Figure 1.1

Mouse Embryonic Mesoderm and Haematopoietic Development

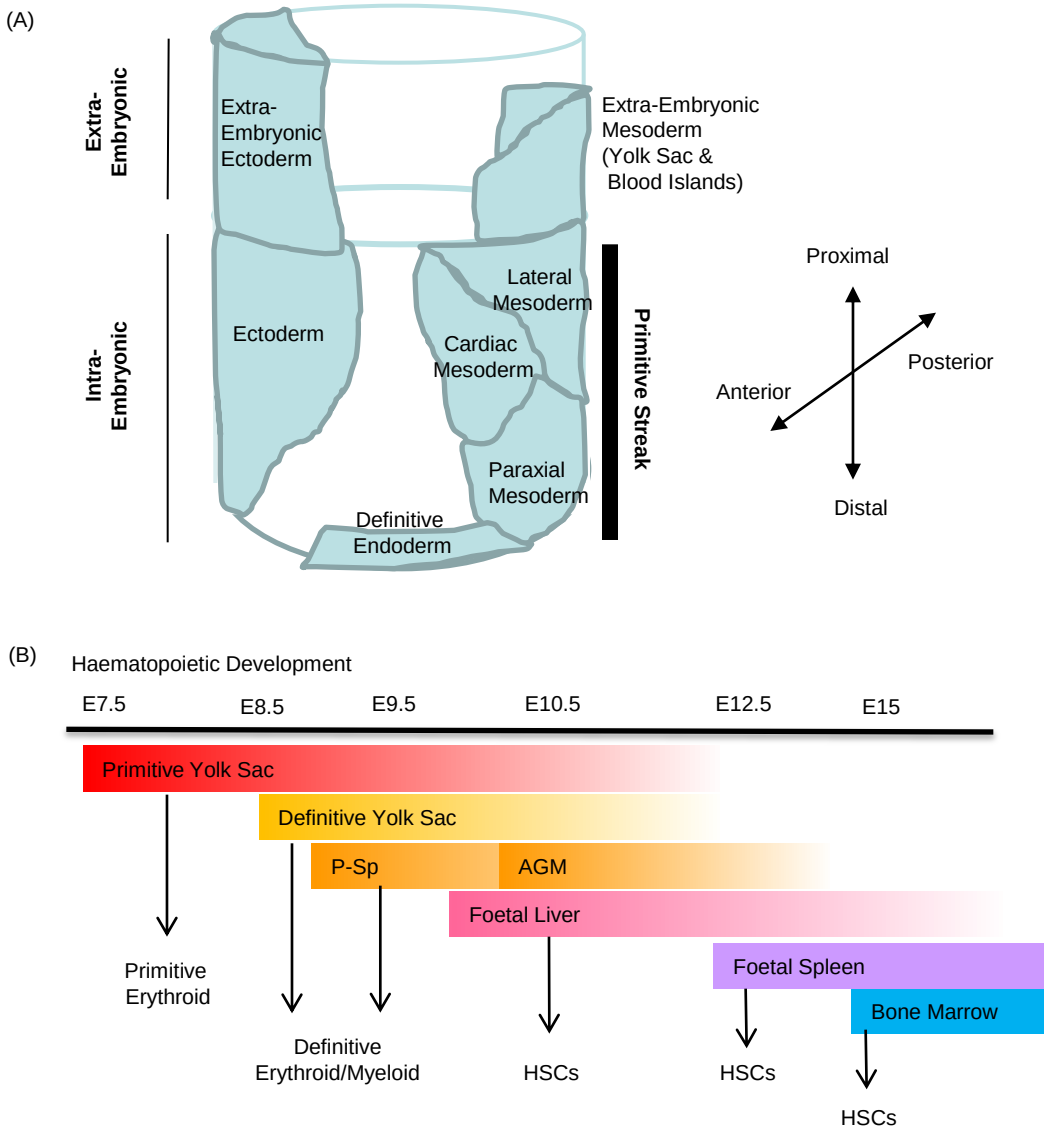


Figure 1.1 Mesoderm and haematopoietic development in the mouse embryo. (A) Schematic representing the regionalisation of developing mesoderm populations, from the primitive streak, during early stages of mouse embryogenesis (E5.5 to E7). (B) Timeline of haematopoietic development within the mouse embryo (adapted from Dzierzak *et al.* 2008).

Figure 1.2

Haematopoietic & Cardiac Development in the Mouse Embryo

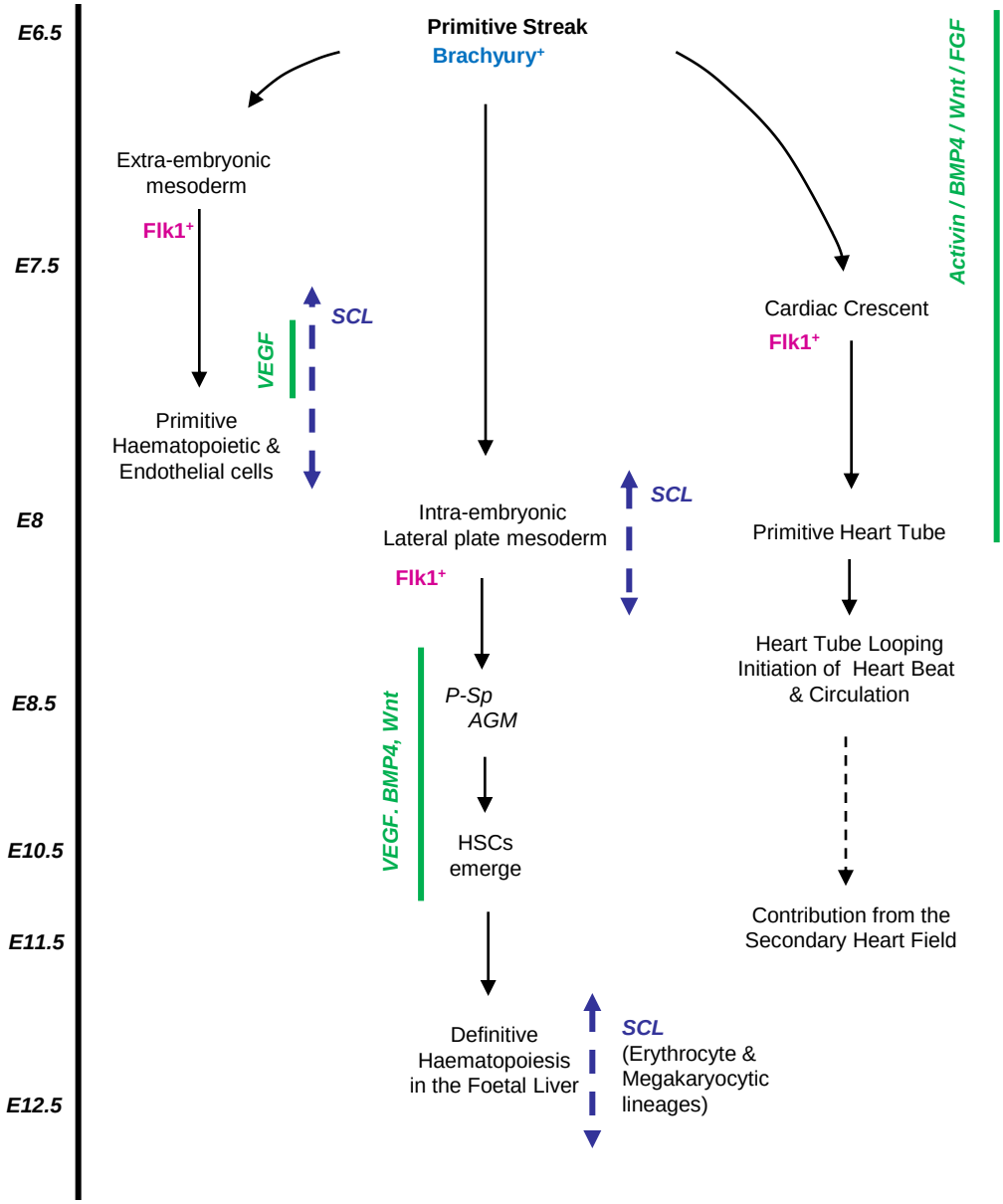


Figure 1.2 Flow diagram representing the successive waves of primitive streak derived haematopoiesis and cardiac development in the mouse embryo. Stages which require key signalling pathways (Wnt, Activin/Nodal, BMP, VEGF) are indicated in green. Expression of important cell surface markers and transcription factors are indicated in red and blue respectively. .

Figure 1.3

Haematopoietic & Cardiac Development in the Zebrafish Embryo

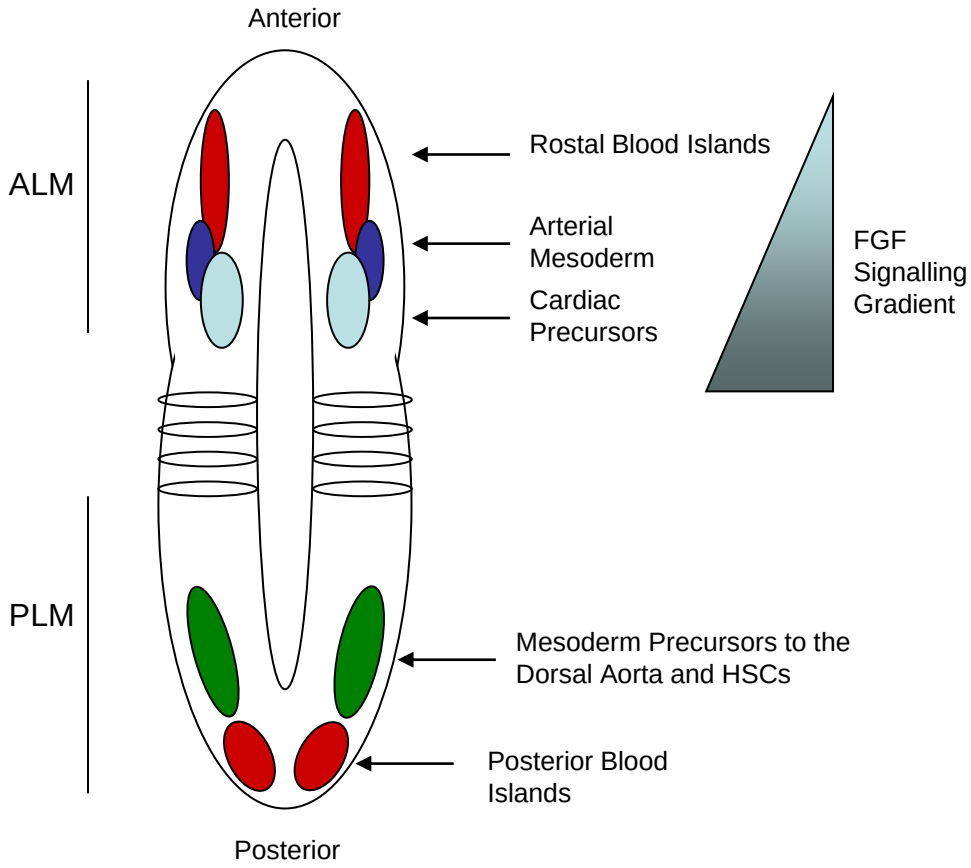


Figure 1.3 Schematic showing the location of distinct mesoderm populations within the anterior lateral plate mesoderm (ALM) and posterior lateral plate mesoderm (PLM) in the zebrafish embryo at the 7-somite stage. The triangle highlights the gradient of FGF expression in the AML , which controls the differentiation of anterior haemangioblasts into blood, arterial or cardiac precursor cells (with high levels of FGF resulting in cardiac differentiation and low levels resulting in haematopoietic differentiation). Figure adapted from Monterio *et al*, 2011 and Simones *et al*, 2011.

Figure 1.4

Using the Mouse ES/EB System to Model Development

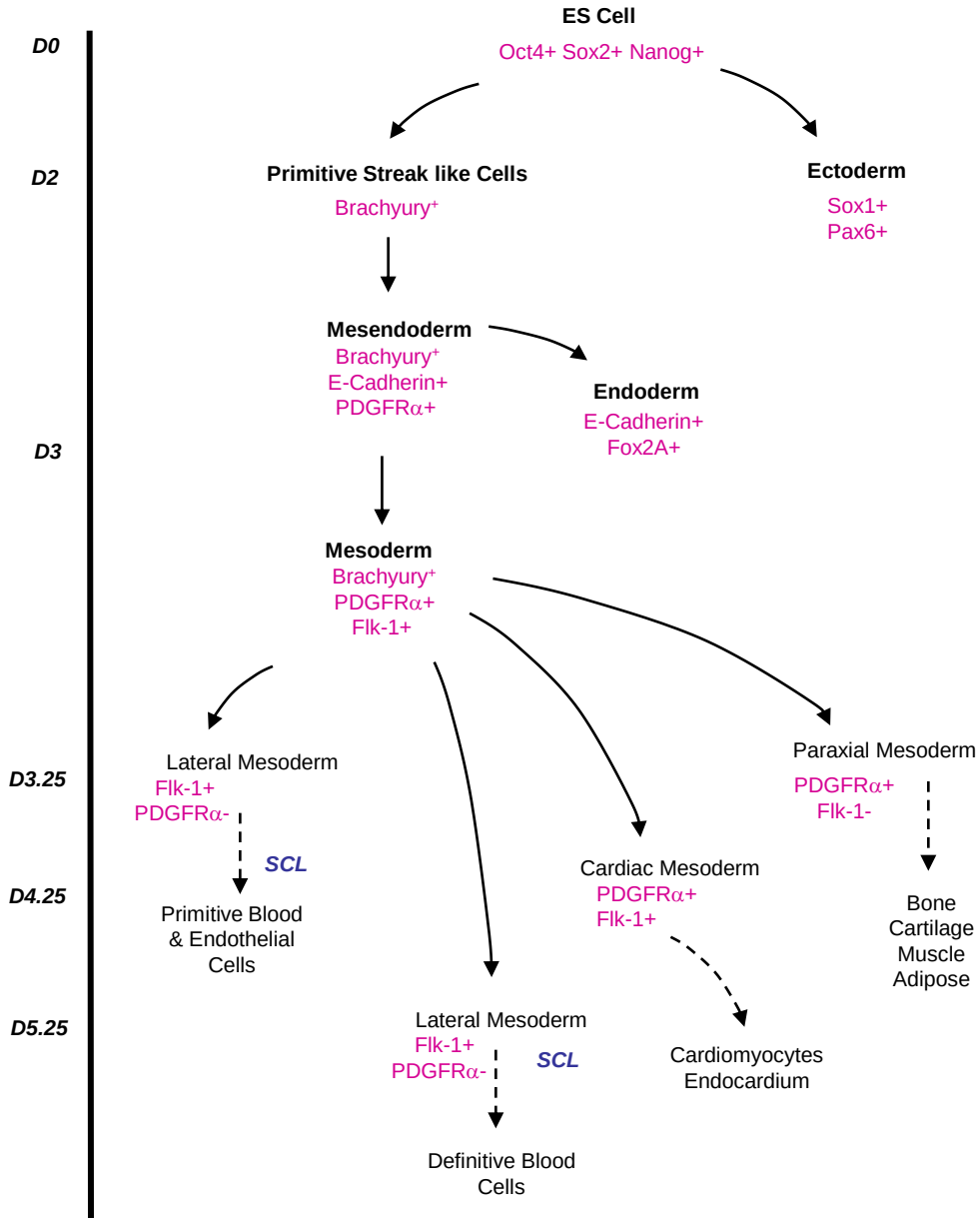


Figure 1.4 Flow diagram representing the successive waves of primitive streak derived haematopoiesis and cardiac development in the mouse embryo. Expression of key lineage markers are indicated in red. Expression of the SCL at and key time points in development is indicated in blue.

Figure 1.5

The Haematopoietic Transcription Factor SCL

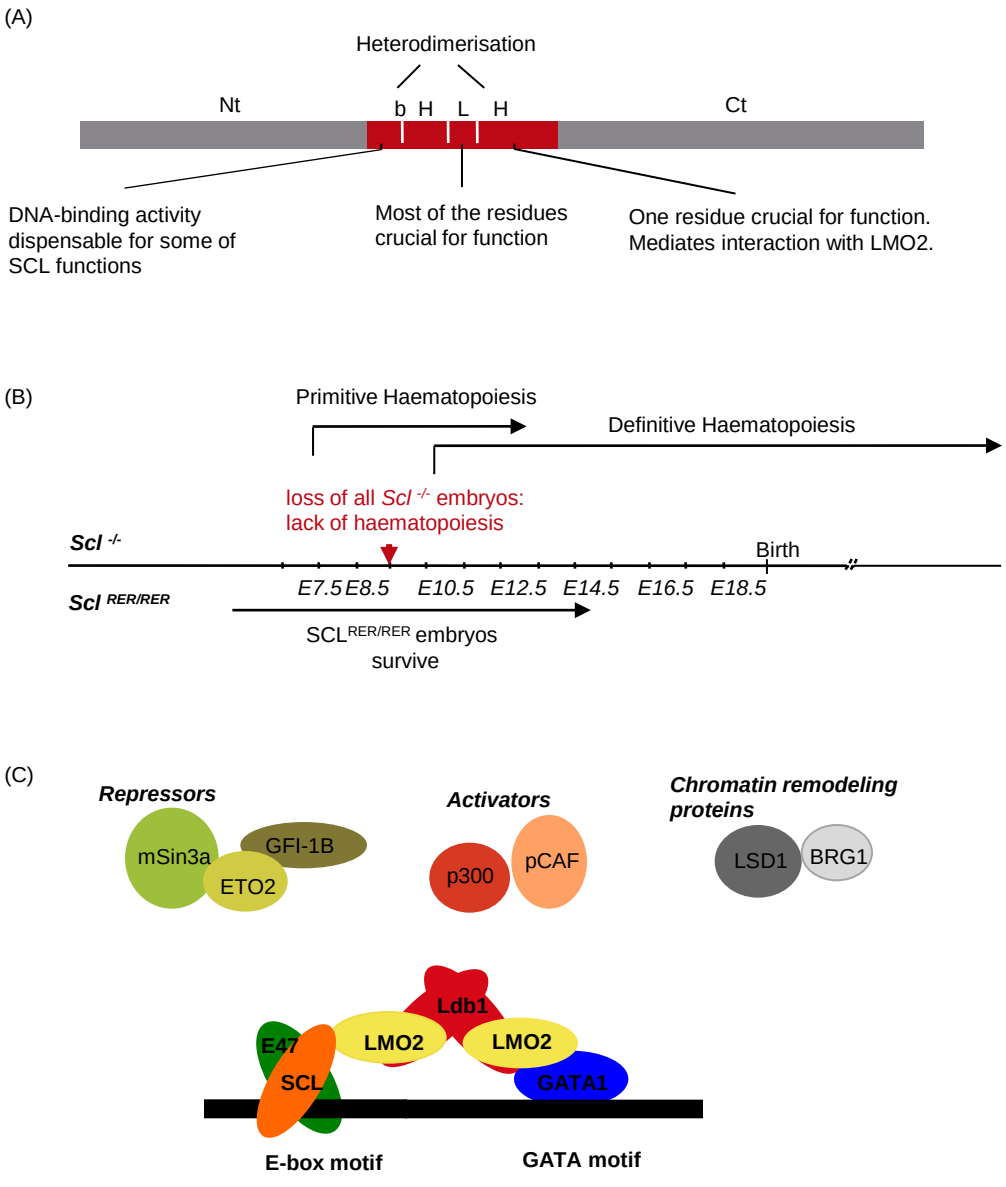


Figure 1.5 Characteristics of SCL. (A) Schematic representing the full length SCL protein, highlighting the basic helix-loop-helix (bHLH) domain which is essential for protein-DNA and protein-protein interactions. (B) Timeline indicating the embryonic lethality of *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ mice. (C) Schematic showing SCL bound in a pentameric complex with E47, LMO2, LDB1 and GATA1 to DNA. SCL associated co-repressors, co-activators and chromatin remodelling proteins are indicated.

