinsky *et al.*, 1993; Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996; Tavian *et al.*, 1996; Wood *et al.*, 1997). Mice lacking Flk-1, an early endothelial marker, fail to generate both vasculature and haematopoietic cells (Shalaby *et al.*, 1995). Flk-1 null embryonic stem (ES) cells also fail to contribute to either endothelium or haematopoiesis in chimaeric mice, showing that Flk-1 is essential for the formation of both lineages (Shalaby *et al.*, 1997). Consistent with this concept are observations demonstrating that VEGF, the ligand for Flk-1, can stimulate formation of multipotent haematopoietic stem cells from ES cells (Kennedy *et al.*, 1997). Furthermore, single Flk-1-positive (Flk-1⁺) cells from avian embryos can develop into either haematopoietic or endothelial colonies (Eichmann *et al.*, 1997). Moreover, in the zebrafish mutant *cloche*, the numbers of both endothelial and haematopoietic cells are severely reduced (Liao *et al.*, 1997). These studies are all consistent with the notion of a common precursor for haematopoietic and endothelial cells, the haemangioblast.

The *SCL* gene (also known as *TAL-1*) was first identified as a T-cell oncogene (Begley *et al.*, 1989a,b; Finger *et al.*, 1989; Chen *et al.*, 1990). It encodes a basic helix–loop–helix (bHLH) protein normally expressed in blood, endothelium and brain (Green *et al.*, 1992; Hwang *et al.*, 1993; Kallianpur *et al.*, 1994; Drake *et al.*, 1997). Anti-sense and overexpression studies have suggested that *SCL* modulates proliferation and self-renewal of multipotent haematopoietic cells (Green *et al.*, 1991) and also acts as a positive regulator of erythroid differentiation (Aplan *et al.*, 1992). *SCL* null mice lacked yolk sac haematopoiesis (Robb *et al.*, 1995; Shivdasani *et al.*, 1995) but initially were reported to develop morphologically normal endothelium and defects of brain development were not seen (Shivdasani *et al.*, 1995). *SCL* null ES cells also failed to contribute to any definitive haematopoietic lineage

(Porcher *et al.*, 1996; Robb *et al.*, 1996). These data established an essential and cell-autonomous role for SCL in the formation or subsequent behaviour of embryonic haematopoietic stem cells. However, the role of SCL in endothelial and neuronal development remained obscure.

In this study, we have isolated the zebrafish homologue of the *SCL* gene product and demonstrate that it is co-expressed initially with markers for haematopoietic, endothelial and pronephric duct progenitors in the lateral mesoderm. Ectopic expression of SCL resulted in perturbation of vasculogenesis with excessive production of blood and endothelial precursors at the expense of somitic and pronephric duct cells. These data provide the first evidence that SCL acts to specify haemangioblast development from early mesoderm.

Results

Cloning of the *SCL* cDNA from zebrafish

To clone the *Danio rerio* (zebrafish) *SCL* gene, an embryonic cDNA library was screened for cDNAs encoding SCL-like bHLH transcription factors. The fragment used to screen the library had been amplified under low stringency conditions on genomic zebrafish DNA using two primers defined according to the 5' and 3' ends of the bHLH region of the *SCL*-related *Fugu rupripes* gene *SLP1* (Gottgens *et al.*, 1998). Four different cDNAs were isolated and sequenced. All of them encoded bHLH proteins but only one exhibited extensive similarities to SCL proteins in other vertebrates. Its sequence of 324 amino acids shared 57–59% identical residues with the human, murine and chicken SCL proteins (compared with 36 and 40% identity with the closely related murine and human LYL-1 proteins, respectively) (Figure 1a and b). Its bHLH domain (residues 185–244) was identical to that of the other vertebrate SCL proteins (Figure 1a). Outside the bHLH domain, significant conservation was also observed in both N- and C-terminal regions, suggesting the existence of additional functionally important domains. Deletion of 53 amino acids including the conserved C-terminal domain was associated with enhanced apoptosis of transformed human T cells (Leroy-Viard *et al.*, 1995). The N-terminal region has been reported to contain a transactivation domain, the activity of which was modulated by phosphorylation of two serine residues (Figure 1a, asterisks; Cheng *et al.*, 1993; Prasad and Brandt, 1997). Of these, Ser122 was conserved in zebrafish, whereas the second one, Ser172 (Prasad and Brandt, 1997), was replaced by an alanine residue.

Expression profile of *SCL* in early zebrafish development

Whole-mount *in situ* hybridization analysis of zebrafish embryos was used to characterize SCL expression (Figure 2). SCL mRNA was first detected in 1–3 somite embryos (11 hpf) in two pairs of stripes (Figure 2a–d).

One pair of stripes was situated in the body, initially in the lateral margins of the mesoderm. These trunk stripes extended anteriorly and posteriorly (compare Figure 2a with e, c with g, and d with h). While the anterior third of these trunk stripes stayed lateral (Figure 2i–n, white arrowheads), the posterior two-thirds moved towards the midline (compare Figure 2d, h and j). At this stage in

development, cells of the lateral mesoderm are known to migrate to the midline to form the ICM just ventral to the notochord (Al-Adhami and Kunz, 1977). We therefore believe that the moving SCL expression pattern reflects migration of cells rather than a wave of *SCL* gene expression traversing the mesoderm. The migrating stripes fused anteriorly and posteriorly at both ends at the 14 somite stage (15.5 hpf), resulting in a γ -shaped pattern [shown in a 15 somite stage embryo (16.5 hpf) in Figure 2j]. The cells of the loop of this γ -structure subsequently 'zipped up' in the craniocaudal direction to form a single stripe in the position of the ICM (Figure 2j, l and m, black arrowheads). This process was complete at the 20 somite stage (19 hpf) (data not shown). The ICM (between black arrowheads in Figure 2m) is known to give rise to haematopoietic cells which enter the circulation between 24 and 26 hpf (Zon, 1995). SCL expression was observed in circulating blood cells at 30 hpf (Figure 2o, arrowhead indicates SCL-positive blood cells within the heart), but subsequently declined to undetectable levels by 50 hpf (Figure 2p). While these data suggest that SCL-expressing cells within the ICM became blood cells, it was not clear from these initial results what the function of the SCL-positive (SCL^+) cells in the arms of the γ -structure might be.

The second pair of stripes of SCL-expressing cells was located in the head in the position of the head mesenchyme (Figure 2a, b, e and f). The most anterior of these cells ceased to express SCL between the 10 and the 15 somite stage (13.5 and 16.5 hpf) (compare Figure 2e and i), while the cells posterior to the eye anlagen continued to express SCL until ~23 hpf (Figure 2n). At first, the nature of these cells was unclear. At 21 hpf, SCL was also noted transiently in the position of primary motoneurons of the spinal cord (data not shown) and at 50 hpf in the location of proliferative neuronal cells in the diencephalon, in the midbrain and in a few cells of the hindbrain (Figure 2p, arrowhead).

During mouse development, SCL has also been shown to be expressed in haematopoietic progenitors (yolk sac, AGM, fetal liver) as well as in the midbrain, hindbrain and the spinal cord (Green *et al.*, 1992; Kallianpur *et al.*, 1994; Silver and Palis, 1997). The pattern of SCL expression during development is therefore highly conserved from teleost fish to mammals.

SCL is expressed in multipotent progenitors in early lateral mesoderm

In zebrafish, the lateral mesoderm of the trunk and tail gives rise to at least three different tissues: the haematopoietic, endothelial and pronephric lineages. To investigate the relationship of the progenitors for these three lineages within the lateral mesoderm, the expression patterns of haematopoietic [GATA-2 (Detrich *et al.*, 1995; A.R.F.Rodaway, unpublished data)], endothelial [Flk-1 (Fouquet *et al.*, 1997; Liao *et al.*, 1997; Sumoy *et al.*, 1997)] and pronephric duct markers [Pax-2 (Krauss *et al.*, 1991)] were studied in pairwise comparisons with that of SCL. In double whole-mount *in situ* hybridization experiments, embryos of different stages were first labelled for SCL-expressing cells in red and then stained for GATA-2, Flk-1 or Pax-2 expression in blue. Cells co-expressing both SCL and the second marker therefore

a

HUMAN	1	-MTE-RPPS-----EAARSDFQLEGR--DAAEASMAFF---HLVLLNGVAKETS
MOUSE	1	-MTE-RPPS-----EAARSDFQLEGR--DAAEASMAFF---HLVLLNGVAKETS
CHICKEN	1	-MTMDRPPAPPPSSSDPRDARRHDFEADATSEPDSSRGGMEFPAE--FQLLLNGAKEAG
ZEBRAFISH	1	MMEK LKSEQ--FHLSPSAEGCASPFRGDDPAR-GKQEGTTAETGEHRIPEELNGVAKETA
HUMAN	43	RAAAAEF---PVIELGARCGPGGGPAGGGGAARDKRCRDATTAEARHRVPTTELCPPPG
MOUSE	43	RAAAAEF---PVIELGARCGPGGGPAGGGGAARDKRCRDATTAEARHRVPTTELCPPPG
CHICKEN	58	RPSGPFRAAAVPIELVRGG-----SLDTSKR-SAAEAMORAPGAEPCr---
ZEBRAFISH	58	HHATELKK-EVAVIELSRGG-----SADTKGR-ELKAELSRVQTTELCPPI
HUMAN	99	PAPAPAPASVTAEPLPGDGRMVQLSPPALAAFAAFGRALLYS-LSOPLASLGSFFGEPDA
MOUSE	99	PAPAPAPASAPAEPLPGDGRMVQLSPPALAAFAAFGRALLYS-LSOPLASLGSFFGEPDA
CHICKEN	103	-----A--AAACEARMVQLSPPALPLQP-PGRAMLYN-LGQPLGTIGSFFGEPDS
ZEBRAFISH	105	ELLEL----PRDPLSDTRMVQLSPPAFPLPA--RAMLYSNMTTPLATINSGLAGDAEQ
HUMAN	158	FFMFTNNNRVKRRPSPYEMEITDGPHTKVVRRIFTNSRERWRQQNVNGAFAE LRKLIPTH
MOUSE	158	FFMFTNNNRVKRRPSPYEMEITDGPHTKVVRRIFTNSRERWRQQNVNGAFAE LRKLIPTH
CHICKEN	151	FSMYGSN-RVKRRPSPYEMEITDGPHTKVVRRIFTNSRERWRQQNVNGAFAE LRKLIPTH
ZEBRAFISH	157	YGMYPSEN-RVKRRPAPYEVEINDGSQPKVVRRIFTNSRERWRQQNVNGAFAE LRKLIPTH
HUMAN	218	PPDKKLSKNEILRLAMKYINFLAKLLNDQE--EEGTQRAKTGKDPVVGAGGGGGGGGGA
MOUSE	218	PPDKKLSKNEILRLAMKYINFLAKLLNDQE--EEGTQRAKFGKDPVVGAGGGG--AGGGI
CHICKEN	210	PPDKKLSKNEILRLAMKYINFLAKLLNDQE--EEGNORGKVNKD-----SGI
ZEBRAFISH	216	PPDKKLSKNEILRLAMKYINFLAKLLNDQEDMVGGEAPARAND-----SR-----DATE
HUMAN	276	PPDLLQDVLSPNSSCGSSLDGAASPDSTEEFAPKHT---ARSLHFAMLPAA D GAGPR
MOUSE	274	PPEDLLQDVLSPNSSCGSSLDGAASPDSTEEFAPKHT---SRSLHFAMLPAA D GAGPR
CHICKEN	255	VQEDLLQDMLSPNSSCGSSLDGAASPDSTEEHDTLDSKH--ARNLHFAILPVEGSAQR-
ZEBRAFISH	266	VRD DLLQEMLSPNSSCGSSLDGDASPESPTEDQSSVESRPSARGLHSSLP LDGNAQR-

b

Fig. 1. Zebrafish SCL protein sequence comparisons. (a) Alignment of vertebrate SCL proteins. Within the basic helix-loop-helix domain, the basic sequence is marked with a white bar, the helices with black bars. Two serine residues reported to be important for the regulation of mammalian SCL activity are marked with asterisks. The nucleotide sequence of the zebrafish SCL cDNA is available from DDBJ/EMBL/GenBank under accession No. DR_SCL AF038873. (b) A radial phylogenetic tree of SCL-related and other bHLH proteins. The zebrafish SCL protein clearly falls into the family of vertebrate SCL proteins. The DDBJ/EMBL/GenBank accession Nos of the proteins included in the tree are: z-SCL (AF038873), c-SCL (S20085), m-SCL (A37864), h-SCL (A36358), d-SCL (L20771), h-LYL-1 (A30988), m-LYL-1 (S16678), h-Tal-2 (A41629), h-NSCL-1 (A45075), h-NSCL-2 (B45075), m-ASH-1 (S28186), z-ASH-a (I50507), z-MyoD (Z36945), m-MyoD (M84918), z-NeuroD (AF017302) and m-NeuroD (Q60867). Abbreviations: d, *Drosophila*; h, human; m, mouse; z, zebrafish.

appeared purple. In addition, sequential staining of thin (10 µm) sections was performed to confirm co-expression of genes in single cells. For this purpose, double *in situ* hybridizations were performed on embryos. The embryos were stained for SCL in red, sectioned, and the sections

documented, before proceeding to stain the same section for expression of the second gene.

At the six somite stage (12.5 hpf), SCL and the haematopoietic marker GATA-2 were co-expressed in the lateral mesoderm (cells between black arrowheads in

Figure 3a). This was expected as both genes are known to be expressed in haematopoietic progenitors. However, SCL⁺ cells could also be stained for expression of the endothelial marker Flk-1 (Figure 3d, cells between black arrowheads) and the pronephric duct marker Pax-2 (Figure 3j). Sequential staining of embryos indicated that at the six somite stage, cells expressing SCL and Pax-2 (compare Figure 3i and j, white arrowheads) were medial to cells expressing Pax-2 only (Figure 3j, black arrowhead). This is seen more easily in 10 µm sections, which also confirm at the single cell level that SCL was expressed in a subpopulation of the Pax-2-positive (Pax-2⁺) cells (compare Figure 3k and l). The co-expression of SCL, GATA-2, Flk-1 and Pax-2 within the same population of cells suggests that at this stage SCL⁺ cells have not yet committed themselves to the blood lineage and might still be multipotent.