Figure 3.1
Mesoderm Populations in Wild Type EBs

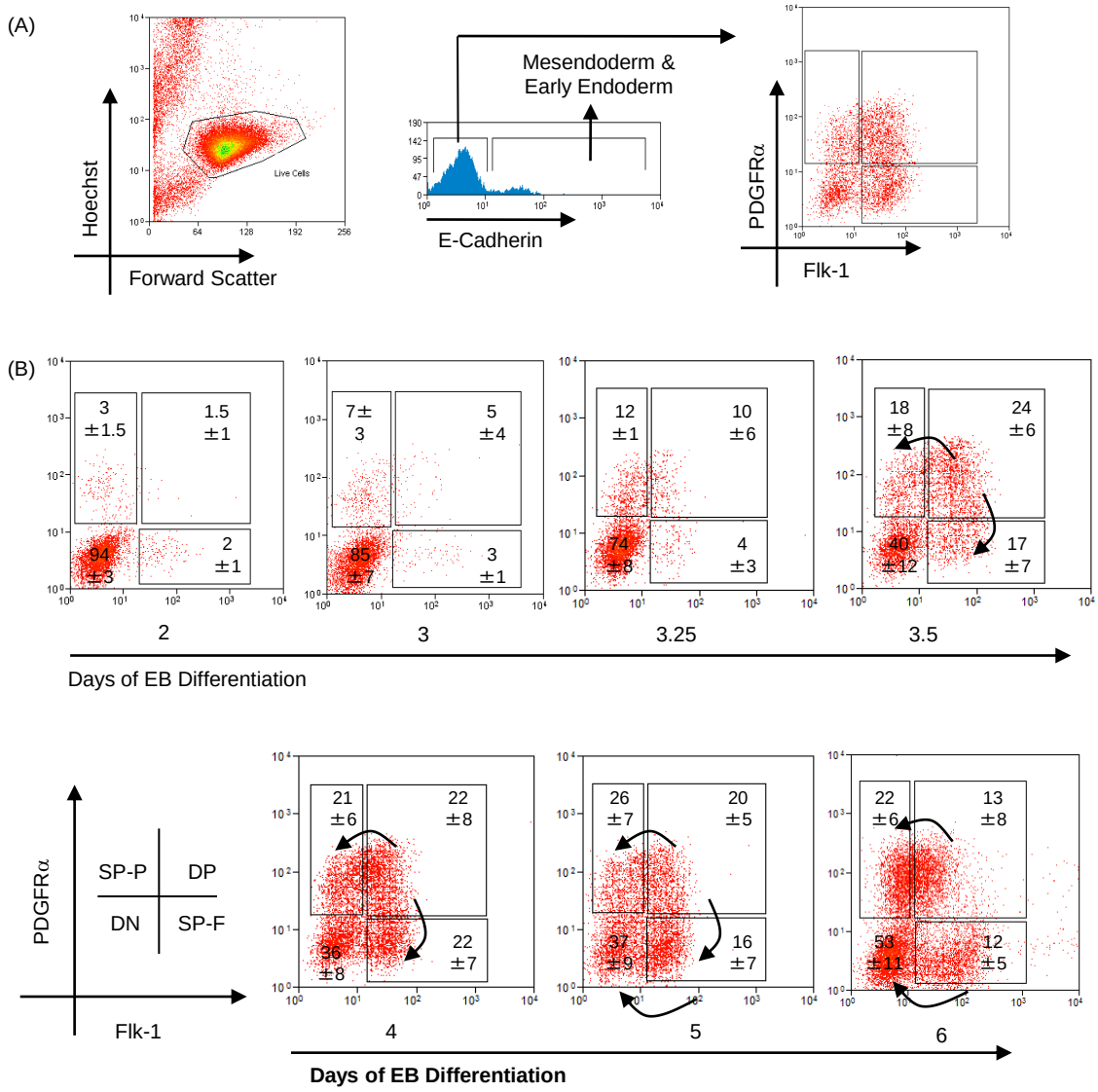


Figure 3.1 Mesoderm populations in differentiating EBs can be defined by immuno-phenotype using E-Cadherin, Flk-1 and PDGFRα as cell surface markers. (A) FACS protocol used to fractionate mesoderm populations, where live cells (Hoechst negative) are separated according to E-Cadherin, Flk-1 and PDGFRα expression. (B) Flk-1 and PDGFRα expression exhibited by E-Cadherin negative cells in wild type EBs after 2-6 days of differentiation. Values indicate the mean percentage of live cells that express the indicated cell surface proteins, ±standard deviation, where n ≥ 6. Arrows indicate gain and loss of marker expression as populations differentiate over time (see Figure 3.2).

Figure 3.2
Immuno-phenotypic Differentiation Potential of Day 3.5 Flk-1 / PDGFRα Populations

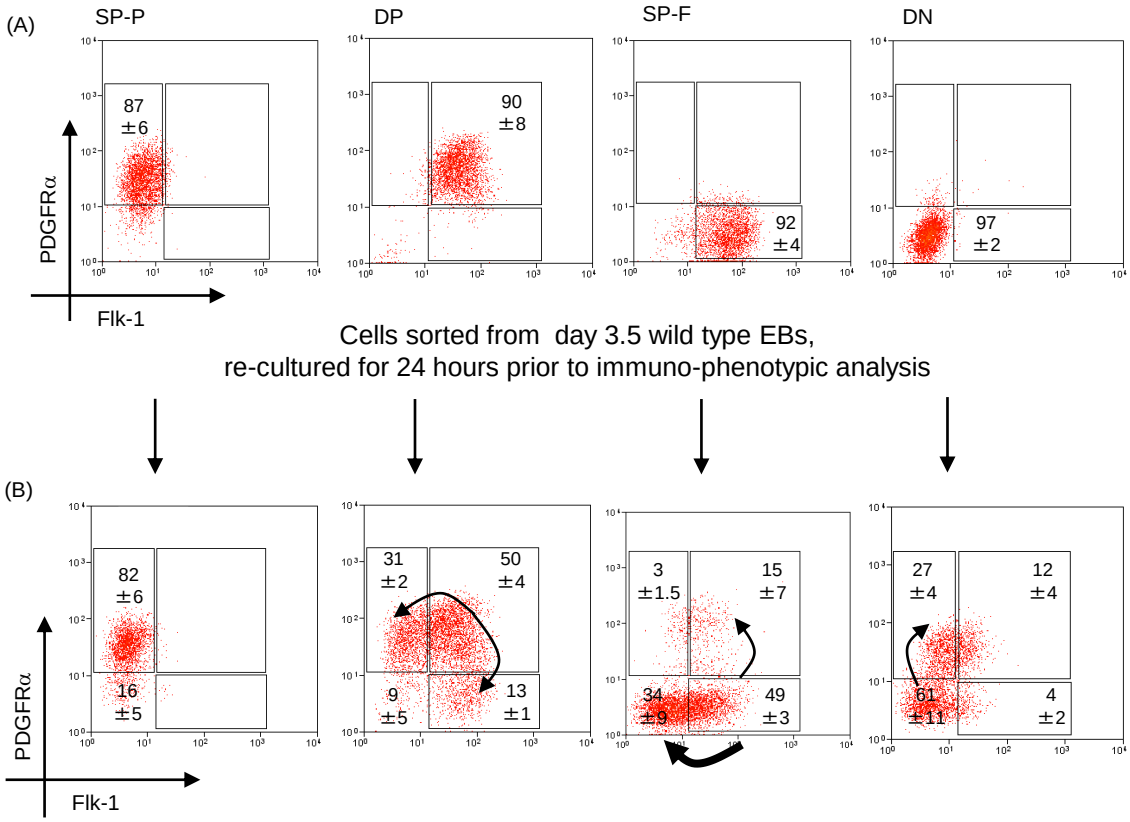


Figure 3.2 The relationship between Flk-1/PDGFRα mesoderm populations was determined by sorting wild type EB cells (based on their immuno-phenotype), re-aggregating cells into EB-like structures for 24 hours (to allow further differentiation) and re-analysed by FACS. (A) FACS plots indicate the purity of mesoderm populations sorted from wild type day 3.5 EBs (SP-P = PDGFRα^{+} Flk-1^{-} , DP = PDGFRα^{+} Flk-1$^{+,}$ SP-F = PDGFRα^{-} Flk-1$^{+,}$ DN = PDGFRα^{-} Flk-1^{-}). (B) Immuno-phenotype of sorted populations after an additional 24 hours of differentiation. Values represent the percentage of cells expressing the indicated cell surface protein, ± standard deviation (where n=2). Arrows indicate gain and loss of cell surface marker expression as populations expand and differentiate.

Figure 3.3
Cellular Fate Re-plating Assays

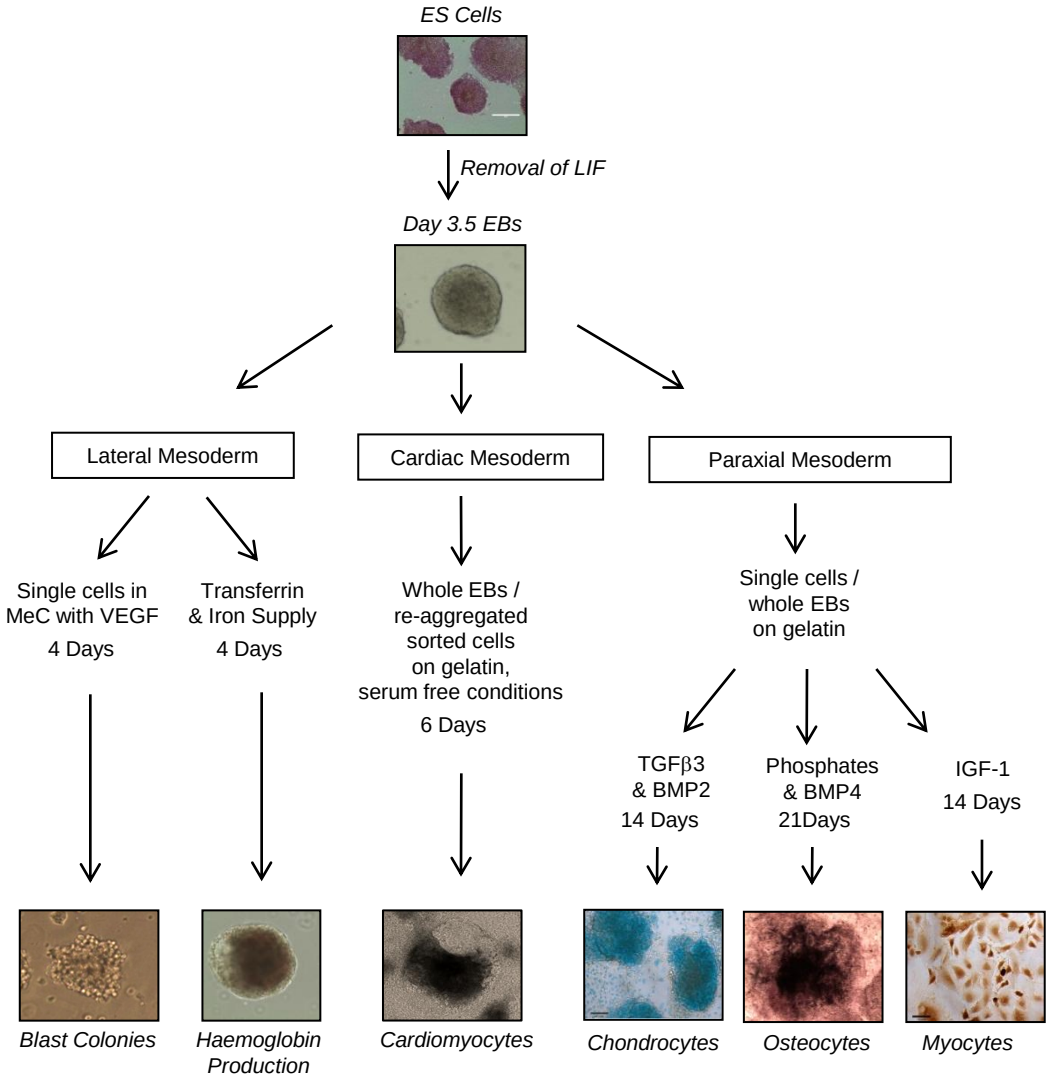


Figure 3.3 Schematic showing *in vitro* cell fate assays used to determine the differentiation potential of ES cells. Morphology and pluripotent potential of ES cell colonies is highlighted by alkaline phosphatase staining. Differentiations assays were usually performed using day 3.5 EBs. Specific reagents, growth factors and culture conditions are indicated for each assay. Blast colonies and red cells were identified by morphology. Cardiomyocytes were identified by spontaneous contraction of colonies. Chondrogenic nodules were identified by Alican Blue staining for cell surface proteoglycans, osteoblasts by Alizarin Red staining for calcium deposits, and myocytes by immuno-histochemical / HRP staining for Myogenin.

Figure 3.4
Cell Fate Potential of Day 3.5 Mesoderm Populations

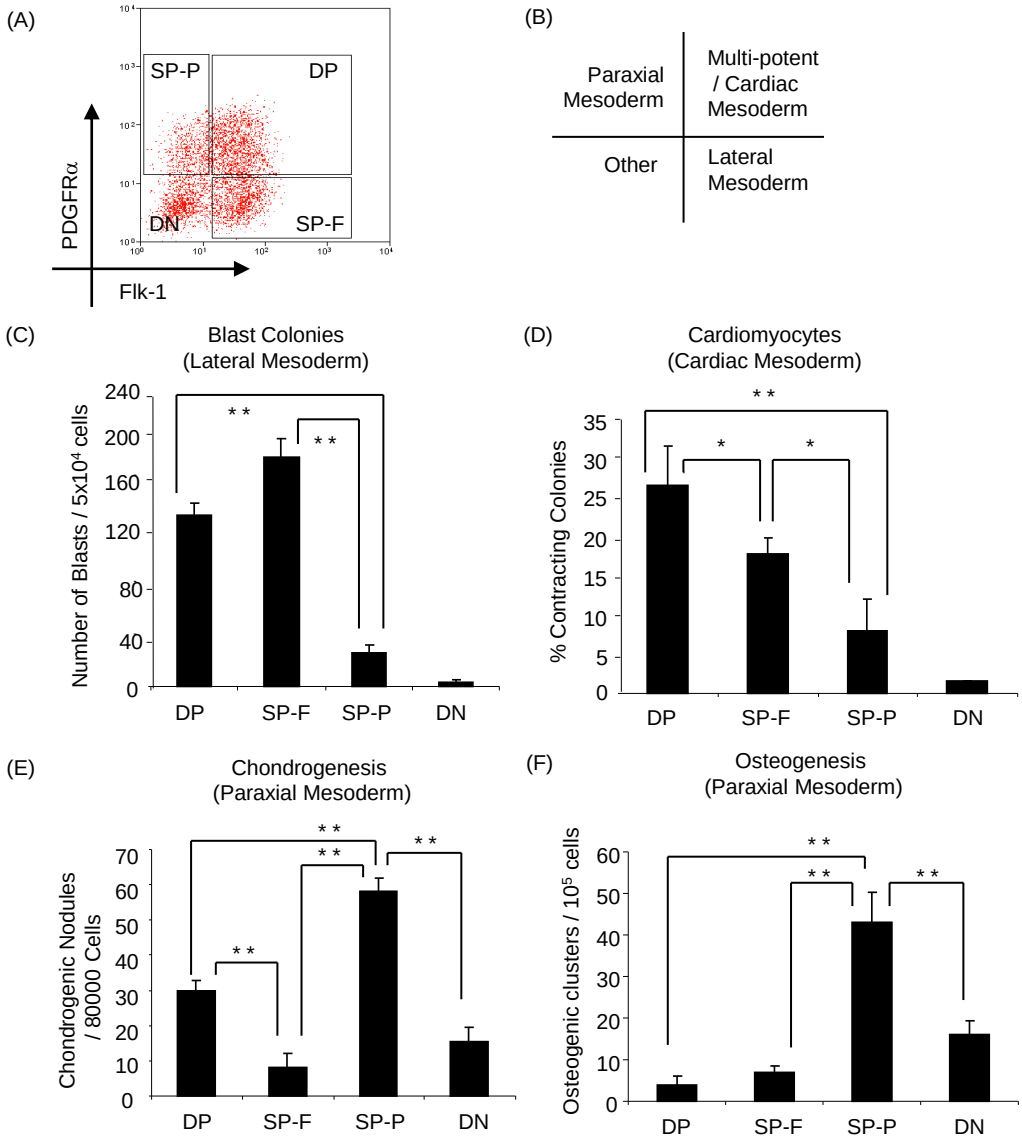


Figure 3.4 To assess the *in vitro* cell fate potential of early Flk-1/PDGFRα mesoderm populations, day 3.5 wild type EB cells were sorted based on their immuno-phenotype (A) and re-plated under conditions that support the development of mesoderm lineages. (B) Mesoderm populations associated with Flk-1/PDGFRα expression, based on cell fate potential and gene expression analysis (Figure 3.3). (C) Haematopoietic and endothelial potential was measured by ability to generate blast colonies. (D) Cardiac potential was measured by ability to generate spontaneously contracting colonies. (E-F) Paraxial mesoderm potential was assessed by differentiation into (E) chondrocytes and (F) osteoblasts. See Figure 3.3 for cell fate assays. Graphs represent results from three experiments. Error bars represet the standard deviation of resutlts, with * indicating a P-value < 0.05, and** indicating a P-value < 0.01.

Figure 3.5
Gene Expression in Day 3.5 Flk-1 / PDGFRα Populations

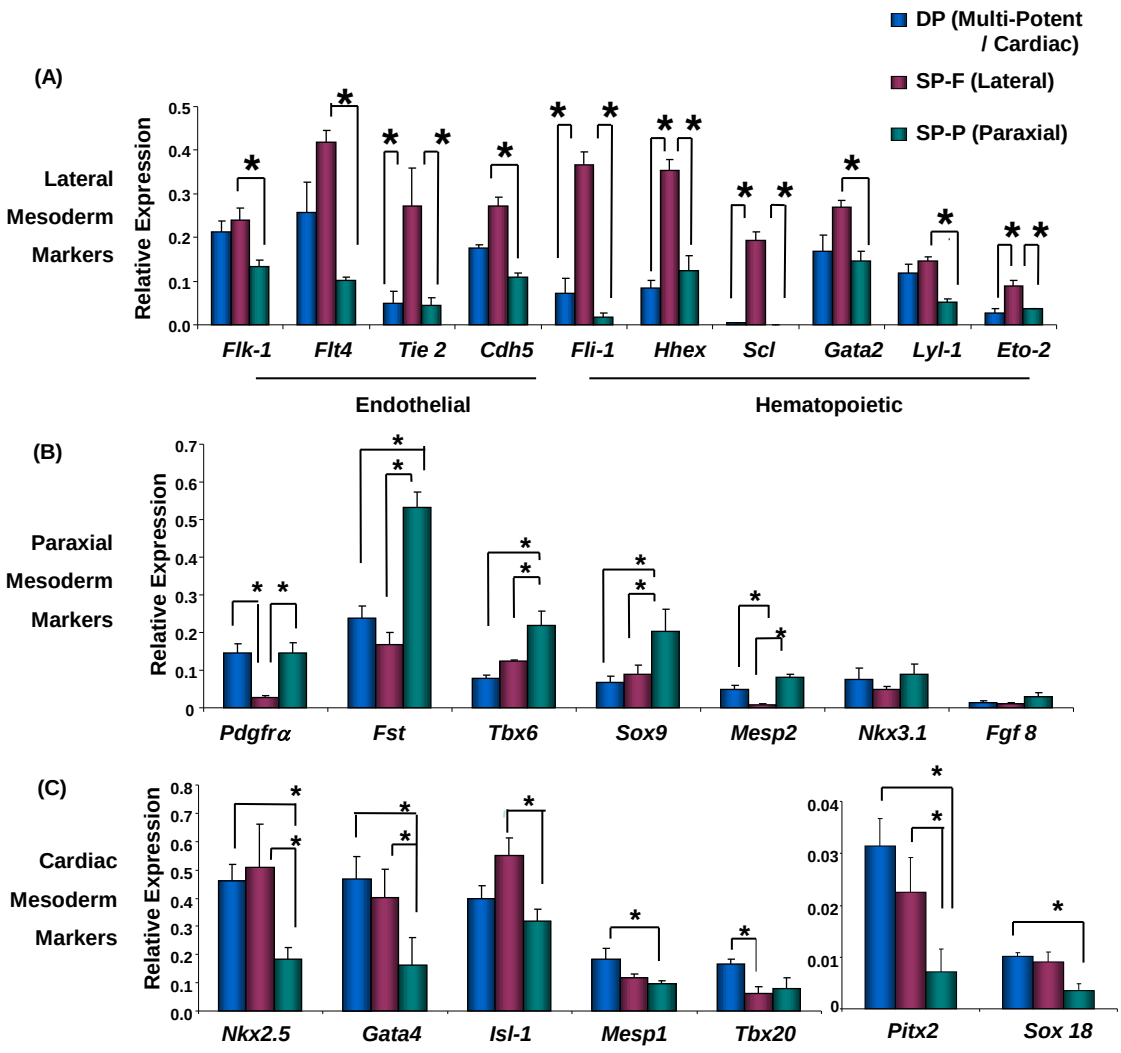


Figure 3.5 Expression of (A) lateral, (B) paraxial and (C) cardiac mesoderm-associated genes was analysed within day 3.5 wild type EB Flk-1/PDGFRα populations. Total RNA from each population was reverse transcribed into cDNA and gene expression quantified by real time qPCR. Gene expression was normalised to *Gapdh* and *18S* expression. Graphs represent results from three biological repeats, with each PCR being performed in duplicate. * indicates P-value < 0.05.

Figure 3.6
Cell Fate Potential of Day 6 Flk-1 / PDGFRα Populations

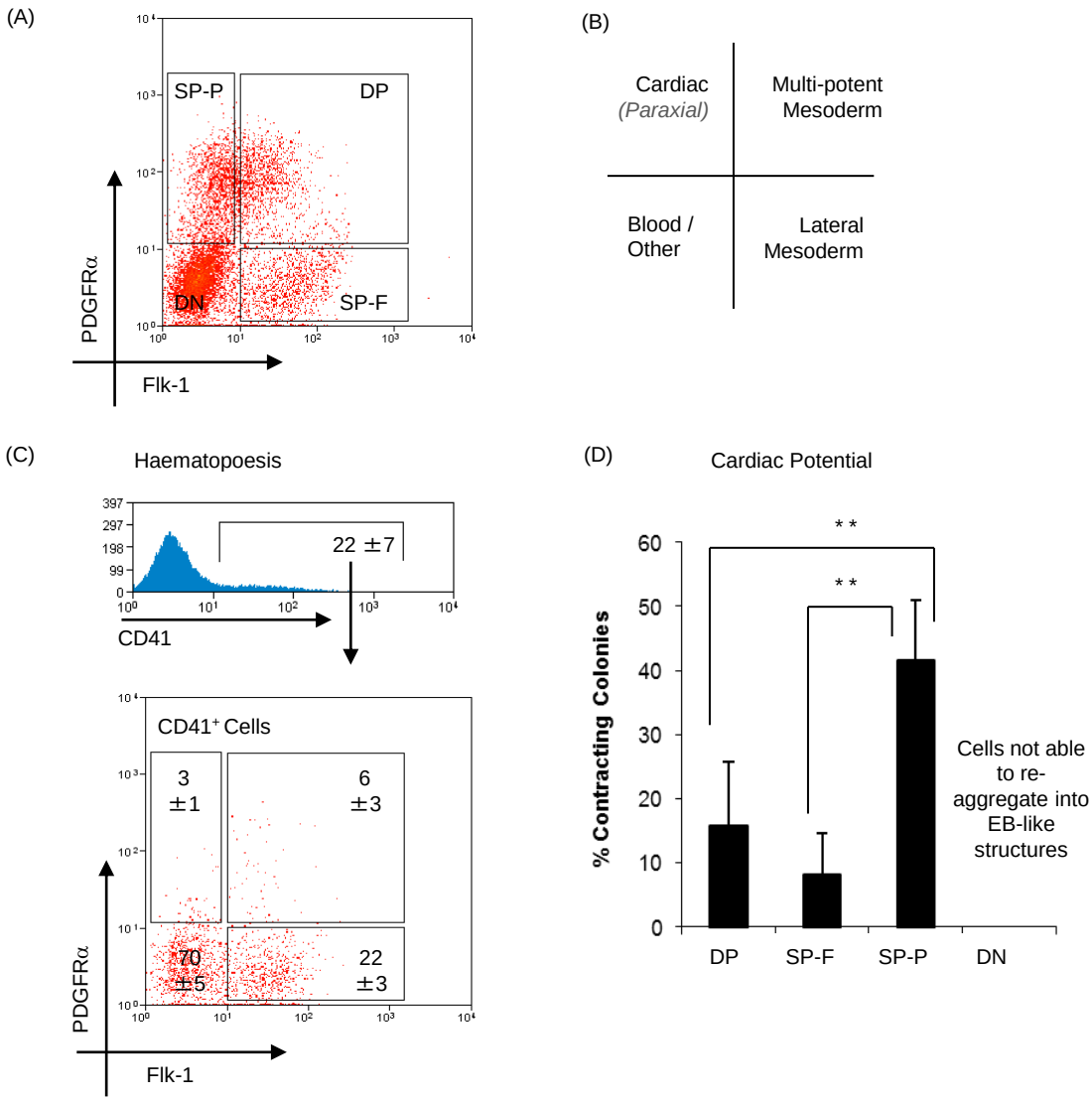


Figure 3.6 The cell lineage potential of Flk-1/PDGFRα populations in day 6 wild type EBs was determined by cell surface marker expression and *in vitro* cell fate assays. (A) Flk-1/PDGFRα immuno-phenotype exhibited by day 6 EBs. (B) Mesoderm-type potential in each Flk-1/PDGFRα compartment. (C) Top plot shows the percent of cells expressing CD41, (the first markers of committed haematopoietic cells) in day 6 wild type EBs. Bottom plot shows Flk-1/PDGFRα co-expression within the CD41⁺ population. Values represent the mean percentage of cells which express the indicated cell surface markers, ±standard deviation, where n=5. (D) *In vitro* cardiac potential was measured by the ability of cells to generate spontaneously contracting colonies. Graph represents results from two independent experiments. ** indicates a P-value < 0.01**.**

Figure 3.8
Flk-1/PDGFRα Expression within SCL⁺ Populations

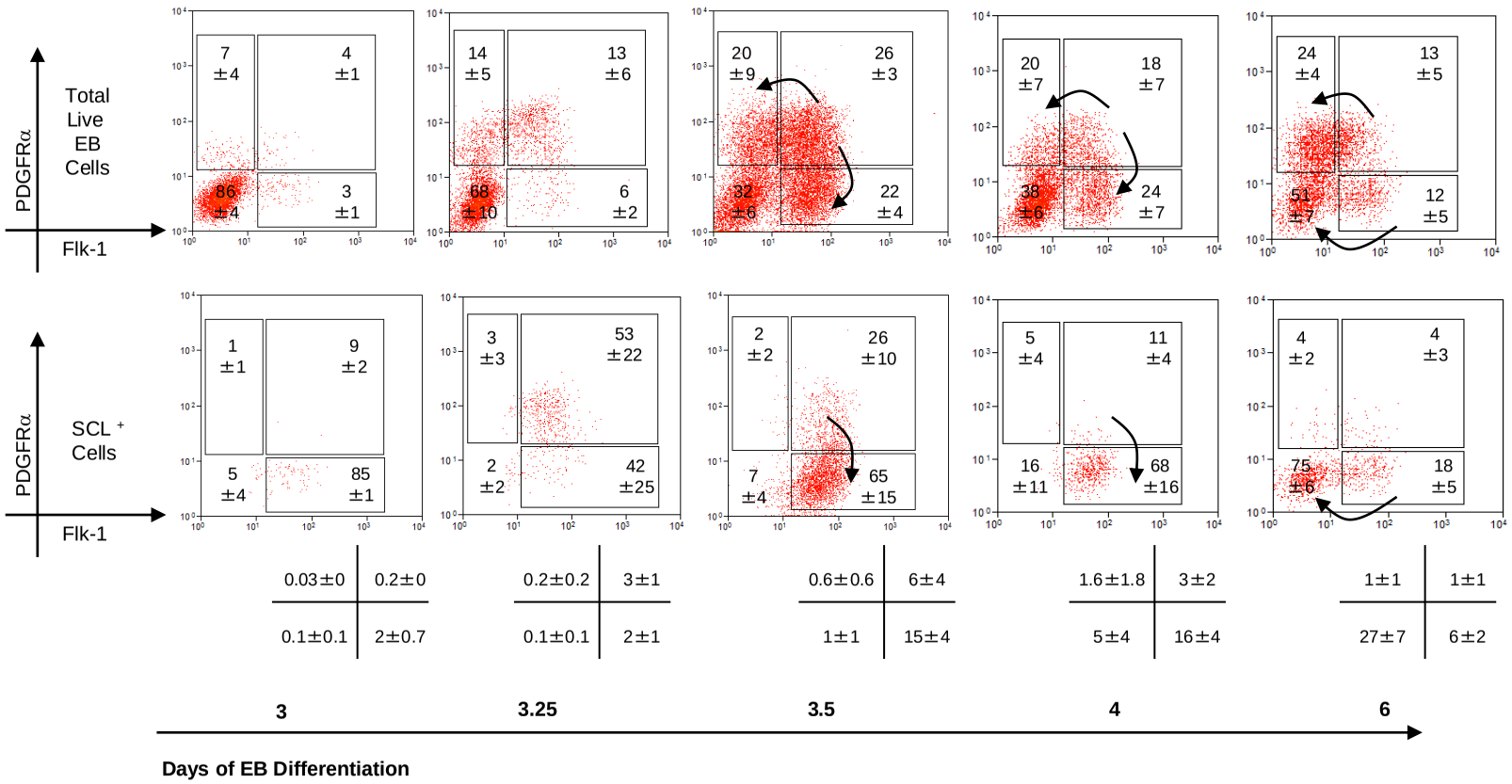


Figure 3.8 Flk-1/ PDGFRα immuno-phenotype of SCL expressing cell populations in differentiating wild type EBs (determined using the FACS protocol outlined in Figure 3.6A). Top panel shows the Flk-1/PDGFRα immuno-phenotype of total EB cells between days 3 and 6 of differentiation. Bottom panel shows back-gating of SCL expressing cells, identifying co-expression with Flk-1 and/or PDGFRα. Values represent the mean percentage of total live cells (top plots), or percentage of SCL⁺ cells (bottom plots), which fall within the indicated gates, ± standard deviation, where n ≥ 3. Percentage of total EBs cells that co-express SCL and Flk-1/PDGFRα is indicated in the quadrants below. Arrows indicate gain and loss of Flk-1 and/or PDGFRα expression as cell populations differentiate (see Figure 3.2).

Figure 3.9
Comparison of *Scl* Gene and SCL Protein Expression

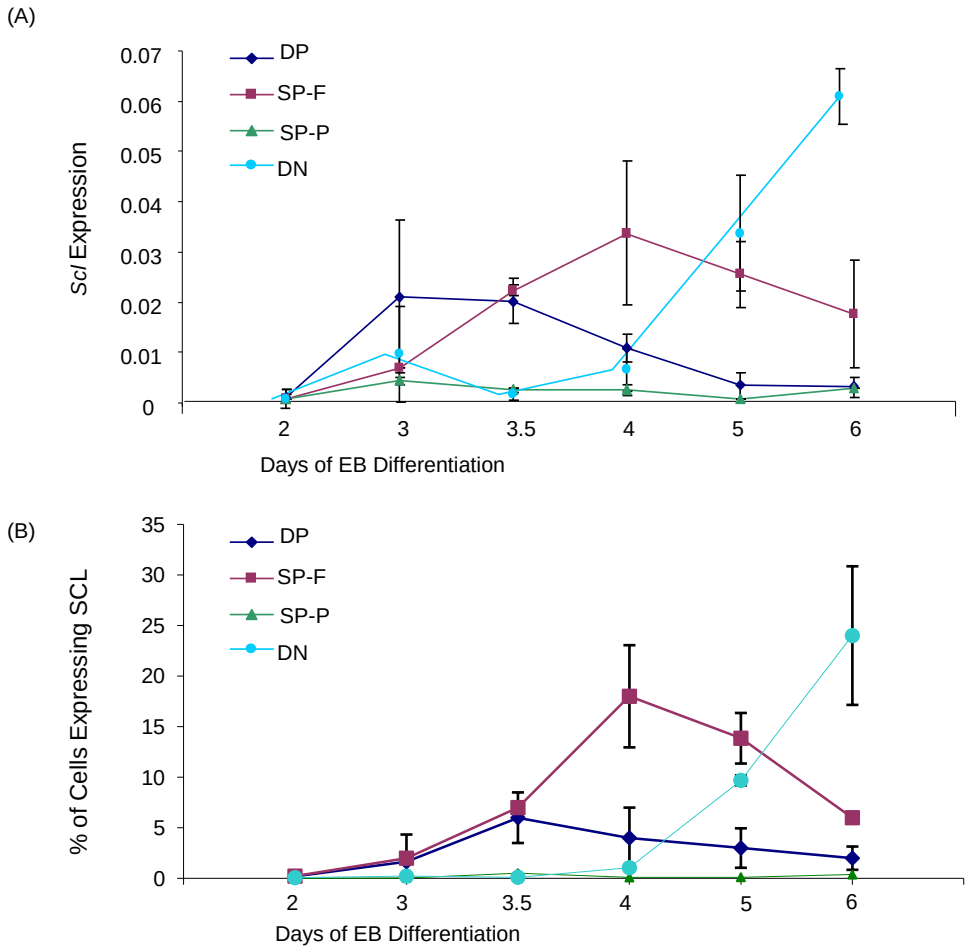


Figure 3.9 Comparison of *Scl* gene expression and SCL protein expression in wild type EB mesoderm populations. (A) Quantitative real-time PCR analysis of *Scl* gene expression in Flk-1/PDGFRα mesoderm populations at different stages of EB differentiation. *Scl* expression was normalised to *Gapdh* and *18S* expression. (B) Percentage of cells expressing SCL protein in Flk-1/PDGFRα mesoderm populations, as determined by intracellular FACS. Graph represents results from at least three repeats**.**

Figure 4.1

Mesoderm Populations in Wild Type, Scl $^{-/-}$ and Scl $^{RER/RER}$ EBs

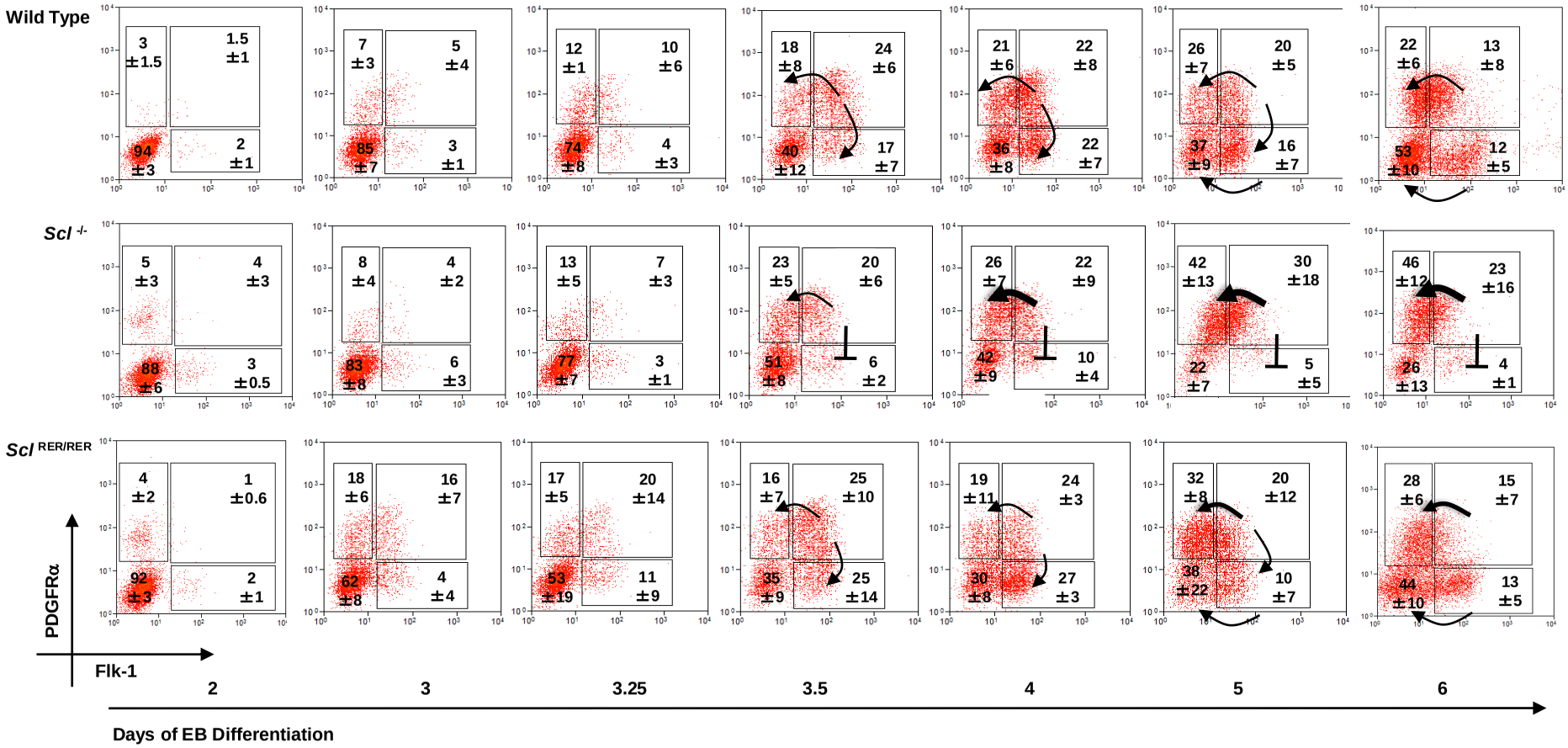


Figure 4.1 Immuno-phenotype of Flk-1/PDGFRα mesoderm populations in differentiating wild type (top panel), *Scl* $^{-/-}$ (middle panel) and *Scl* $^{RER/RER}$ (bottom panel) EBs. Values represent the mean percentage of cells found in each quadrant (±standard deviation, where n ≥ 3. Arrows indicate gain or loss of protein expression as cell populations differentiate.