At the 10 somite stage (13.5 hpf), SCL and Pax-2 expression patterns had already begun to separate (data not shown) and were clearly distinct at the 15 somite stage (Figure 3m). By contrast, SCL, GATA-2 and Flk-1 were still co-expressed in 10 somite stage embryos. Sequential staining of 10 µm sections for SCL and Flk-1 expression confirmed co-expression of both genes in single cells (compare Figure 3e and f). During the period of extension of the tail and convergence of the lateral mesoderm, SCL and GATA-2 were still co-expressed (Figure 3b and c) whereas distinct expression domains of SCL and Flk-1 became apparent (Figure 3g). At the 18 somite stage (18 hpf), Flk-1⁺ cells that no longer expressed SCL had reached the midline below the notochord where the dorsal aorta forms (the blue stripe indicated by the black arrow in Figure 3g). A stripe of cells expressing Flk-1 and SCL remained in a more lateral position (purple cells indicated by the black arrowhead in Figure 3g). They were probably the progenitors of the axial vein that later ceased to express SCL (data not shown). Between the two stripes of Flk-1-expressing endothelial progenitors, a population of cells expressing SCL but not Flk-1 was discernible on close inspection (the red stripe indicated by a white arrow in Figure 3g). These cells later came to lie in the centre of the ICM between the endothelial precursors, differentiated into red blood cells and entered circulation by 24–26 hpf.

These data suggest that the SCL⁺/Flk-1⁺ cells seen in the 10 somite stage embryos are able to give rise to SCL⁺/Flk-1⁺ haematopoietic and SCL⁺/Flk-1⁺ endothelial cells and, thus, represent common progenitors for both blood and endothelium, the proposed haemangioblast.

The double *in situ* hybridization experiments described above also shed light on the nature of the SCL⁺ cells in

the head mesenchyme and in the arms of the γ-structure. These cells were likely to be functionally different from those SCL⁺ cells that reached the ICM, in that previous studies have shown that, unlike in the ICM, there is no GATA-1 expression and no blood formation in these more anterior areas (Detrich *et al.*, 1995; Figure 5g). Our results showed that most of these more anterior cells also expressed GATA-2 (white arrowheads in Figure 3a and b) and Flk-1 (white arrowheads in Figure 3d, g and h) and therefore were likely to be endothelial progenitors involved in the formation of the head vessels and the vessels of the Ducti Cuvieri (Fouquet *et al.*, 1997; Liao *et al.*, 1997; Sumoy *et al.*, 1997) that link the venous system to the heart. In the region of the Ducti Cuvieri, SCL⁺ cells (white arrowhead in Figure 3n) were adjacent to Pax-2⁺ pronephric progenitors (black arrowhead in Figure 3n). This observation suggests that these SCL⁺ cells may participate in the vascularization of the pronephros or, alternatively, in the initiation of definitive haematopoiesis in the pronephros (Al-Adhami and Kunz, 1977).

Overexpression of SCL in zebrafish embryos causes overproduction of blood

Microinjection of mRNA into *Xenopus* and, more recently, into zebrafish embryos has been used to produce ectopic expression of transcription factors that normally exhibit a restricted expression pattern (Hopwood and Gurdon, 1990; Hopwood *et al.*, 1991; Ferreira *et al.*, 1994; Turner and Weintraub, 1994; Lee *et al.*, 1995; Blader *et al.*, 1997). To investigate the function of SCL in the development of early lateral mesoderm, SCL mRNA was injected into 2- or 4-cell zebrafish embryos. Varying amounts of SCL mRNA were injected in pilot studies to determine the RNA concentration that produced a consistent phenotype with minimal non-specific toxicity. By 30 hpf, the majority of embryos injected with 70 pg of SCL mRNA exhibited a dramatic abnormal phenotype (Table I). As shown in Figure 4, this was characterized by a marked excess of haemoglobinized blood cells in the ICM (Figure 4b, black arrowhead), a striking absence of blood circulation despite a beating heart (Figure 4b) and an abnormally curved axis. The presence of red blood cells and normal pigmentation (not visible in Figure 4 because embryos were treated with 1-phenyl-2-thiourea to suppress pigmentation after 24 hpf) showed that the injected embryos were not retarded. The embryos were not ventralized since head and notochord structures which are the first to be affected in ventralized embryos (Neave *et al.*, 1997) were morphologically normal. By 50 hpf, half of the affected embryos were able to establish restricted or normal blood circulation

Fig. 2. SCL expression in early zebrafish development. Views are lateral (lat.) with anterior left and dorsal top, anterior (ant.) with dorsal top, dorsal (dors.) with anterior left, and posterior (post.) with dorsal top. (a–d) At the three somite stage (10–11 hpf), expression of SCL was first seen in two pairs of stripes. The head pair was located in the head mesenchyme, the trunk pair in the posterior lateral mesoderm. (e–h) At the 10 somite stage (13.5 hpf), the trunk stripes extended anteriorly and posteriorly, and started to move towards the midline. Convergence of SCL-expressing cells was also observed in the head. (i–n) The most anterior cells in the head ceased to express SCL between the 10 and 15 somite stage (16.5 hpf) (compare e and i). Cells just behind the eye anlagen continued to express SCL up to 23 hpf (i, k, m and n). The trunk stripes formed a γ-shaped pattern. Anterior cells stayed lateral (white arrowheads in i–n), while the posterior two-thirds of the stripes formed a loop with cells meeting anteriorly and posteriorly (black arrowheads in i and j). The cells of this loop subsequently ‘zipped up’ craniocaudally (cells between black arrowheads in i–m), forming a single stripe of SCL-positive cells in the intermediate cell mass (ICM) by the 20 somite stage (19 hpf) as shown in a 21 and a 23 hpf embryo (m and n). (o) By 30 hpf, SCL expression could be seen in circulating blood cells in the heart and around the yolk (arrowhead), but disappeared in the head mesenchyme (compare n and o). (p) By 50 hpf, SCL expression was no longer detectable in the blood but clearly visible in cells of the diencephalon, mid- and hindbrain (arrowhead). (q) A diagram illustrating the spatial and temporal pattern of SCL expression between the three somite and 21 hpf stage in a schematic drawing of a dorsal view.

while the other half of the embryos never gained circulation, developed severe oedema and died after 6–7 days.

Sections through the severely affected 50 hpf embryos revealed high numbers of haemoglobinized cells in the ICM. Strikingly, the trunk vessels, dorsal aorta and axial vein, as well as one or both pronephric ducts, frequently were missing. In addition, a massive reduction of somitic tissue was observed, often more marked on one side of the embryo (Figure 4, compare c with d). By contrast, notochord and neural tube always appeared normal. Control injections were performed using similar amounts of mRNA for *lacZ*, green fluorescent protein (GFP), zebrafish E12 (Wulbeck *et al.*, 1994) and a fusion protein consisting of the bHLH region of a neuronal bHLH protein fused to VP16. None of these produced a similar phenotype (data not shown). The specificity of the phenotype described here is also evidenced by the fact that it was not reported following injection of mRNA for multiple other bHLH proteins into frog or zebrafish embryos in other laboratories (Hopwood and Gurdon, 1990; Hopwood *et al.*, 1991; Ferreira *et al.*, 1994; Turner and Weintraub, 1994; Lee *et al.*, 1995; Blader *et al.*, 1997).