Figure 4.2

SCL and CD41 Expression in *Scl* $^{RER/RER}$ EBs

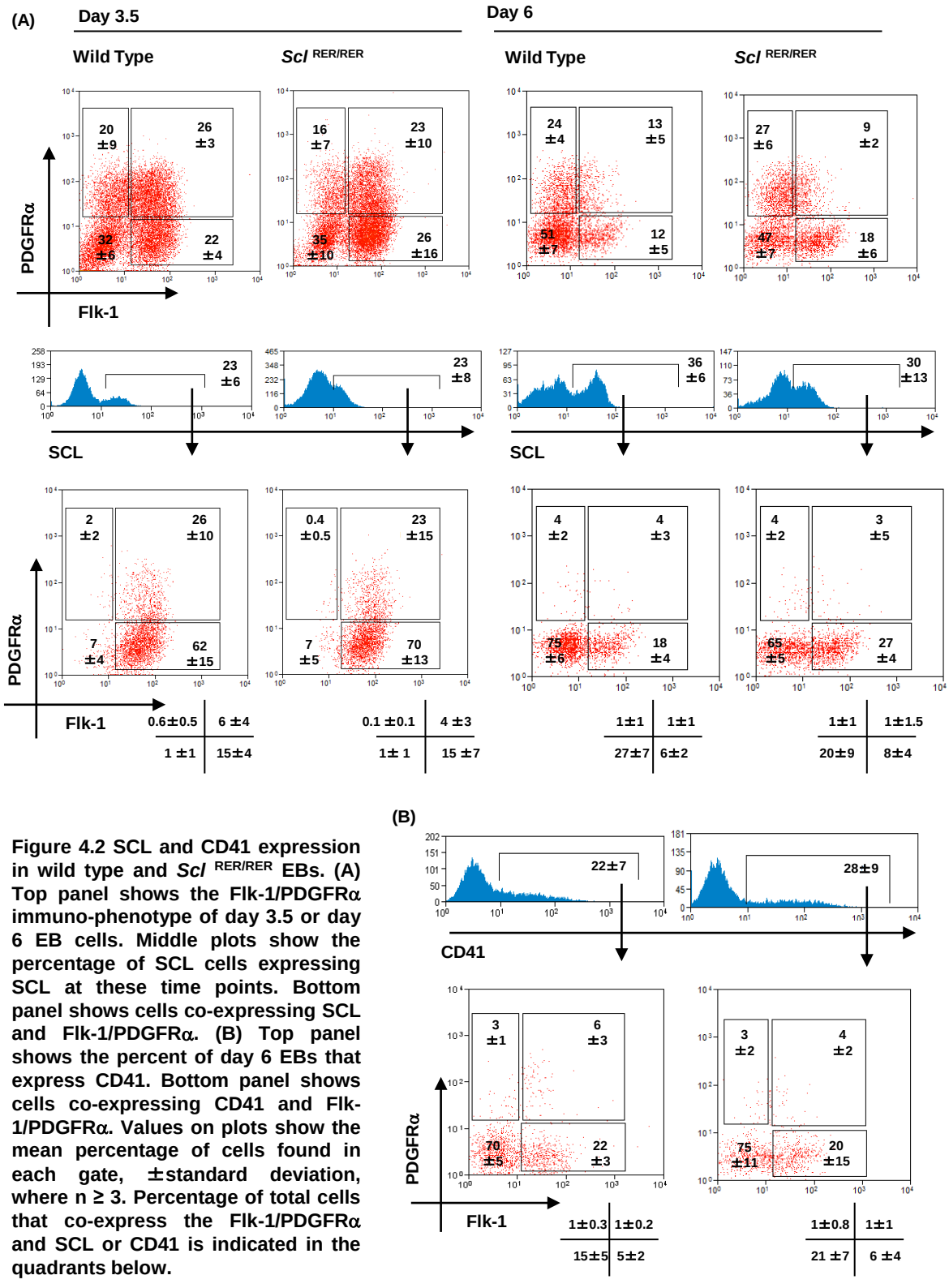


Figure 4.2 SCL and CD41 expression in wild type and *Scl* $^{RER/RER}$ EBs. (A) Top panel shows the Flk-1/PDGFRα immuno-phenotype of day 3.5 or day 6 EB cells. Middle plots show the percentage of SCL cells expressing SCL at these time points. Bottom panel shows cells co-expressing SCL and Flk-1/PDGFRα. (B) Top panel shows the percent of day 6 EBs that express CD41. Bottom panel shows cells co-expressing CD41 and Flk-1/PDGFRα. Values on plots show the mean percentage of cells found in each gate, ±standard deviation, where n ≥ 3. Percentage of total cells that co-express the Flk-1/PDGFRα and SCL or CD41 is indicated in the quadrants below.

Figure 4.3
Apoptosis & Cell Cycle Analysis of day 3.5 Flk-1/PDGFRα Populations in Wild Type and *Scl* $^{-/-}$ EBs

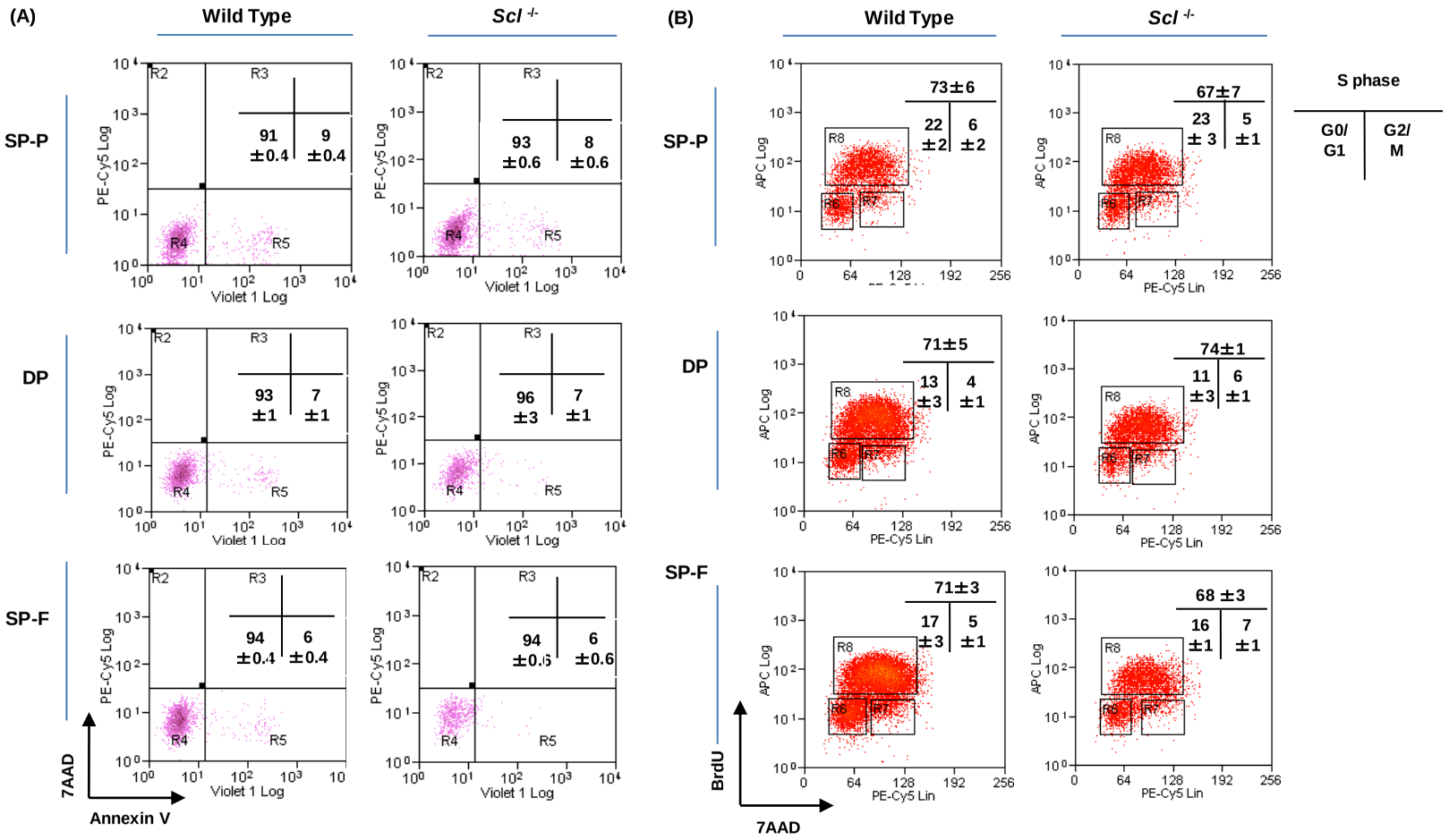


Figure 4.3 Cell Death and Proliferation was unaltered between wild type and *Scl* $^{-/-}$ SP-P, DP and SP-F day 3.5 EB populations. (A) Annexin V and 7AAD staining, where Annexin V positive populations represent the percentage of cells undergoing apoptosis. (B) Cell cycle analysis, determined by Brdu incorporation and 7AAD staining. Values represent the mean percentage of SP-P, DP and SP-F cells which fall within the indicated gates, ±standard deviation, where n=3.

Figure 4.4

Haematopoietic Potential of Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs

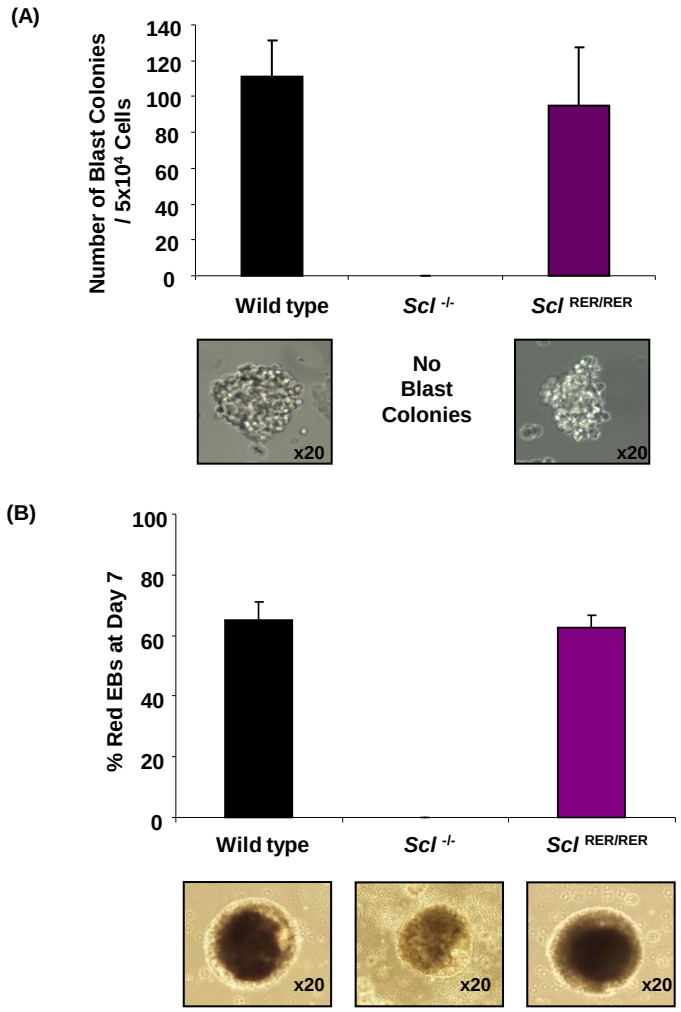


Figure 4.4 Lateral mesoderm potential of wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EB cells was measured by ability to generate (A) blast colonies and (B) hemoglobin (see Figure 3.3). (A) Number of blast colonies generated from re-plating 5x10^4 day 3.5 EB cells. (B) Percentage of EBs which contained visible red cells after 7 days of differentiation. Graphs represent results from three experiments. Photos show the morphology of colonies/EBs formed by each cell type.

Figure 4.5

Chondrogenic Potential of Wild Type and *Scl* $^{-/-}$ EBs

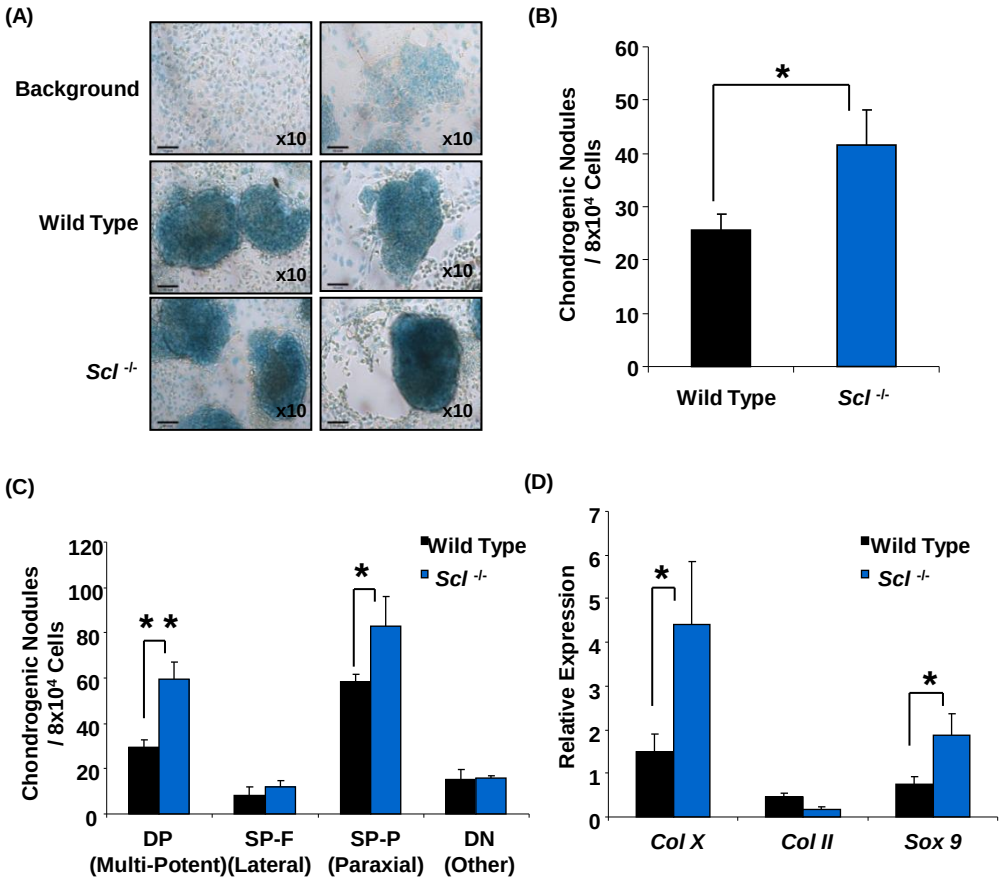


Figure 4.5 **The chondrogenic potential of day 3.5 EBs was assessed by re-plating cells with chondrogenic promoting cytokines (see Figure 3.3). (A) Morphology and Alican Blue staining of chondrogenic nodules formed. (B) Number of chondrogenic nodules formed from re-plating 8x10^4 unsorted EB cells. (C) Number of chondrogenic nodules formed from re-plating Flk-1/PDGFRα sorted cells. (D) Expression of chondrocyte associated genes in nodules harvested after 14 days differentiation. Gene expression was determined by real-time PCR and normalised to GAPDH. PCR reactions were run in duplicate. Graphs represent two independent biological repeats, with experiments being repeated in tripicate. * indicates a P-value < 0.05, ** indicates a P-value < 0.01.**