The increased number of haemoglobinized cells in SCL-injected embryos suggested an excess of blood in the region of the ICM that could reflect either excessive blood formation or abnormal distribution of blood cells. To distinguish between these possibilities, *in situ* hybridization on embryos prior to circulation was performed using markers for blood progenitors (SCL and GATA-1). Endogenous SCL expression was assessed using a probe from a region of the 3' untranslated region (UTR) absent from the injected SCL mRNA, and which therefore discriminates between endogenous and exogenous SCL mRNA. In SCL-injected embryos, the number of cells expressing endogenous SCL in the lateral mesoderm was increased at the 10 somite stage (13.5 hpf) and was often asymmetrical (compare Figure 5a with d; Table I). The increased expression of endogenous SCL was also visible in whole mounts and sections just before the onset of

circulation (compare Figure 5b with e, and c with f; Table I). SCL-injected embryos also contained increased numbers of cells positive for GATA-1 (compare Figure 5g with h; Table I). These observations therefore demonstrate that ectopic SCL expression resulted in overproduction of haematopoietic progenitors capable of differentiating into haemoglobinized cells.

SCL overexpression increased the number of haemangioblast-like cells

The failure of circulation and lack of vessel formation in SCL-injected embryos suggested that early endothelial development might be abnormal. To investigate this issue, SCL-injected and control embryos were hybridized to probes for Flk-1 (Fouquet *et al.*, 1997; Liao *et al.*, 1997;

Table I. The phenotype of zebrafish embryos after injection of SCL RNA

Abnormal phenotype	Dev. stage	Normal	Abnormal	Total
No circulation and increased number of red blood cells in the ICM	30 hpf	33	64	97
Increased SCL expression	50 hpf	28	13	41
	10 s	7	21	28
	22 hpf	4	11	15
Increased GATA-1 expression	22 hpf	5	31	36
Increased Flk-1 expression	10 s	4	11	15
	15 s	16	23	39
	22 hpf	5	32	37
	30 hpf ^a	1	15	16
Increased Fli-1 expression	22 hpf	1	14	15
Fewer Paraxis-expressing cells	6 s	12	10	22
Fewer MyoD-expressing cells	15 s	24	30	54
Fewer Pax2-expressing cells in the region of the pronephric ducts	10 s	48	2	50
	24 hpf	10	9	19
	30 hpf ^a	3	13	16

^a*In situ* hybridizations were only performed on embryos without circulation and with an increased number of red blood cells in the ICM.

Fig. 3. SCL is expressed in multipotent progenitors in the lateral mesoderm. Views of embryos are lateral (lat.) with anterior left and dorsal top, posterior (post.) with dorsal top, and dorsal (dors.) with anterior left. (a–c) Dual colour *in situ* hybridization for SCL and GATA-2. At the six somite stage (12 hpf), SCL (red) and GATA-2 (blue) were widely co-expressed (purple) in the posterior lateral mesoderm (black arrowheads in a) and in the head mesenchyme (white arrowhead in a). Some cells, that were only positive for SCL, are marked with a white arrow (a). At the 17 somite stage (17.5 hpf), GATA-2 was co-expressed with SCL in the cells that form the arms (white arrowheads in b) and the loop (black arrowheads in b) of the γ -shaped SCL expression pattern. A section through the trunk of the 17 somite stage embryo revealed cells expressing SCL and GATA-2 that are migrating towards the midline (cells between white arrowheads in c). Note that the cells between the purple cells and the notochord are not expressing either of the two genes (black arrowhead in c). (d–h) Dual colour *in situ* hybridization for SCL and Flk-1. At the six somite stage (12 hpf), SCL (red) and Flk-1 (blue) were co-expressed in the head mesenchyme (white arrowhead in d) and the posterior lateral mesoderm (black arrowheads in d). Flk-1 expression was also observed in part of the hindbrain and the tailbud (white arrows in d), as described previously (Liao *et al.*, 1997). In a 10 somite stage embryo (13.5 hpf), sequential staining of 10 μ m sections (taken from the embryo shown in Figure 5a) confirmed SCL and Flk-1 expression in single cells in the lateral mesoderm (compare cells marked by black arrowhead in e and f). By the 18 somite stage (18 hpf), precursors of the dorsal aorta had migrated to the midline, lost SCL expression and had reached a position below the notochord (the blue stripe indicated by the black arrow in g). They were followed by a wave of SCL⁺ cells (the red stripe indicated by the white arrow in g) and a separate wave of SCL⁺/Flk-1⁺ cells (the purple stripe indicated by the black arrowhead in g), which became the precursors of the axial vein. The anterior SCL⁺/Flk-1⁺ cells of the caudal stripes remained lateral (white arrowheads in g and h) in the position of the Ducti Cuvieri. (i–n) Dual colour *in situ* hybridization for SCL and Pax-2. At the six somite stage (12 hpf), sequential staining of embryos (compare i and j) and sections (compare k and l) showed that SCL (red) and Pax-2 (blue) expression patterns overlapped in the lateral mesoderm, with SCL⁺/Pax-2⁺ cells (white arrowheads in i–l) being medial to cells that only express Pax-2 (black arrowhead in j and l). Pax-2⁺ cells at the midbrain–hindbrain border are marked by a white arrow in (j). By the 15 somite stage (16.5 hpf) (m), there were clearly separate stripes of posterior SCL⁺ (the red stripe indicated by the white arrowhead in m) and Pax-2⁺ cells (the blue stripe indicated by the black arrowhead in m). The former migrated into the position of the ICM while the latter stayed behind to form the pronephric ducts. At 22 hpf, the anterior SCL⁺ precursors of the trunk stripes (white arrowhead in n) were lateral to the Pax-2⁺ cells of the pronephros (black arrowhead in n), suggesting that the former might contribute to the vascularization of the pronephros or be involved in the induction of definitive haematopoiesis in the kidney. The black arrows in (n) mark spinal cord cells stained for Pax-2. (o) A diagram of the expression patterns of SCL, GATA-2, Flk-1 and Pax-2 of transverse sections through the trunk of six, 10 and 18 somite stage embryos. ICM, intermediate cell mass; LM, lateral mesoderm; NC, notochord; NT, neural tube; PM, paraxial mesoderm; SM, somitic mesoderm.

Fig. 4. SCL overexpression in zebrafish embryos produced consistent phenotypic abnormalities. Views of embryos are lateral (lat.) with anterior left and dorsal top. (a) and (b) Gross phenotype of SCL-injected and uninjected embryos 50 hpf. Embryos were grown in 0.003% 1-phenyl-2-thiourea to prevent pigmentation, anaesthetized, fixed and stained for haemoglobin with diaminofluorene (DAF). SCL-injected embryos had no circulation of haemoglobinized cells. There was an increased number of DAF-stained blood cells in the ICM (black arrowhead in b) and almost no blood cells in the heart and vessels around the yolk sac which contained most of the blood cells in uninjected embryos (black arrowhead in a). Oedema was already visible in injected embryos (white arrowhead in b). The axis of many SCL-injected embryos was kinked (compare a and b). (c) and (d) Sections through the trunk of 50 hpf uninjected and SCL-injected embryos. SCL-injected embryos revealed an elevated number of blood cells (DAF-stained brown cells in d, arrowhead indicates an example of blood cells in an abnormal site), absence of dorsal aorta, axial vein and pronephric ducts, as well as severely reduced somitic tissue (c and d). AV, axial vein; DA, dorsal aorta; NC, notochord; NT, neural tube; PD, pronephric ducts; SM, somitic mesoderm.