Figure 4.6

Osteogenic Potential of Wild Type and *Scl* $^{-/-}$ EBs

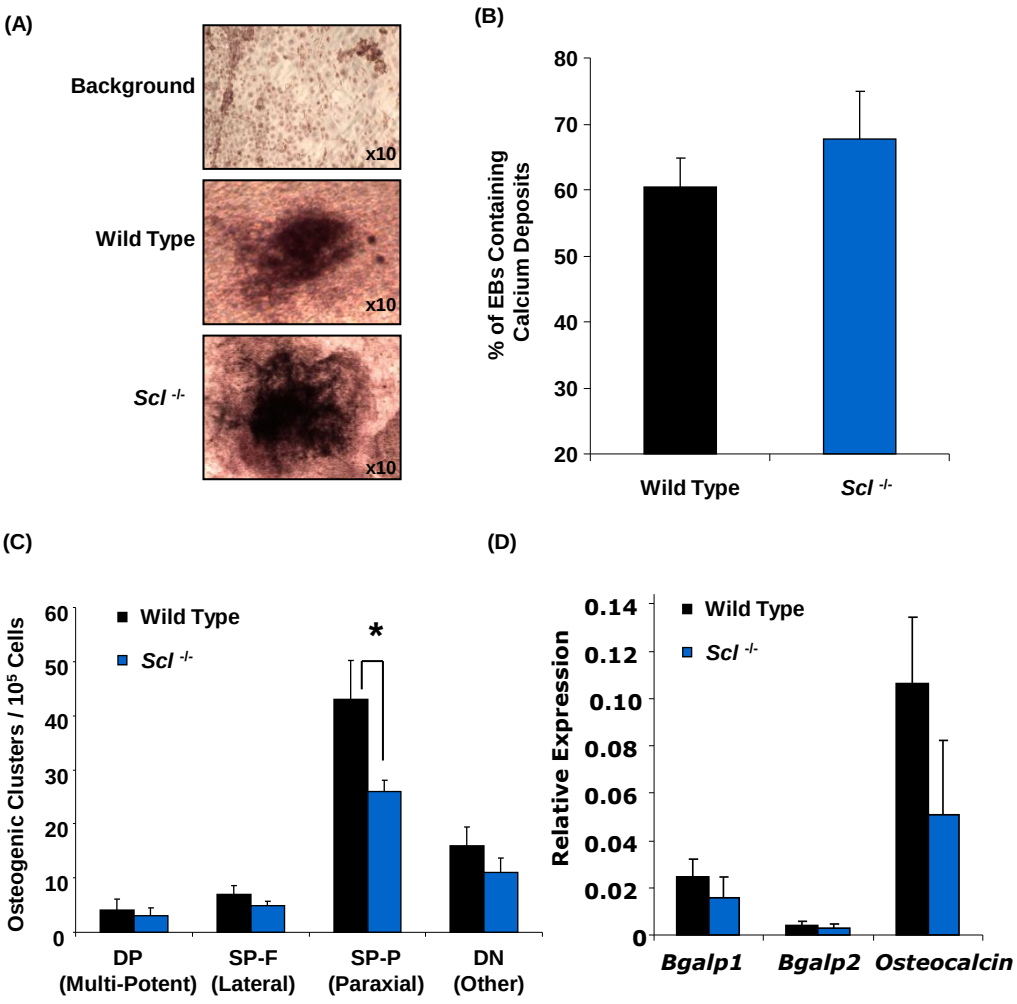


Figure 4.6 **The osteogenic potential of day 3.5 EBs (was assessed by re-plating under osteogenic conditions (see Figure 3.3). (A) Morphology and Alizarin Red staining of calcium deposits in EB derived cell clusters. (B) Percentage of cell clusters contained calcium deposits. (C) Number of osteogenic clusters formed from 10^5 Flk-1/PDGFRα sorted cells. (D) Expression of osteoblast associated genes in cell clusters harvested after 21 days. Gene expression was determined by qPCR and normalised to GAPDH. Graphs represent two independent biological repeats, with experiments being performed in triplicate. * indicates a P-value < 0.05.**

Figure 4.7

Myogenic Potential of Wild Type and *Scl* $^{-/-}$ EBs

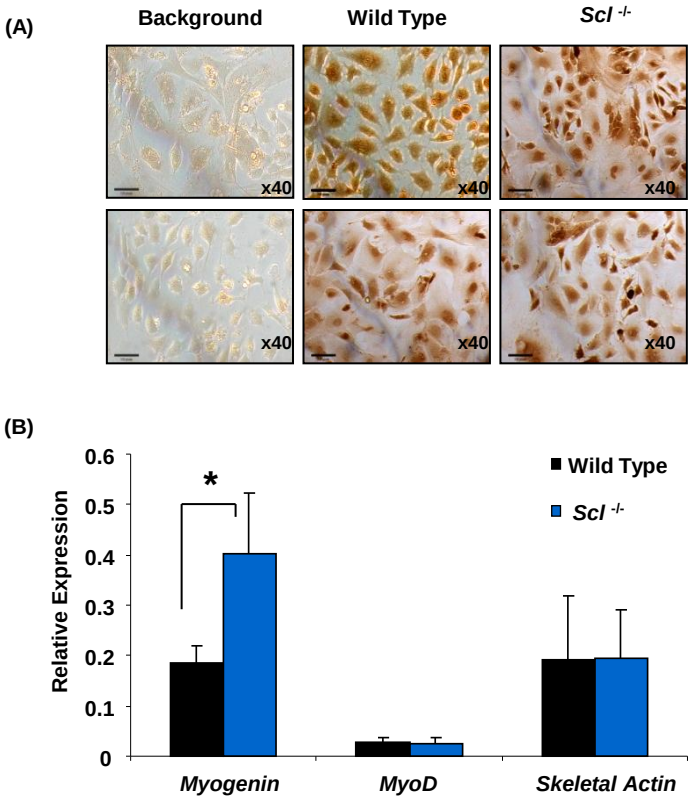


Figure 4.7 Myogenic potential of day 3.5 EB cells was assessed by re-plating EB derived cells with myogenesis promoting cytokines (see Figure 3.3). (A) Myocyte generation was confirmed by immuno-histochemical staining for Myogenin, an early marker of muscle formation. (B) Relative expression of myogenic genes in day 14 cultures, performed using qPCR. Gene expression is normalised to GAPDH. Graph represents two independent biological repeats. * indicates a P-value < 0.05

Figure 4.8

Cardiac Potential of Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs

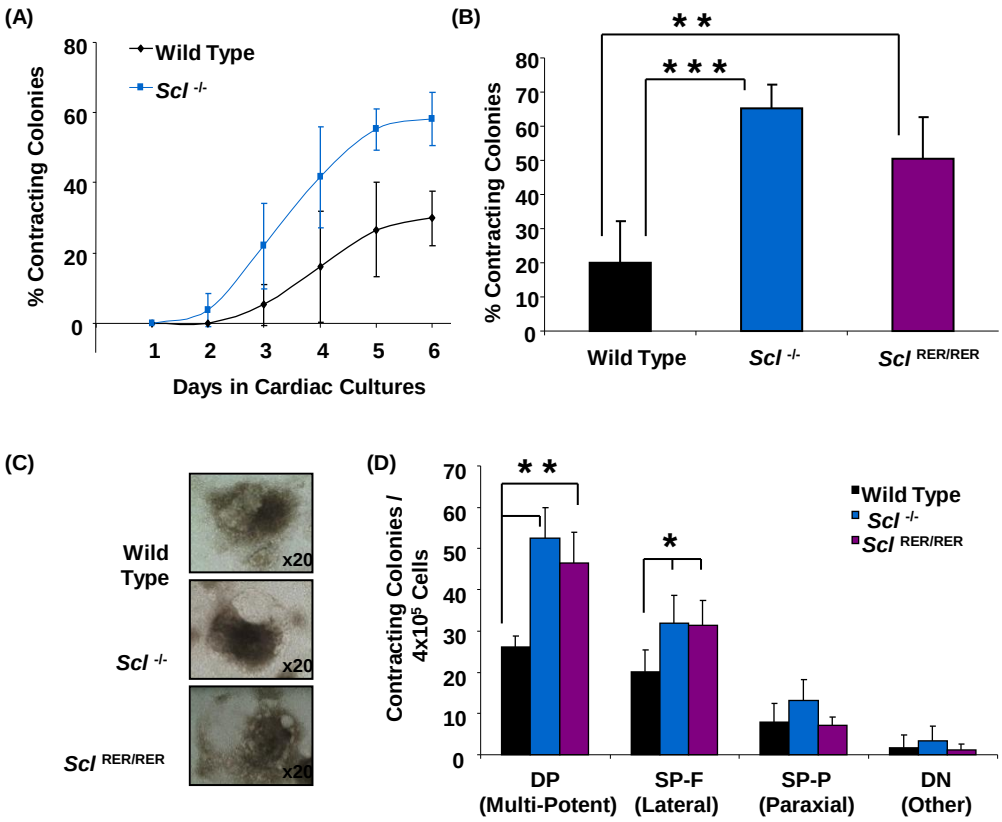


Figure 4.8 Cardiac potential was assessed by ability to generate spontaneously contracting colonies under serum-free conditions (Figure 3.3). (A) Percentage of wild type and *Scl* $^{-/-}$ EBs, re-plated at day 4 into cardiac conditions, that spontaneous contracted after 1 to 6 days (5 to 10 days total differentiation). (B) Percentage of wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs which spontaneously contracted after 6 days of culture in cardiac conditions. (C) Morphology of contracting colonies. (D) Number of contracting colonies generated from $4x10^5$ day 3.5 Flk-1/PDGFRα sorted cells. Graphs represent at least three independent experiments. * indicates a P-value < 0.05, ** indicates a P-value < 0.05.

Figure 4.9

Cardiac Troponin T (cTnT) Expression in Cardiac Colonies

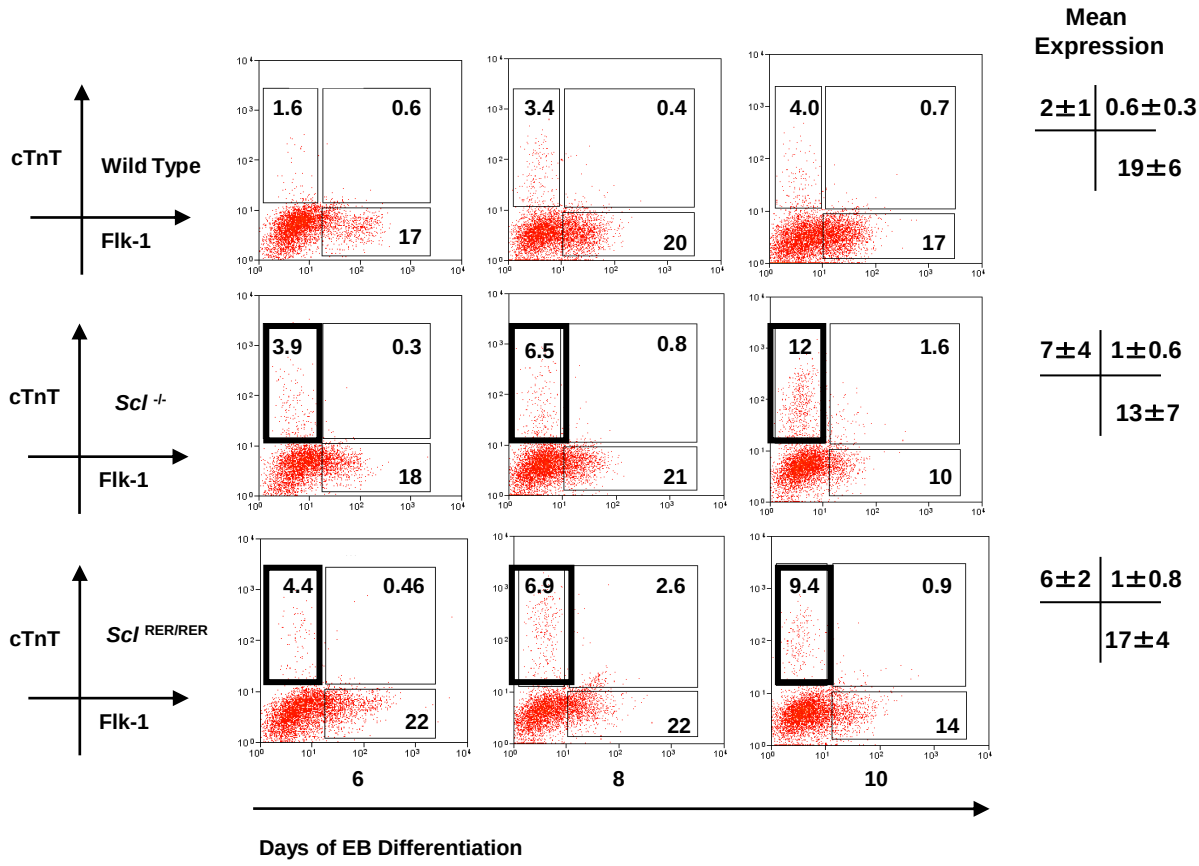


Figure 4.9 **FACS plots show expression of cTnT and Flk-1 in wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ cardiac colonies, generated by re-plating day 4 EBs under serum free, cardiac inducing conditions (see Figure 3.3) for 2-6 days (total 6-10 days differentiation from ES cells). Values represent the percentage of live cells in the indicated gates. Mean values for Day 10 cardiac colonies (± standard deviation, where n=6) are shown in the adjacent quadrants.**

Figure 4.10

Cardiac Populations in Differentiating EBs

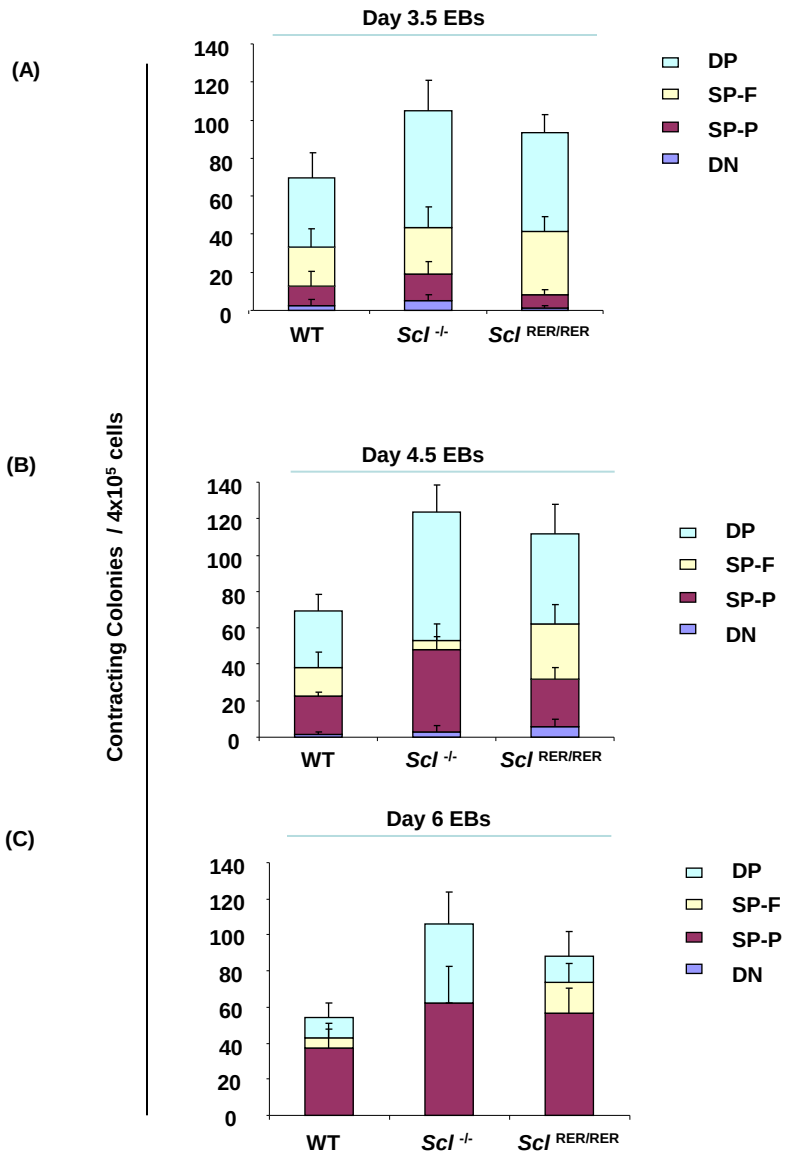


Figure 4.10 Number of contracting cardiac colonies generated from 4x10^5 Flk-1/PDGFRα sorted cells from day 3.5 (A), day 4.5 (B) and day 6 (C) EBs after 6 days in cardiac culture. Graphs represent results from three experiments.

Figure 4.11

Haematopoietic and Cardiac Potential in Temporally Distinct Flk-1^{+} Populations

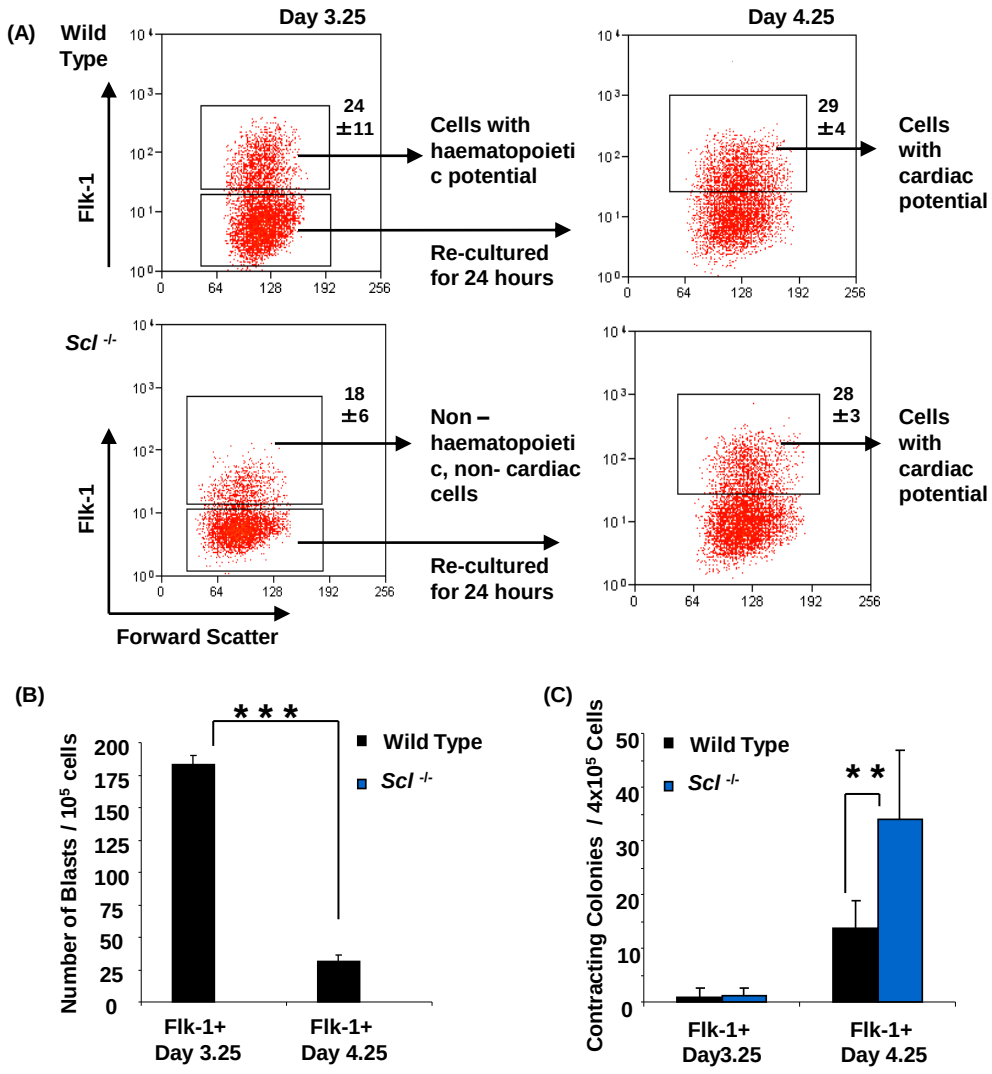


Figure 4.11 **Haematopoietic and cardiac potential of mesoderm cells associated with the first and second wave of Flk-1 expression. The first wave of Flk-1^{+} cells was isolated from day 3.25 wild type and *Scl* $^{-/-}$ EBs (A; left panel). Flk-1 negative cells were re-aggregated into EB-like structures for a further 24 hours differentiation, and re-sorted to isolate the second wave of Flk-1 expressing cells (A; right panel). Values in FACS plots represent the percentage of live cells that express Flk-1, ± standard deviation, at the given time point. Cells were re-plated in haematopoietic (B; blast colony assay) or cardiac (C; spontaneous contraction) inducing conditions. Results represent two experiments. ** indicated a P-value < 0.01, *** indicates a P-value < 0.001.**

Figure 4.12

Gene Expression in Flk-1^{+} Wild Type, *Scl* $^{-/-}$ and Scl $^{RER/RER}$ EB Cells

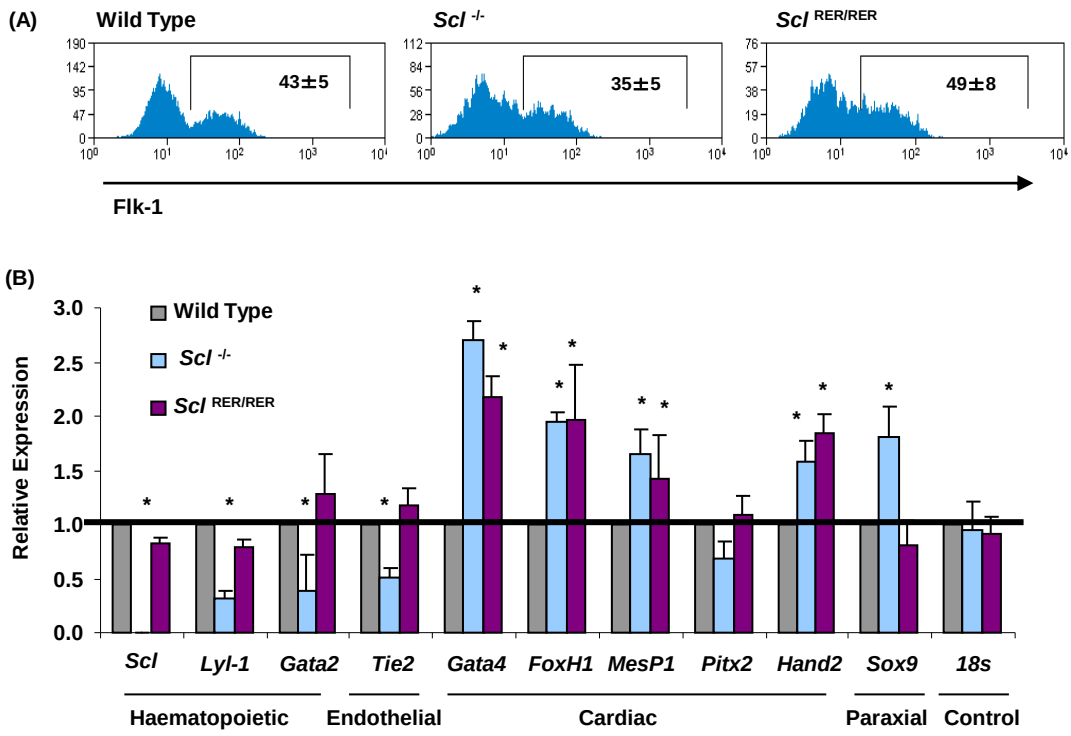


Figure 4.12 **Gene expression profiles in Flk-1 expressing populations in day 3.5 wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs. (A) FACS plots show Flk-1^{+} populations sorted from day 3.5 EBs. Values represent the percentage of live cells expressing Flk-1, ±standard deviation, from three biological repeats. (B) Real-time PCR results comparing levels of gene expression within sorted Flk-1^{+} populations. Gene expression is normalised to GAPDH and is displayed as a fold change over wild type expression (where wild type expression = 1). * indicates expression which is significantly different to wild type, with a P-value < 0.05.**

Figure 4.13
SCL Over-Expressing ES Cell Lines

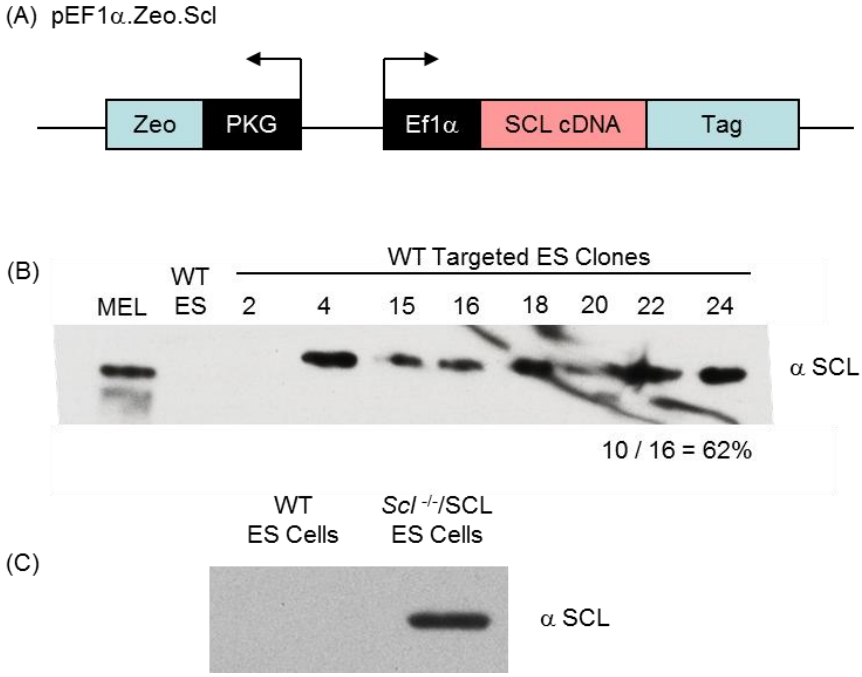


Figure 4.13 ES clones/cells lines that ectopically express SCL were created by electroporation of wild type and *Scl* $^{-/-}$ ES cells with a construct designed to drive SCL expression under an Ef1α promoter (creating SCL over-expression and *Scl* $^{-/-}$/SCL ES cell lines respectively). (A) Schematic of the construct. (B-C) Western blot showing SCL expression in Zeocin resistant ES clones. (B) On a wild type background. (C) On a *Scl* $^{-/-}$ background. MEL cells, which express endogenous SCL, and wild type ES cells were used as positive and negative controls. The size difference in MEL vs ES cell clone SCL is due to the presence of a Biotin-tag in transfected SCL cDNA. Percentage indicates the total number of Zeocin resistant ES cell clones found to express SCL.

Figure 4.14
Mesoderm Differentiation of SCL Over-Expressing EBs

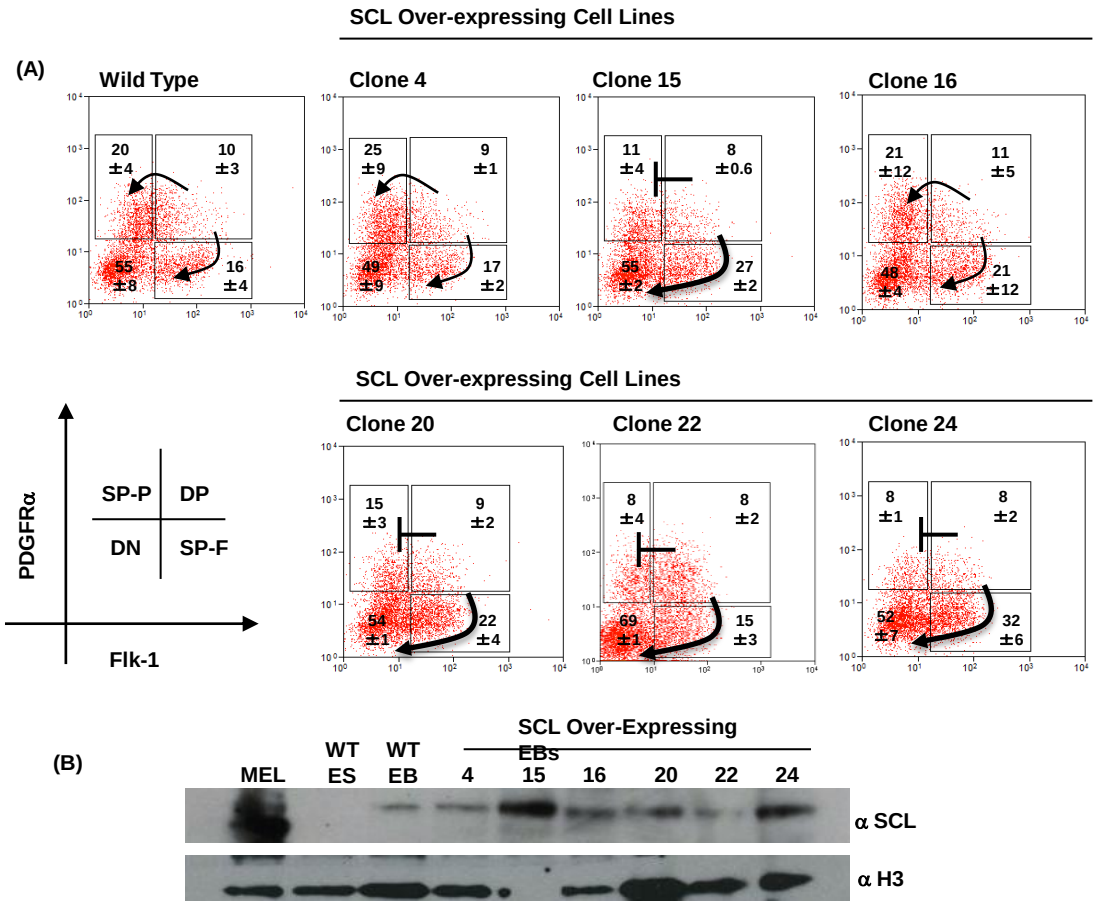


Figure 4.14 **Targeted ES clones that over-expressed SCL were differentiated *in vitro* for 6 days. (A) Day 6 Flk-1/PDGFRα immuno-phenotype of SCL over-expressing EBs. Values represent the mean percentage of live cells which express the indicated cell surface markers, ± standard deviation, where n=3. Arrows indicate gain and loss of cell surface marker expression anticipated with differentiation. (B) Western blot showing SCL expression in day 6 EBs derived from SCL over-expressing ES cells. MEL cells and wild type day 4 EBs were used as positive controls. Wild type ES cells were used as a negative control.**

Figure 4.15
Haematopoietic Potential of SCL Over-Expressing EBs

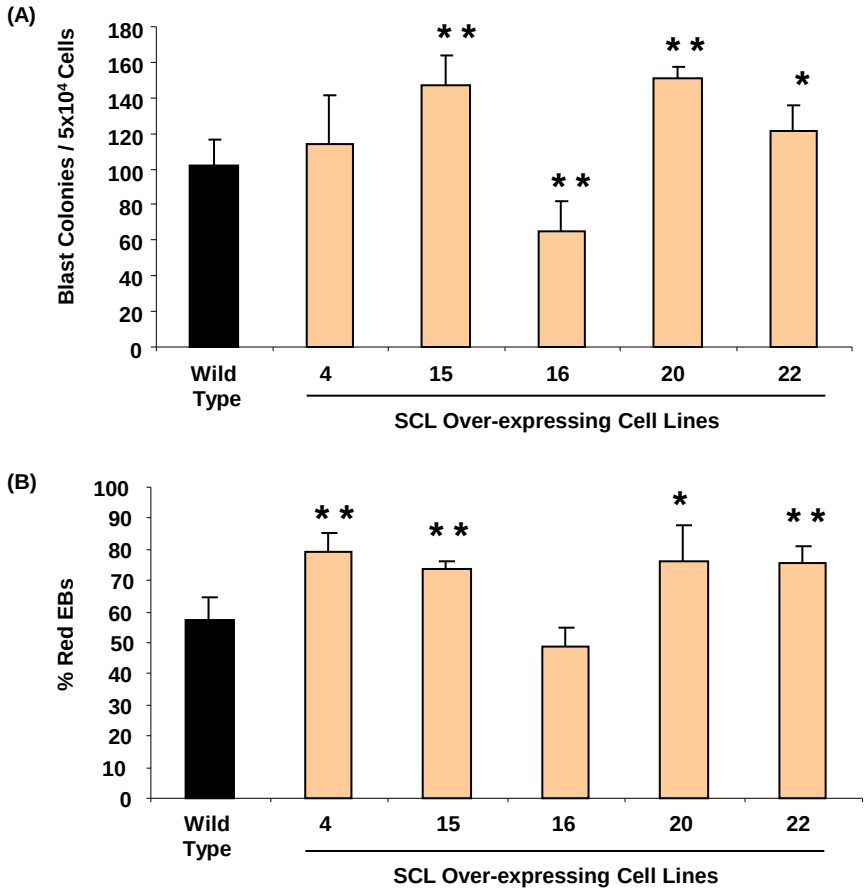


Figure 4.15 Haematopoietic potential of SCL over-expressing ES cell lines was assessed by ability to generate (A) blast colonies and (B) haemoglobin compared to wild type cells (see Figure 3.3). (A) Number of blast colonies generated from re-plating cells from day 3.5 EBs. (B) Percentage of EBs which turned red, due to hemoglobin production, after 7 days of differentiation. Graphs represent results from three independent experiments. * indicates a statistically significant change compared to wild type, where * indicates a P-value < 0.05 and ** indicates a P-value < 0.01.

Figure 4.16
Cardiac Potential of SCL Over-Expressing EBs

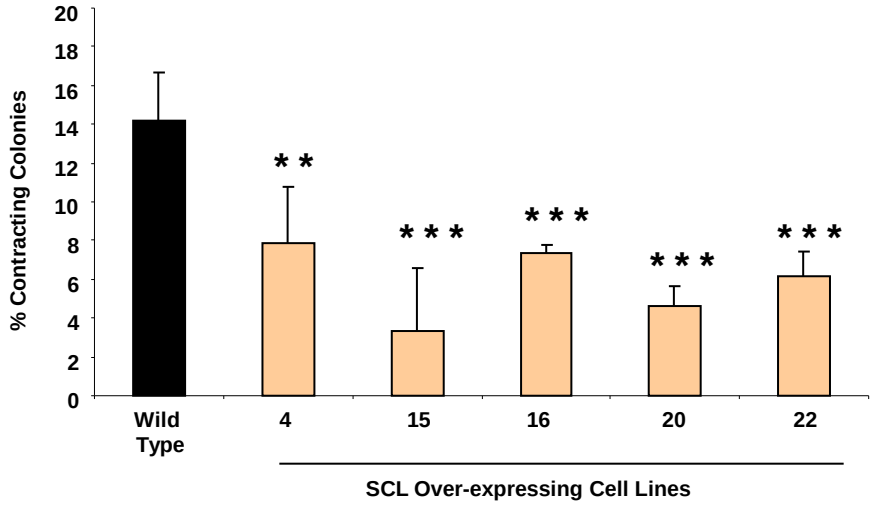


Figure 4.16 Cardiac potential of SCL over-expressing ES cells was assessed by ability to generate spontaneously contract colonies (see Figure 3.3). (A) Percentage of contracting colonies generated from day 4 EBs after re-plating for 6 days under cardiac inducing conditions (10 days total differentiation). Graphs represent results from three independent experiments. * indicates a statistically significant change compared to wild type, where ** indicates a P-value P<0.01 and * indicates a P-value <0.001.**

Figure 4.17
Rescue of *Scl* $^{-/-}$ Haematopoietic Phenotype

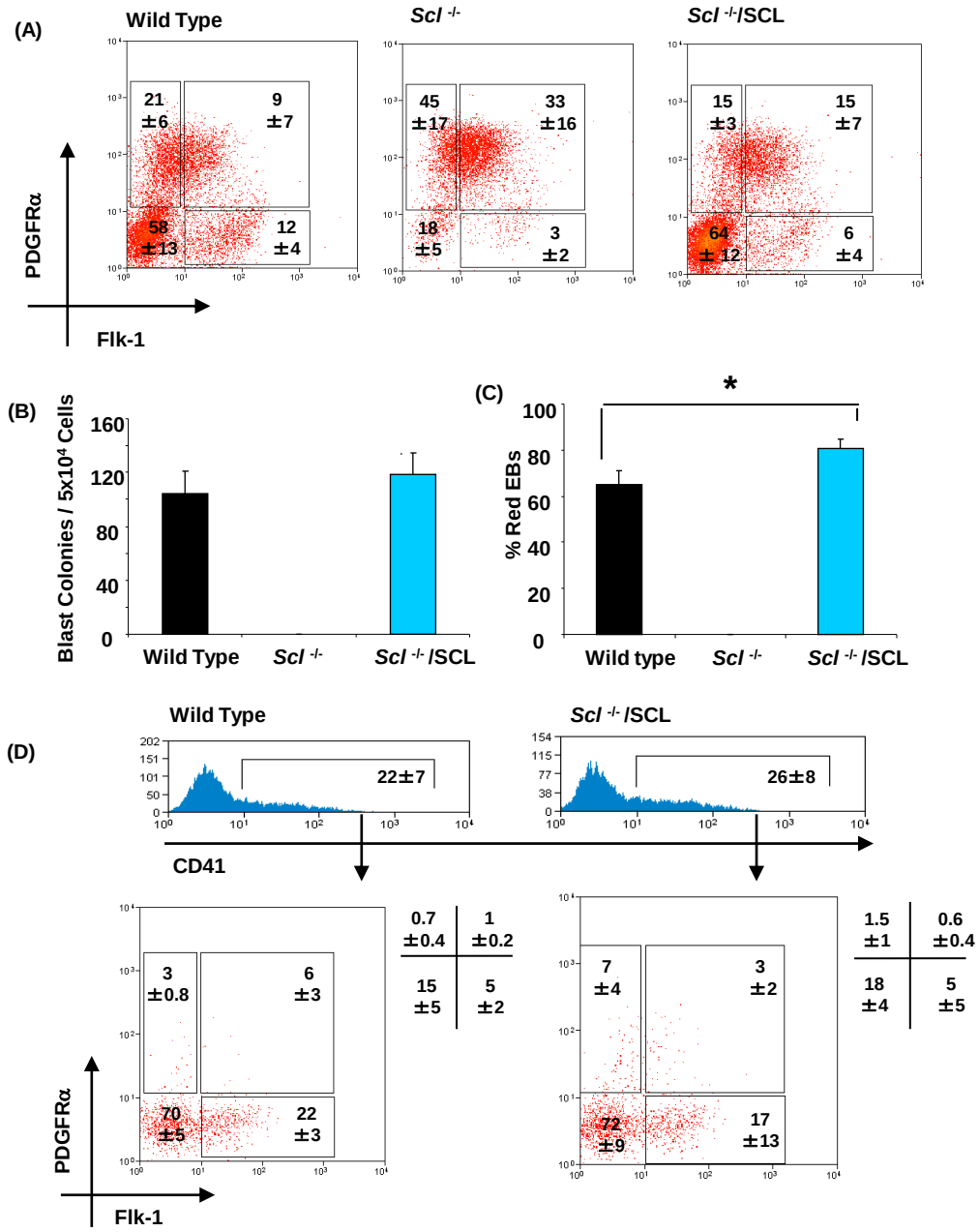


Figure 4.17 **Haematopoietic potential of *Scl* $^{-/-}$ /SCL ES cells was assessed by FACS analysis and *in vitro* cell fate assays (see Figure 3.3). (A) Day 6 Flk-1/PDGFRα immuno-phenotype. (B) Number of blast colonies generated per 5x10^4 day 3.5 EBs cells. (C) Percentage of day 7 EBs with visible haemoglobin production. Graphs represent results from three experiments. * indicates a P-value < 0.05. (D) Top plots show the percentage of live cells expressing CD41. Bottom plots show the percentage of CD41 expressing cells co-expressing Flk-1 and/or PDGFRα. Percentage of total cells to co-express CD41 and/or Flk-1/PDGFRα is indicated in the adjacent quadrants. Values show the mean and standard deviation where n = 3.**