Sumoy *et al.*, 1997) and Fli-1 (L.Brown, T.F.Schilling, A.R.F.Rodaway, D.C.Hickleton, T.Jowett, P.W.Ingham, R.K.Patient and A.D.Scharrocks, in preparation), both early markers of endothelial development in zebrafish. In SCL-injected embryos, there was a striking increase in the number of Fli-1⁺ and Flk-1⁺ cells (compare Figure 6a with b, and c with d; Table I) which were distributed aberrantly (compare Figure 6e with f and g).

The increased number of haematopoietic and endothelial progenitors in SCL-injected 22 hpf embryos suggested that ectopic expression of SCL might be expanding a population of common precursors for both lineages. This would predict increased numbers of SCL⁺/Flk-1⁺ cells in 10 somite stage embryos (13.5 hpf). To test this prediction, 10 μ m sections of an SCL-injected 10 somite stage embryo (shown in Figure 5d after staining of the whole mount for SCL) were stained sequentially for SCL and Flk-1. These sections revealed a massive unilateral increase in the number of SCL-expressing cells in the non-axial mesoderm

(Figure 6i). The vast majority of these SCL⁺ cells also expressed Flk-1 (Figure 6j). This suggests that ectopic SCL expression increased the number of early SCL⁺/Flk-1⁺ haemangioblast-like progenitors.

To follow these cells later during development of the SCL-injected embryo, we sequentially stained 22 hpf embryos for Flk-1 (in red) and either SCL or GATA-1 (in blue). Flk-1 and SCL expression patterns were still overlapping at this stage (compare Figure 6k and l), but cells immediately above the yolk cell extension proved to express only SCL but not Flk-1 (black arrowheads in Figure 6k and l). Cells in the same position were also found to express GATA-1 (Figure 6n, black arrowhead), suggesting that these cells are the progenitors of the haemoglobinized cells found in injected embryos after 50 hpf (Figure 4b). The whole mounts indicated that Flk-1 and GATA-1 expression patterns were not overlapping (compare Figure 6m and n), and this was confirmed by sections through the trunk (Figure 6h).

Fig. 5. SCL overexpression in zebrafish embryos increased the number of blood cells. Views are dorsal (dors.) with anterior bottom and lateral (lat.) with anterior left and dorsal top. (a–f) Exogenous SCL injection dramatically increased the number of cells expressing endogenous SCL in 10 somite stage (13.5 hpf) embryos (compare a and d) and in 24 hpf embryos (compare b and e). Sections through the trunk of 22 hpf embryos confirmed this finding and often showed that the migration towards the midline of these SCL-positive cells had been prevented (compare c and f). (g) and (h) SCL overexpression increased the number of GATA-1-positive cells in 22 hpf embryos.

Our data therefore suggest that the cells which initially co-expressed Flk-1 and SCL at the 10 somite stage developed into two domains in 22 hpf embryos. A ventral haematopoietic region ($SCL^+/GATA-1^+$) contained cells which were capable of terminal differentiation as witnessed by excess production of haemoglobinized cells in SCL-injected embryos. The more dorsal domain continued to

express SCL and Flk-1, suggesting the continued presence of haemangioblast-like cells. The impairment of vessel formation in SCL-injected embryos might reflect either a direct effect of SCL overexpression on the differentiation programme of individual endothelial precursors, or may be an indirect consequence of the mesodermal disorganization.

SCL directs non-axial mesoderm into haematopoietic and endothelial lineages

Excessive production of haemangioblast-like progenitors with concomitant loss of somitic and pronephric duct cells strongly suggested that ectopic SCL expression was altering the fate of non-axial mesodermal cells. The abnormalities induced by injection of SCL mRNA were often more marked on one or the other side of SCL-injected embryos, an observation likely to reflect asymmetrical distribution of the progeny of injected cells. Consistent with this explanation, co-injection of *lacZ* and SCL mRNA demonstrated frequent unilateral β -galactosidase activity in embryos with ipsilateral morphological abnormalities (Figure 7b). To investigate the timing of the events responsible for the observed abnormalities, embryos were hybridized with probes for somitic mesoderm (MyoD), paraxial mesoderm (Paraxis) or pronephric duct tissue (Pax-2).

MyoD is an early marker for somitic mesoderm (Weinberg *et al.*, 1996). In 15 somite stage embryos (16.5 hpf), MyoD expression was normal on the side of the embryo that displayed little β -galactosidase activity (Figure 7b, white arrowhead), but was markedly reduced on the side that had received most of the injected mRNA (Figure 7b, black arrowhead; Table I). Moreover, Flk-1 expression was up-regulated in the opposite manner—increased expression was confined to the side of the embryo that exhibited β -galactosidase activity [Figure 7c, purple stain, arrowheads indicate injected (black) and uninjected (white) sides]. These results suggest that SCL was altering the fate of cells normally destined to become somitic muscle.

In view of these results, we studied an even earlier marker, one for the paraxial mesoderm. Embryos were hybridized with a probe for Paraxis which is normally expressed both in early paraxial mesoderm and in newly formed somites (Burgess *et al.*, 1995; S.Shanmugalingam and S.W.Wilson, in preparation). In six somite stage (12 hpf) SCL-injected embryos, Paraxis expression in the paraxial mesoderm was markedly reduced, asymmetrically in the example shown, and somite boundaries were hardly visible (compare Figure 7d with e, arrowhead; Table I). These data suggest that ectopic SCL expression converted the fate of paraxial mesodermal cells at a very early stage.

Sections of severely affected SCL-injected embryos showed that there was frequent loss of one or both pronephric ducts (Figure 4d). Pax-2 was therefore used as an early marker for the pronephric lineage (Krauss

et al., 1991). At the 10 somite stage (13.5 hpf), Pax-2 expression appeared normal (Table I), suggesting that ectopic SCL expression was converting early mesoderm into blood and endothelial progenitors but not into pronephric precursors. However, Pax-2 expression was clearly reduced in the region of one or both pronephric ducts by 22 hpf (26 somite stage) (compare Figure 7f and g arrowheads; Table I) and 30 hpf (Table I). These data may reflect diversion of putative multipotent progenitors within the lateral mesoderm away from the pronephric duct lineage, or alternatively may represent an effect of enforced SCL expression on subsequent pronephric duct differentiation.