Figure 4.18
Rescue of *Scl* $^{-/-}$ Cardiac Phenotype

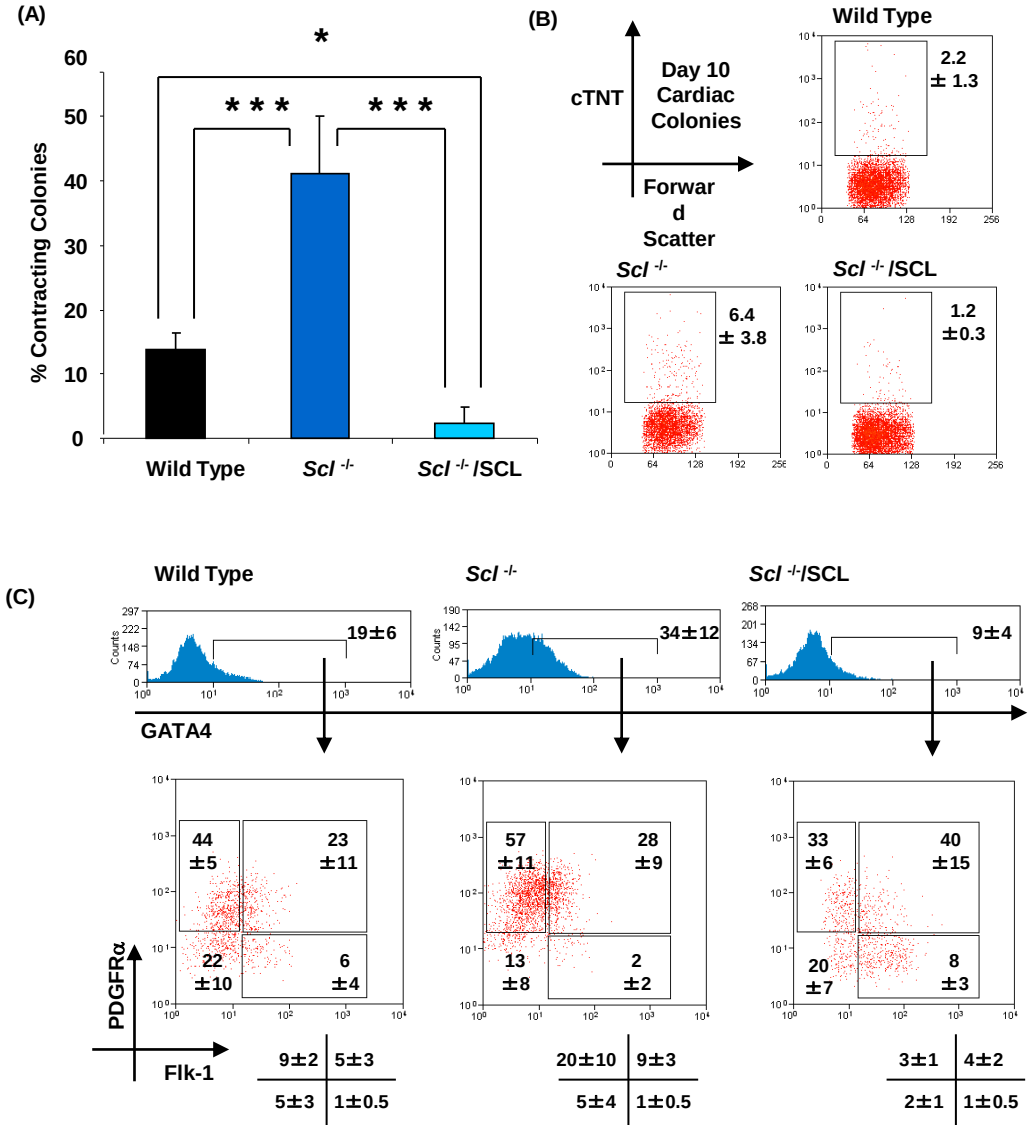


Figure 4.18 **Cardiac potential of *Scl* $^{-/-}$ /SCL ES cells was assessed by ability to (A) generate contracting colonies, and (B) express cTnT. (A) Percentage of colonies, generated from re-plating day 4 EBs for 6 days under cardiac inducing conditions (10 days total differentiation), able to spontaneously contract. Graph represents results from three independent experiments. * indicates a P-value < 0.05 and, *** indicates a P-value < 0.001. (B) Expression of cTnT in day 10 cardiac cultures, values represent mean percentage of live cells expressing cTnT, ± standard deviation, where n ≥ 3. (C) Blue plots show the percentage of day 6 EB cells expressing GATA 4. Red plots show Flk-1/PDGFRα expression patterns within the GATA4^{+} population. Values in plots show the mean percentage of cells expressing the indicated proteins. Percentage of total cells to co-express GATA4 with Flk-1/PDGFRα is indicated in the quadrants below.**

Figure 5.1
Knock-in of a Tetracycline Responsive Element

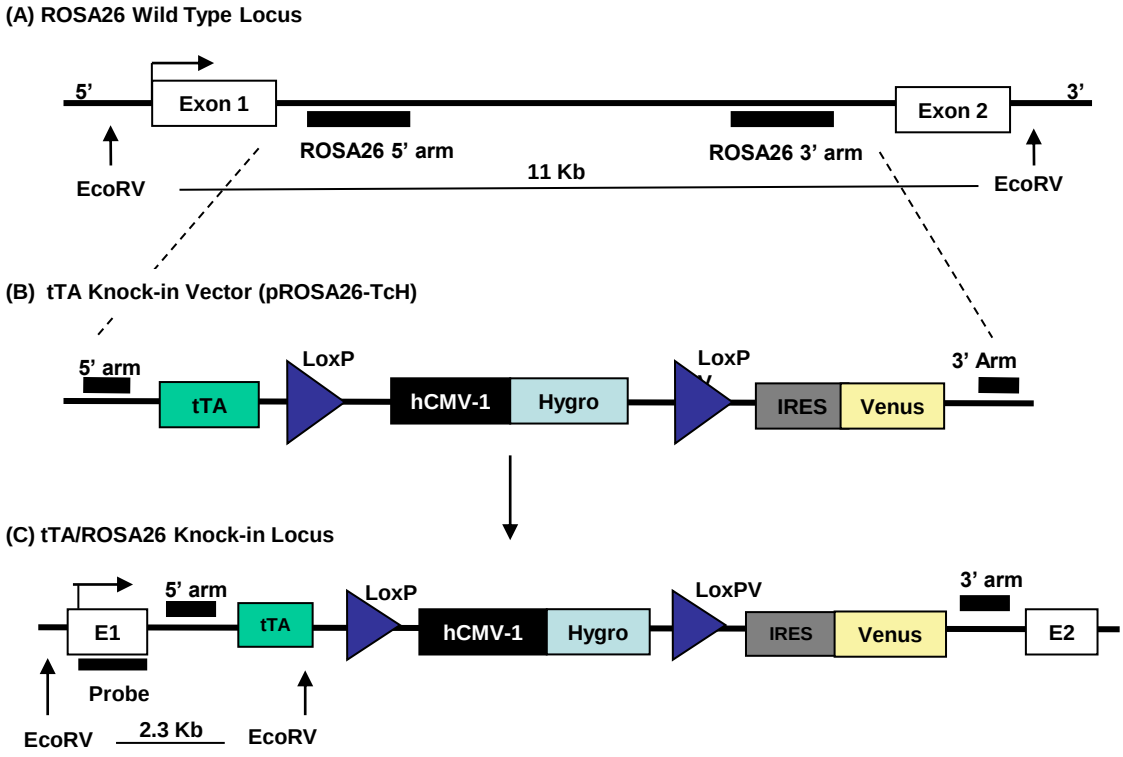


Figure 5.1 Wild type and *Scl* $^{-/-}$ ES cell lines (A) were targeted with a construct (B) designed to introduce a tetracycline trans-activator (tTA) into the ROSA26 locus. To enable selection of correctly targeted clones, an hCMV-1 / hygromycin resistance cassette and an IRES-Venus were also introduced. The hygromycin resistance cassette was flanked with LoxP sites to allow Cre-recombinase mediated excision and replacement with a cDNA under the control of a tetracycline responsive hCMV-1* promoter (see Figure 5.5 for further targeting strategies).

Figure 5.2
Screening for tTA/ROSA26 ES Cells

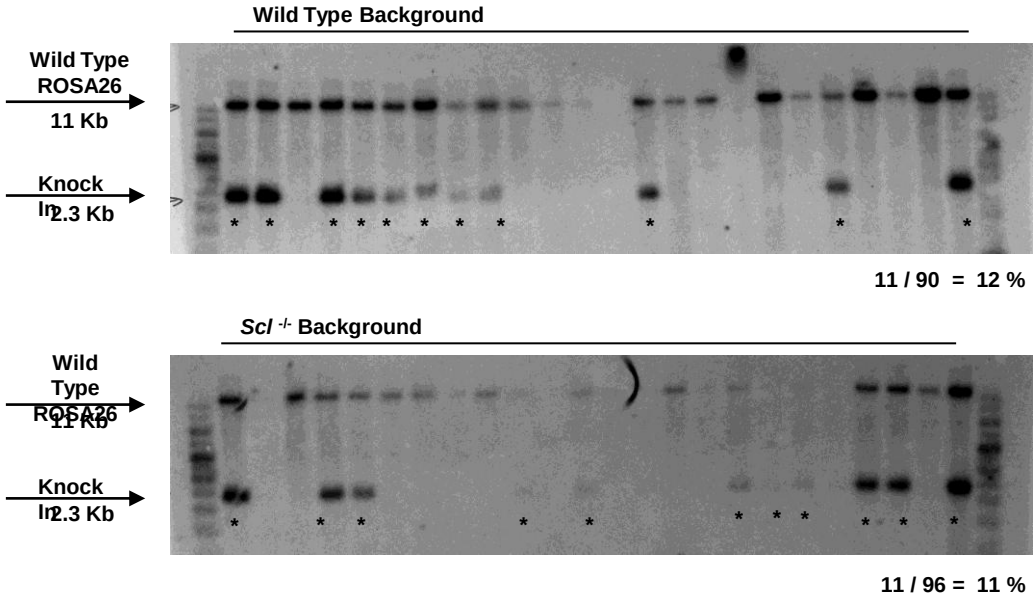


Figure 5.2 Hygromycin resistant ES clones were screened by Southern blot analysis to confirm targeting of the ROSA26 locus. An 11 Kb band is generated from the wild type ROSA26 allele, while a 2.3 Kb band is generated by the presence of mutant knock-in allele. Values and percentages represent the number of positive clones identified from the total number of hygromycin resistant clones tested.

Figure 5.3
tTA/ROSA26 Wild Type ES Cells

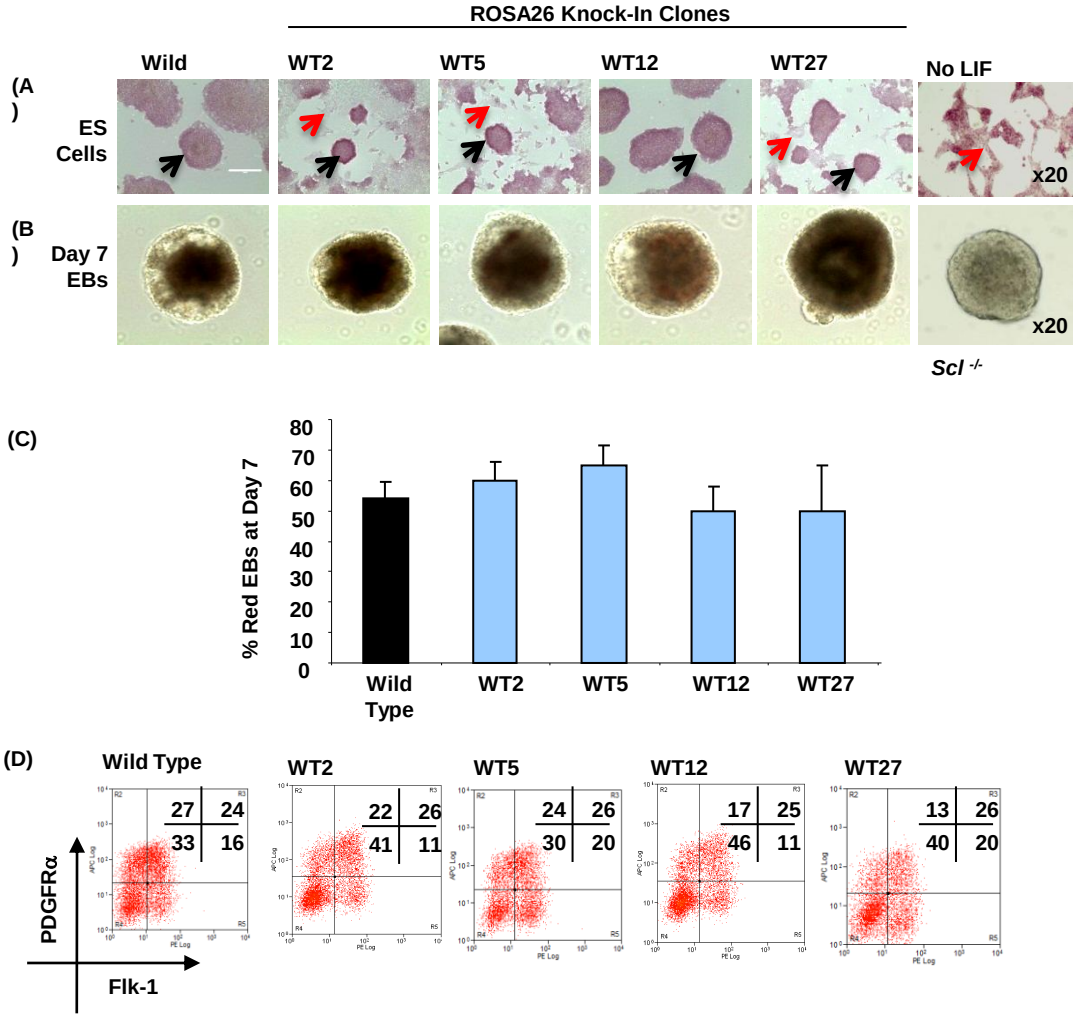


Figure 5.3 Pluri-potency and differentiation potential of tTA/ROSA26 knock-in wild type ES cells. (A) Alkaline phosphatase staining showing morphology and pluripotent potential of ES cell colonies. ES cells cultured without LIF are shown as a control. Black arrows indicate tight ES colonies, and red arrows indicate differentiating cells. (B) Morphology and haemoglobin production in day 7 EBs. *Scl* $^{-/-}$ EBs are shown as a control. (C) Percentage of day 7 EBs showed visible haemoglobin production (~200 EBs counted) Graph represents results from two independent ES cell differentiations, performed in triplicate. (D) Flk-1/PDGFRα immuno-phenotype of day 4 EBs. Numbers represent the percentage of live cells found in corresponding quadrants. Plots show representative plots from two *n vitro* differentiations.

Figure 5.4
tTA/ROSA26 *Scl* $^{-/-}$ ES Cells

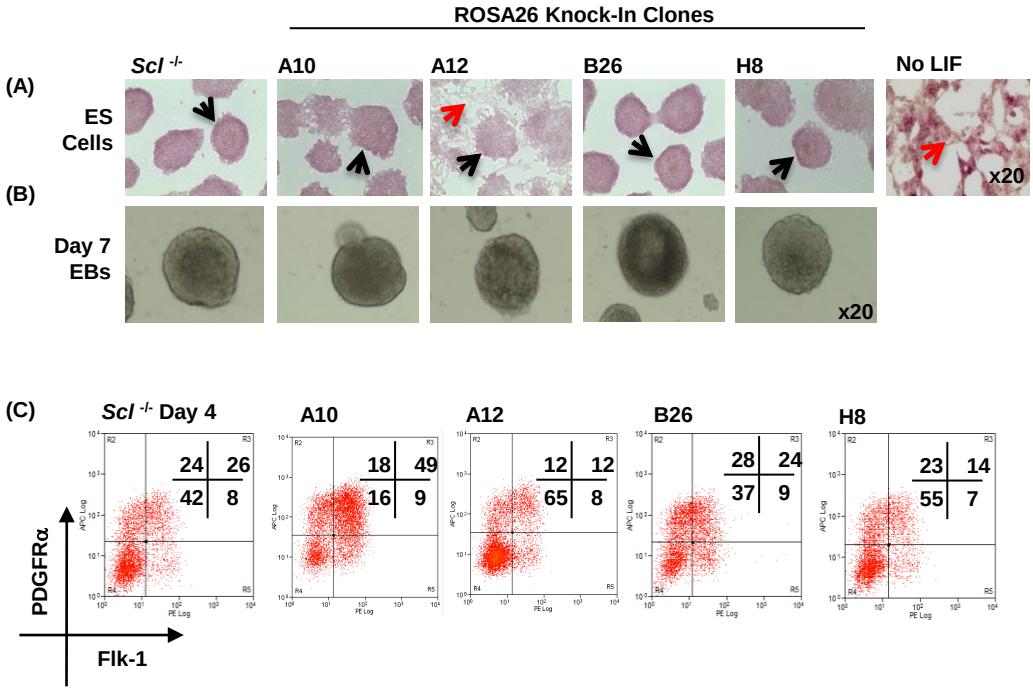


Figure 5.4 Pluripotency and differentiation potential of tTA/ROSA26 knock-in *Scl* $^{-/-}$ ES cells, (A) Alkaline phosphatase staining showing morphology and pluripotent potential of ES cell colonies. Black arrows indicate tight ES colonies, and red arrows indicate differentiating cells. (B) Morphology of day 7 EBs (no red cells due to *Scl* $^{-/-}$ background). (B) Flk-1/PDGFRα immuno-phenotype of day 4 EBs. Numbers represent the percentage of live cells found in corresponding quadrants. Plots show representative plots from two *n vitro* differentiations. .