These results are in accord with the concept that ectopic SCL expression converted non-axial mesodermal cells into haematopoietic and endothelial lineages at the expense of somitic and pronephric duct fates. This suggests that the role of SCL in haematopoiesis and vasculogenesis is analogous to that of MyoD and NeuroD, which are able to convert fibroblasts into muscle cells *in vitro* (Davis *et al.*, 1987) and non-neural ectodermal cells into neuronal cells in *Xenopus* embryos (Lee *et al.*, 1995), respectively. However, it was possible that instead of converting cell fate, SCL expression in non-axial mesoderm resulted in apoptosis of somitic and pronephric duct progenitors followed by a secondary proliferation of normal haematopoietic and endothelial precursors. Two lines of evidence argue against this interpretation. First, the TUNEL assay was used to look for evidence of apoptosis in embryos at time points that preceded or coincided with the earliest molecular changes that we had observed. Only slightly increased apoptosis was observed in SCL-injected embryos, but it was never particularly localized in the non-axial mesoderm (Figure 7h–k). Secondly, we frequently observed aberrant expression of haematopoietic or endothelial markers in locations spatially separate from the main domain of haematopoietic/endothelial cells. This is difficult to reconcile with a model involving secondary proliferation of blood and endothelial progenitors, but is fully consistent with alterations in mesodermal cell fate. Interestingly, while in SCL-injected embryos we consistently observed SCL⁺ (Figure 5f, arrowhead), Fli-1⁺ (Figure 6b, arrowhead) or Flk-1⁺ (Figure 6g, arrowhead) cells that were well separated from the main domain of SCL, Fli-1 or Flk-1 expression, we only rarely observed ectopic cells positive for the later haematopoietic marker GATA-1 (Figure 5h), suggesting that environmental cues not present at the most ectopic locations are likely to be

Fig. 6. SCL overexpression in zebrafish embryos caused an overproduction of haemangioblast-like cells. Views of embryos are lateral (lat.) with anterior left and dorsal top. Sections are shown with dorsal top. (a–g) Overproduction of Fli-1-expressing (a and b) and Flk-1 expressing (c and d) endothelial progenitors in SCL-injected 22 hpf embryos. Some clusters of Fli-1-expressing cells (arrowhead in b) were clearly separate from the main domain of Fli-1-expressing cells. Flk-1 *in situ* hybridizations showed that the formation of the dorsal aorta (DA in c) and the axial vein (AV in c), easily recognizable in the uninjected embryos (c), was perturbed in SCL-injected embryos. Transverse sections through the trunk of embryos (e–g) revealed variability in the abnormal pattern of Flk-1 expression (compare e with f and g), presumably reflecting the different distribution of the progeny of injected cells. The reduction of somitic mesoderm was often more marked on one side of the embryo and led to a kinked axis as indicated by the position of the neural tube (NT) and the notochord (NC) (f). Ectopic Flk-1 expression separate from the main domain of Flk-1-expressing cells was observed in a number of sections (arrowhead in g). (i and j) Massive increase of SCL⁺/Flk-1⁺ cells in SCL-injected 10 somite stage embryos (13.5 hpf). Sections of SCL-injected 10 somite stage embryo (sections taken from the embryo shown in Figure 5d) showed a massive unilateral increase in the number of cells expressing endogenous SCL (i). Subsequent staining of the same 10 μ m section demonstrated co-expression of SCL and Flk-1 (j). (h) and (k–n) Development of SCL⁺/Flk-1⁺ cells into two domains in SCL-injected 22 hpf embryos. Flk-1 and SCL expression domains were mostly overlapping (i and j), whereas Flk-1 and GATA-1 did not seem to be co-expressed (k and l). Transverse sections through the trunk of an embryo stained for Flk-1 and GATA-1 confirmed that the Flk-1 and GATA-1 expression domains were indeed separate (h). Cells just above the yolk cell extension that were Flk-1 negative (black arrowheads in k and m) proved to be SCL and GATA-1 expressing (black arrowheads in l and n, respectively), suggesting that they are true blood progenitors. AV, axial vein; DA, dorsal aorta; LM, lateral mesoderm; NC, notochord; NT, neural tube; PM, paraxial mesoderm.

necessary for full haematopoietic differentiation. Taken together, these observations argue strongly for a dominant effect of SCL expression on mesodermal cell fate.

Discussion

Previous studies have shown that SCL is a critical regulator of haematopoiesis, and is essential for the development of all haematopoietic lineages (Robb *et al.*, 1995, 1996; Shivdasani *et al.*, 1995; Porcher *et al.*, 1996). However, these loss-of-function studies were unable to show whether SCL functioned to specify the formation of haematopoietic stem cells from mesoderm or whether SCL was required for the survival and subsequent differentiation of haematopoietic stem cells. The gain-of-function experiments reported here demonstrate for the first time that SCL can programme early mesodermal cells to form haematopoietic and endothelial progenitors at the expense of other non-axial mesodermal cell fates (Figure 8).

We show that SCL is expressed during zebrafish development in haematopoietic progenitors, as evidenced by the co-expression of SCL and GATA-2, and was also expressed in circulating blood cells. The haematopoietic expression of SCL is therefore conserved in mouse (Green *et al.*, 1992; Kallianpur *et al.*, 1994), *Xenopus* (Turpen *et al.*, 1997; E.M.Read and R.K.Patient, unpublished data) and zebrafish. We also show co-expression of SCL and the early endothelial marker Flk-1 in the posterolateral mesoderm and the head mesenchyme in early zebrafish embryos. We provide evidence that in SCL-injected embryos the population of SCL⁺/Flk-1⁺ cells is massively increased in the posterior mesoderm. Studies in uninjected and SCL-injected embryos suggest that these SCL⁺/Flk-1⁺ cells are the progenitors for both blood and endothelium. In uninjected embryos, a separation of SCL and Flk-1 expression domains coincides with segregation of cells into blood and endothelial precursors. This segregation occurs during their migration towards the midline where they form the ICM just below the notochord. Whereas SCL⁺ blood cells enter circulation, Flk-1⁺ endothelial cells line the major vessels of the trunk. In SCL-injected embryos, a separation of the apparently uniform population of early SCL⁺/Flk-1⁺ cells into separate haematopoietic (SCL⁺/GATA-1⁺/Flk-1⁻) and endothelial/haemangioblast (SCL⁺/Flk-1⁺) domains also supports the view that the early cells are bipotent. Thus, our results strongly suggest that SCL is capable of specifying haemangioblast formation, a concept which accords well with several lines of circumstantial evidence for the existence of haemangioblasts during development (Choi *et al.*, 1998; Nishikawa *et al.*, 1998 and references therein).

Our data also demonstrate striking similarities between

the role of SCL in haematopoiesis and vasculogenesis, and the functions of other bHLH transcription factors such as MyoD and NeuroD in the development of muscle and neuronal tissues, respectively. Ectopic expression of these proteins was shown to cause a change of fate in certain susceptible tissues. While MyoD converts fibroblasts into myoblasts (Davis *et al.*, 1987), NeuroD was reported to induce the differentiation of neurons from *Xenopus* non-neural ectoderm (Lee *et al.*, 1995). Ectopic SCL expression in zebrafish embryos resulted in expansion of haematopoietic and endothelial precursors at the expense of somitic and pronephric duct tissues. Since only a slight increase in apoptosis was observed and cell death was not particularly concentrated in the non-axial posterior mesoderm, we believe that the reduction in somitic and pronephric duct tissues was not a consequence of cell death and replacement by proliferation but rather was due to the diversion of cells into the haematopoietic and endothelial lineages. This interpretation is supported by the expression of endogenous SCL, Flk-1 and Flt-1 in ectopic positions clearly separate from the main domains of haematopoietic and endothelial cells in SCL-injected embryos.

Loss of SCL function in mice abolished the development of all haematopoietic lineages but did not prevent the formation of morphologically normal endothelial cells (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). Defects in the formation of the nervous system, where SCL is expressed as well, were also not reported. These data suggested an essential role for SCL in haematopoietic stem cell formation or function, but could not ascribe a function to SCL in vasculogenesis or neurogenesis. The differences between the loss-of-function phenotype and the gain-of-function experiments reported here further emphasize the parallels between our data and the role of bHLH proteins in the control of cell fate in other tissues. In myogenesis and neurogenesis, it is clear that determination and differentiation are controlled by a cascade of bHLH proteins with overlapping expression patterns (Jan and Jan, 1993; Weintraub, 1993). Thus, ectopic expression of the myogenic bHLH proteins, MyoD, Myf5, MRF4 and myogenin, all induced muscle differentiation in transfected cells (Weintraub, 1993). MyoD and Myf-5 individually were dispensable for skeletal muscle development (Braun *et al.*, 1992; Rudnicki *et al.*, 1992), but disruption of both genes resulted in the complete absence of skeletal muscle (Rudnicki *et al.*, 1993). Similarly, ectopic expression of any of the four bHLH genes in the *Drosophila* achaete-scute complex produced an identical phenotype, increased numbers of microchaetae and macrochaetae (Rodriguez *et al.*, 1990; Brand *et al.*, 1993; Dominguez and Campuzano, 1993), whereas loss-of-function studies demonstrated different effects for each gene (Martinez and Modolell,

Fig. 7. SCL overexpression increased the number of haemangioblast-like cells at the expense of other non-axial mesodermal fates. Views of embryos are dorsal (dors.) with anterior left, and lateral (lat.) with anterior left and dorsal top. (a–c) Reduced number of MyoD- and increased number of Flk-1-expressing cells in SCL-injected 15 somite stage (16.5 hpf) embryos. SCL- and *nlacZ*-injected 15 somite stage (16.5 hpf) embryos were first stained for β -galactosidase activity (blue) and MyoD expression (red), photographed (b), then stained for Flk-1 expression (purple) (c), and compared with similarly processed uninjected embryos (a). Injected embryos often showed unilateral expression of *nlacZ*. Such embryos exhibited ipsilateral reduced MyoD expression (black arrowhead in b) relative to the uninjected side (white arrowhead in b) together with an ipsilateral increase in Flk-1 expression (black arrowhead in c). (d) and (e) Paraxis expression in SCL-injected embryos. In injected six somite stage (12 hpf) embryos, Paraxis expression was often reduced on one side (arrowhead in e) and the borders of the somites were hardly visible. (f) and (g) Pax-2 expression in SCL-injected embryos. Gaps (arrowheads in g) were often observed in the line of Pax-2-expressing prospective pronephric duct cells on one or both sides of the SCL-injected 22 hpf embryos. (h–k) Loss of non-axial mesoderm did not result from increased apoptosis. Uninjected (h and j) and SCL-injected (i and k) one and 10 somite stage embryos (10 and 13.5 hpf) were subjected to a TUNEL assay. The assay revealed slightly increased apoptosis that was never particularly localized to the non-axial mesoderm.

Fig. 8. SCL specifies haemangioblast-like cells from early posterior mesoderm. Injection of SCL mRNA into zebrafish embryos increased the number of SCL- and Flk-1-co-expressing haemangioblast-like progenitors (thick arrows) at the expense of paraxial mesoderm, somites and pronephric ducts (dashed arrows). A subpopulation of these haemangioblast-like cells was able to differentiate into haemoglobinized blood cells but it remains unclear whether terminal endothelial differentiation occurred. SCL had no influence on notochord formation.

1991; Brand *et al.*, 1993; Dominguez and Campuzano, 1993) In addition, NeuroD-deficient mice developed an apparently normal nervous system (Naya *et al.*, 1997), although NeuroD induced conversion of *Xenopus* non-neural ectoderm into neuronal cells (Lee *et al.*, 1995). These results are all consequences of the unique spatial and temporal patterns with which each bHLH gene is expressed during development. The loss-of-function phenotype associated with any particular bHLH protein is therefore thought to reflect the extent to which its expression pattern overlaps with functionally related bHLH proteins. In the case of SCL, functional redundancy with related bHLH proteins may explain the normal development of endothelial cells in SCL null mice.

Two recent lines of evidence support the novel concept that SCL plays an important role in endothelial development. Visvader *et al.* (1998) have partially rescued the haematopoietic phenotype of SCL null mice and thereby revealed a requirement for SCL in angiogenic remodelling of the yolk sac capillary network. In zebrafish, Liao *et al.* (1998) have shown that ectopic expression of SCL partially rescued the blood and vascular defects of *cloche* mutant embryos. Our results are consistent with both of these reports but, in addition, demonstrate that SCL specifies a common progenitor for both blood and endothelium at the expense of other mesodermal fates.

Our data also raise the issue of the relationship between haematopoietic/endothelial and pronephric precursors. In six somite stage (12 hpf) zebrafish embryos, SCL, GATA-2, Flk-1 and Pax-2 expression domains overlapped in the posterior lateral mesoderm, consistent with the existence of a population of cells capable of giving rise to blood, endothelium and pronephric duct cells. Ectopic SCL expression did not alter the number of Pax-2-expressing cells at the 10 somite stage (13.5 hpf), suggesting that SCL did not change the number of putative multipotent progenitors in early lateral mesoderm. However, the subsequent loss of Pax-2 expression and the abnormalities of pronephric duct development suggest that SCL was diverting the fate of the putative multipotent cells from pronephric duct to blood and endothelial fates or, alternatively, that enforced SCL expression perturbed the differentiation of pronephric duct cells.

The pattern of SCL expression was also remarkably conserved in the nervous system. Our data demonstrate SCL expression in the diencephalon, midbrain and hindbrain, together with transient expression in primary

motoneurons of the spinal cord in zebrafish embryos, an expression pattern very similar to that described in mice (Green *et al.*, 1992; Kallianpur *et al.*, 1994; A.M.Sinclair, B.Gottgens, M.L.Stanley, S.Bahn, M.Gering, L.Pardanaud, M.Klaine, A.Bench, S.Aparicio, M.-J.Sanchez, J.L.Fordham and A.R.Green, in preparation) This remarkable degree of conservation suggests an important role for SCL in the development of the nervous system. The studies reported here have focused on the consequences of ectopic SCL expression for the posterolateral mesoderm development. Future experiments with various specific neuronal markers may reveal aspects of SCL function in the developing brain.

Materials and methods

cDNA cloning and plasmid constructs

To screen zebrafish cDNA libraries for SCL-related bHLH genes, we used a 160 bp DNA fragment amplified under low stringency conditions (annealing temperature of 45°C for 1 min) on zebrafish genomic DNA between primers A (5' GTCTTCACCAACAGCCG 3') and B (5' CCAGGAAGTTGATGTACTT 3') defined according to the 5' and 3' ends of the bHLH region of the SCL-related bHLH gene *SLP-1* (Gottgens *et al.*, 1998), respectively. First, 1.2×10^6 recombinant plaques of a somite stage zebrafish cDNA library (9–16 hpf) (David Grunwald) prepared in the vector λ ZAP II (Stratagene, La Jolla, CA) were screened at moderate stringency (hybridization at 65°C, washes at 65°C in $2 \times$ SSC and 0.1% SDS). From a tertiary screen, Bluescript phagemids were rescued from 13 positive λ ZAPII clones and their inserts partially sequenced. The original 13 clones represented three different cDNA clones and one genomic DNA clone. Only one of the cDNAs (plasmid pZE6 containing a 1.9 kb insert), found three times, encoded a protein very similar to SCL in other vertebrates as judged by a BLAST search (Altschul *et al.*, 1990) of GenBank and EMBL databases. However, it turned out that the cDNA was not full-length. Therefore, a second gastrula and somite stage cDNA library (Qi-ling Xu) was screened under the same conditions described previously with a 400 bp *SmaI-KpnI* subfragment of pZE6 encoding the C-terminus of the SCL protein behind the bHLH region. Two identical cDNA clones were obtained with 2527 bp inserts encoding a full-length SCL reading frame (in plasmid pZN2). The DNA sequence is available from DDBJ/EMBL/GenBank under accession number DR_SCL AF038873.

Computing

DNA sequences were processed with the help of the Wisconsin Sequence Analysis Package of GCG. The BLAST program was applied to compare partial cDNA sequences with GenBank (Altschul *et al.*, 1990) and EMBL databases. Multiple sequence alignments were done with the Clustal_X (Thompson *et al.*, 1997) and Boxshade 3.21 programs. Radial phylogenetic trees generated with the PHYLIP program (version 3.572) (Felsenstein, 1989) were based on ClustalX alignments. Photographs of embryos and sections were scanned and processed using the Adobe Photoshop and Freehand programs.

Whole-mount *in situ* hybridizations

Breeding zebrafish were maintained and embryos were raised and staged according to Westerfield (1993). Whole-mount *in situ* hybridizations were carried out as previously described (Jowett and Yan, 1996). All RNA probes used were labelled with digoxigenin (DIG) except the Flk-1, GATA-2 and Pax-2 probes which were used in double *in situ* hybridizations and were labelled with fluorescein. Detection of the antibody-alkaline phosphatase conjugate was done using BM-Purple, Fast Red or 5-bromo-4-chloro-3-indolylphosphate (BCIP, blue) (Boehringer Mannheim, Germany). After *in situ* hybridization, embryos were re-fixed in 4% paraformaldehyde, transferred into 80% glycerol and photographed. Embryos that were to be sectioned were rehydrated and either embedded in 1.5% agarose/5% sucrose for cryostat sectioning or transferred into ethanol and embedded in JB4 methacrylate (Agar Scientific, UK) for microtome (Leica Jung RM2055) sectioning. Some of the sections were stained with toluidine blue. For sequential staining of thin sections, embryos were co-hybridized with DIG and fluorescein-labelled probes, stained for one marker in red, embedded in agarose and sectioned. Sections were documented and stained for the expression of the second gene in purple using BCIP/NBT (4-nitro blue tetrazolium chloride).

Diaminofluorene staining and TUNEL assay

The staining of haemoglobinized cells with diaminofluorene was done as described by Weinstein *et al.* (1996). TUNEL assays on one and 10 somite stage, as well as 24 and 30 hpf zebrafish embryos, were performed. The assay was based on the original method (Gavrieli *et al.*, 1992) that was adapted for zebrafish embryos by Juliet Williams using the ApoptTag Terminal Transferase Kit (Oncor, USA).

Preparation of mRNA for injection and antisense RNA probes

To generate mRNA for injection, the entire SCL reading frame was first cloned into the vector pBUT-MT2. The vector pBUT-MT2 is a derivative of plasmid pBUT2 which consists of the 5' and 3' UTRs of *Xenopus* β -globin (from pSP64T; Krieg and Melton, 1984) ligated into pBluescript (Stratagene, USA) with a polylinker replacing the *Bgl*II site of pSP64T. pBUT2-MT was derived from pBUT2 by replacing its 3' UTR with the 3' UTR of pSP64T-MEN (a gift from Kathy Weston; Badiani *et al.*, 1994) which also encodes a Myc epitope tag. To clone the SCL gene in-frame with the Myc tag reading frame of the vector, we performed a PCR between the primers C (5' CTCCTCTAGAGCCACCATTGGAAGAACTGAAATCCGAGCAATT 3') and D (5' CTCCTCGAGACCGCTGGGCATTTCCGTCACGCG 3') using Vent DNA polymerase (New England Biolabs, USA). The PCR fragment was gel purified, cut with *Xba*I and *Xho*I that were introduced into the sequence through the primers, and ligated into the *Xba*I- and *Xho*I-digested plasmid. The DNA sequence of the insert was confirmed. The plasmid was linearized with *Eco*RI and the gene was transcribed *in vitro* with the help of the T3 mMessage mMachine Kit (Ambion, USA) yielding capped RNA for injection. The linearized plasmids pCSGFP2 [a derivative of pBGFp/RN3P (Zernicka-Goetz *et al.*, 1996) generated by J.Haseloff and J.Pines] and pSP64T β (a gift from D.Wilkinson) were transcribed with T7 and SP6 (Ambion, USA) to yield synthetic GFP and nuclear *lacZ* RNA for injection, respectively.

To prepare SCL antisense RNA probes, plasmid pZE62 was used. This plasmid was derived from pZE6 by restriction digestion with *Eco*RI and religation to yield pZE61 that was then cut with *Eco*RV and *Xho*I, made blunt-ended with T4 DNA polymerase, and religated. The new plasmid named pZE62 thus only carried the *Eco*RI-*Eco*RV fragment 3' of the SCL reading frame, allowing us to pick up the expression of the endogenous SCL gene only.

To make DIG- and fluorescein-labelled antisense RNA probes, plasmids containing fragments of the SCL (pZE62), GATA-1 (Detrich *et al.*, 1995), Flk-1 (Fouquet *et al.*, 1997), Fli-1 (L.Brown, T.F.Schilling, A.R.F.Rodaway, D.C. Hickleton, T.Jowett, P.W.Ingham, R.K.Patient and A.D.Scharrocks, in preparation), Paraxis (cloned by S.Shanmugalingam, gift from S.W.Wilson), Pax2 (Krauss *et al.*, 1991) and MyoD (Weinberg *et al.*, 1996) cDNAs were linearized, and run-off transcripts were generated using either T3 or T7 RNA polymerase (Promega, USA) and digoxigenin-11-UTP or fluorescein-12-UTP (Boehringer Mannheim, Germany).

Microinjection studies

Two- or 4-cell stage zebrafish embryos were injected with 200 pl of capped SCL RNA in the range of 180–500 ng/ μ l. mRNAs encoding GFP and nuclear *lacZ* were co-injected to monitor injection efficiencies

by fluorescence in blue light (480 nm) using an FITC filter set or staining embryos with X-Gal (Griffin *et al.*, 1995), respectively. Injection of SCL mRNA at a concentration of 350 ng/ μ l was found to be optimal, giving a high number of embryos with the described phenotype and only ~10% clearly abnormal or dying embryos that were not taken for further studies.

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