Figure 5.5
An Inducible SCL Expression System

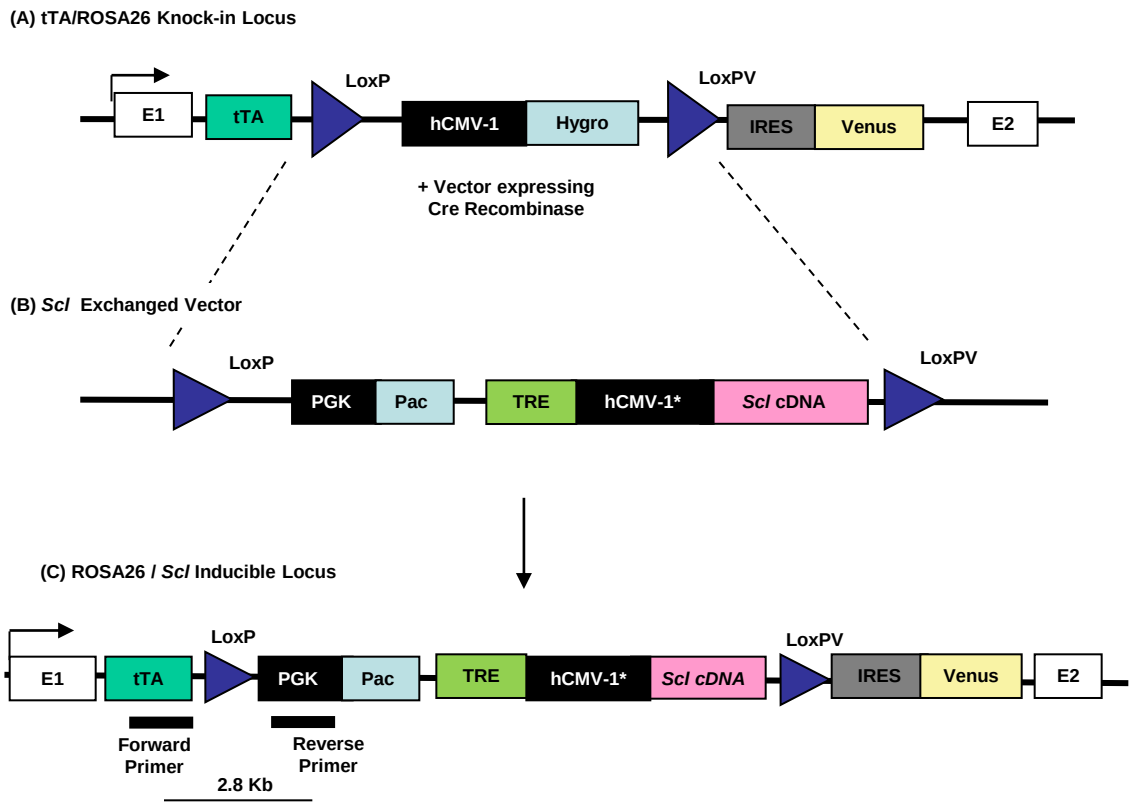


Figure 5.5 tTA/ROSA26 knock-in ES cells (A) were transfected with an exchange vector (B) containing a PGK-Pac puromycin resistance cassette and *Scl* cDNA under the control of a tetracycline responsive (TRE) hCMV-1* promoter. Co-transfection with a Cre-recombinase expressing vector mediates cleavage at LoxP sites, followed by recombination to introduce the exchange fragment into the ES cell genome, (C), thus placing *Scl* expression under the control of the tetracycline responsive element. Location of primers used to confirm ES cell genotype are indicated.

Figure 5.6
Selection of ES Cells with Inducible *Scl* Expression

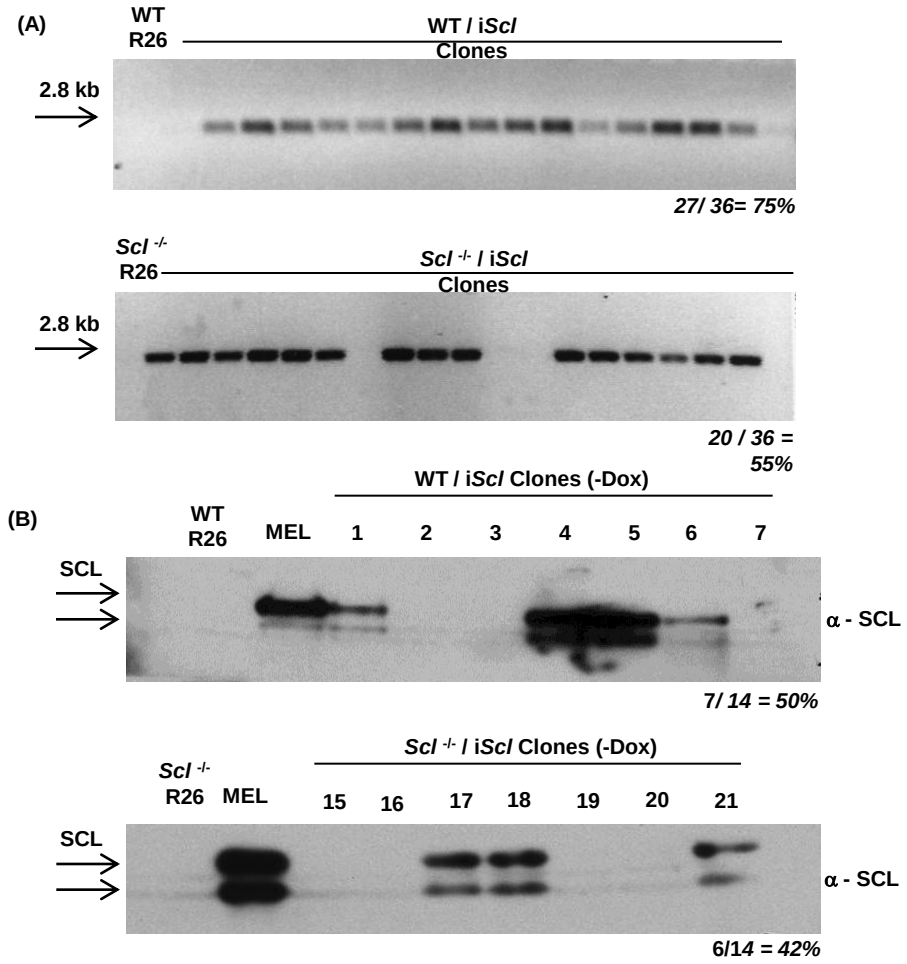


Figure 5.6 (A) Wild type and *Scl* $^{-/-}$ ES cell clones, targeted as outlined in Figure 5.5 and characterised as Puromycin resistant and Hygromycin sensitive, were screened by PCR (primers indicated in figure 5.5) to confirm cassette exchange. Percentages represent the number of positive clones identified from the total number of Puromycin resistant, Hygromycin sensitive, clones tested. Parental tTA/ROSA26 clones, on a wild type (WT R26) or *Scl* $^{-/-}$ (*Scl* $^{-/-}$ R26) background, were used as negative controls. (B) Western blot confirming expression of SCL in correctly genotyped ES cells, cultured in the absence of doxycycline to allow activation of the hCMV-1* promoter. MEL cells and parental ES cells (WT R26 or *Scl* $^{-/-}$ R26) were used as positive and negative controls for SCL expression. The two bands represent two isoforms of SCL. Percentages represent the number of SCL-expressing ES clones identified from the total number of correctly genotyped ES cell clones tested.

Figure 5.7
Doxycycline Control of *Scl* Expression

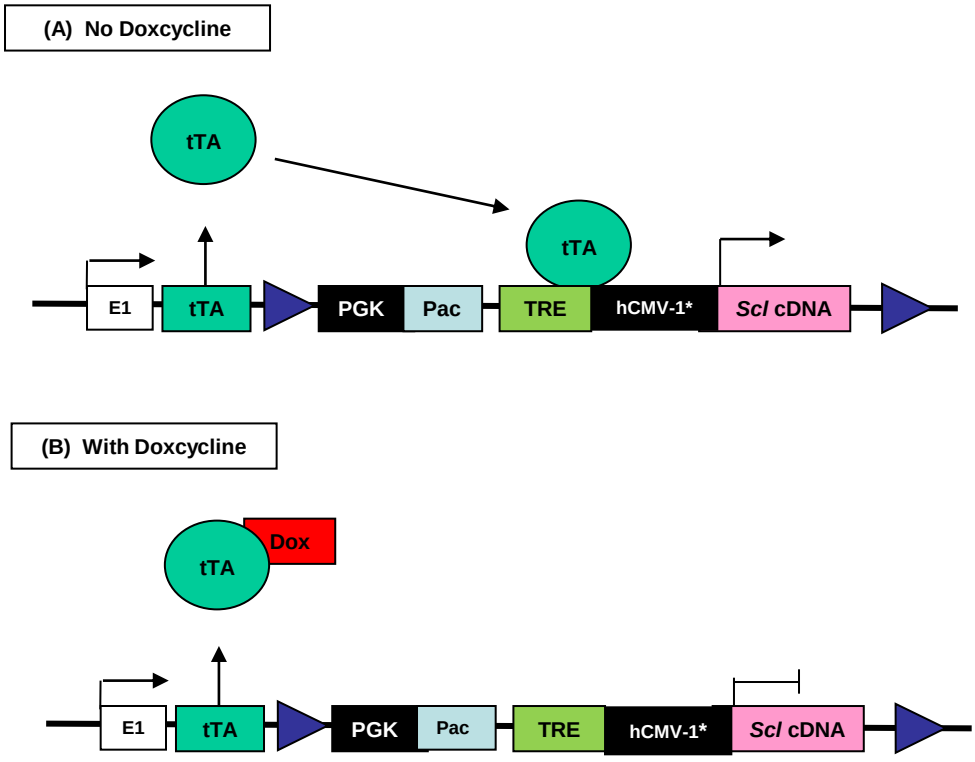


Figure 5.7 Schematic demonstrating the control of SCL expression with doxycycline treatment in WT / i*Scl* and *Scl* $^{-/-}$ / i*Scl* cells lines, where addition of doxycycline (Dox) prevents expression from the hCMV-1* promoter.

Figure 5.8
Doxycycline Control of SCL Expression in ES Cells

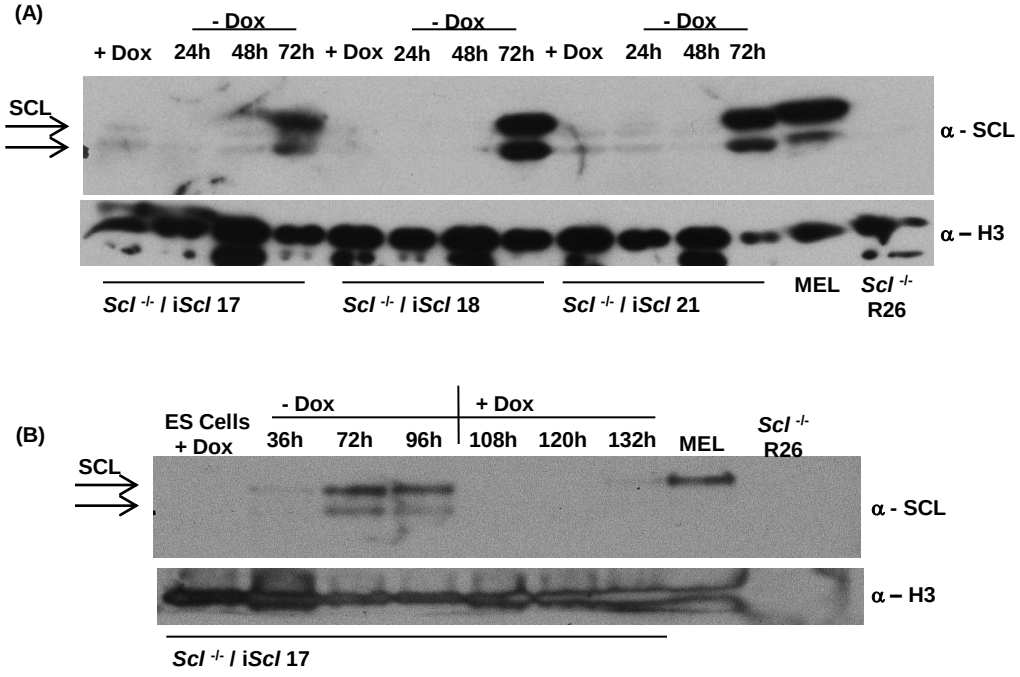


Figure 5.8 Repression and activation of SCL expression in *Scl* $^{-/-}$ / i*Scl* clones 17, 18 and 21, which expressed SCL in the absence of doxycycline (see Figure 5.6). (A) Western Blot showing repression of SCL expression in *Scl* $^{-/-}$ / i*Scl* ES cell lines after two days treatment with doxycycline (+Dox), and re-activation of SCL expression with the removal of doxycycline (-Dox) over 72 hours. (B) Western Blot showing the activation of SCL in *Scl* $^{-/-}$ / i*Scl* 17 ES cells line upon removal of doxycycline, followed by the repression of SCL expression 12 hours after re-introduction of doxycycline into ES cell media. MEL cells and parental ES cells were used as positive and negative controls for SCL expression. Expression of Histone 3 is shown as a loading control.

Figure 5.9
Inducible SCL Expression in Differentiating EBs

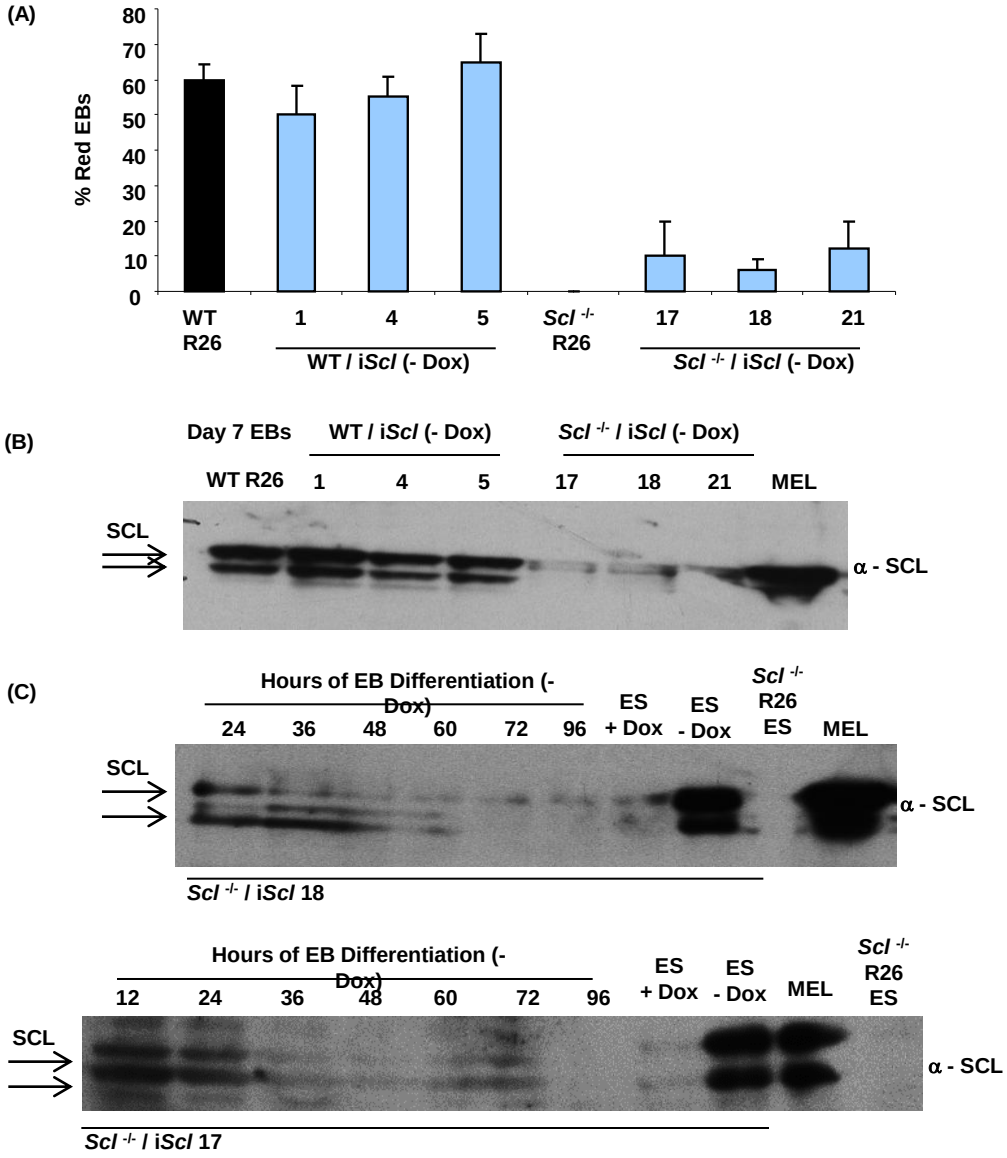


Figure 5.9 EB differentiation of WT / i*Scl* and *Scl* $^{-/-}$ / i*Scl* ES cell clones in absence of doxcycline. (A) Percentage of EBs with visible red cell production after 7 days of differentiation. Values represent results from two experiments performed in triplicate (B) Western blot showing SCL expression in day 7 EBs, compared to endogenous expression levels in parental cell lines (WT R26). (C) Western blot showing SCL expression during the first 4 days of differentiation of *Scl* $^{-/-}$ / i*Scl* 17 and *Scl* $^{-/-}$ / i*Scl* 18. MEL cells and parental *Scl* $^{-/-}$ R26 ES cells were used as positive and negative controls for SCL expression.

Figure 6.1a
Differential Gene Expression between Wild Type and *Scl* $^{-/-}$ Flk-1^{+} Cells

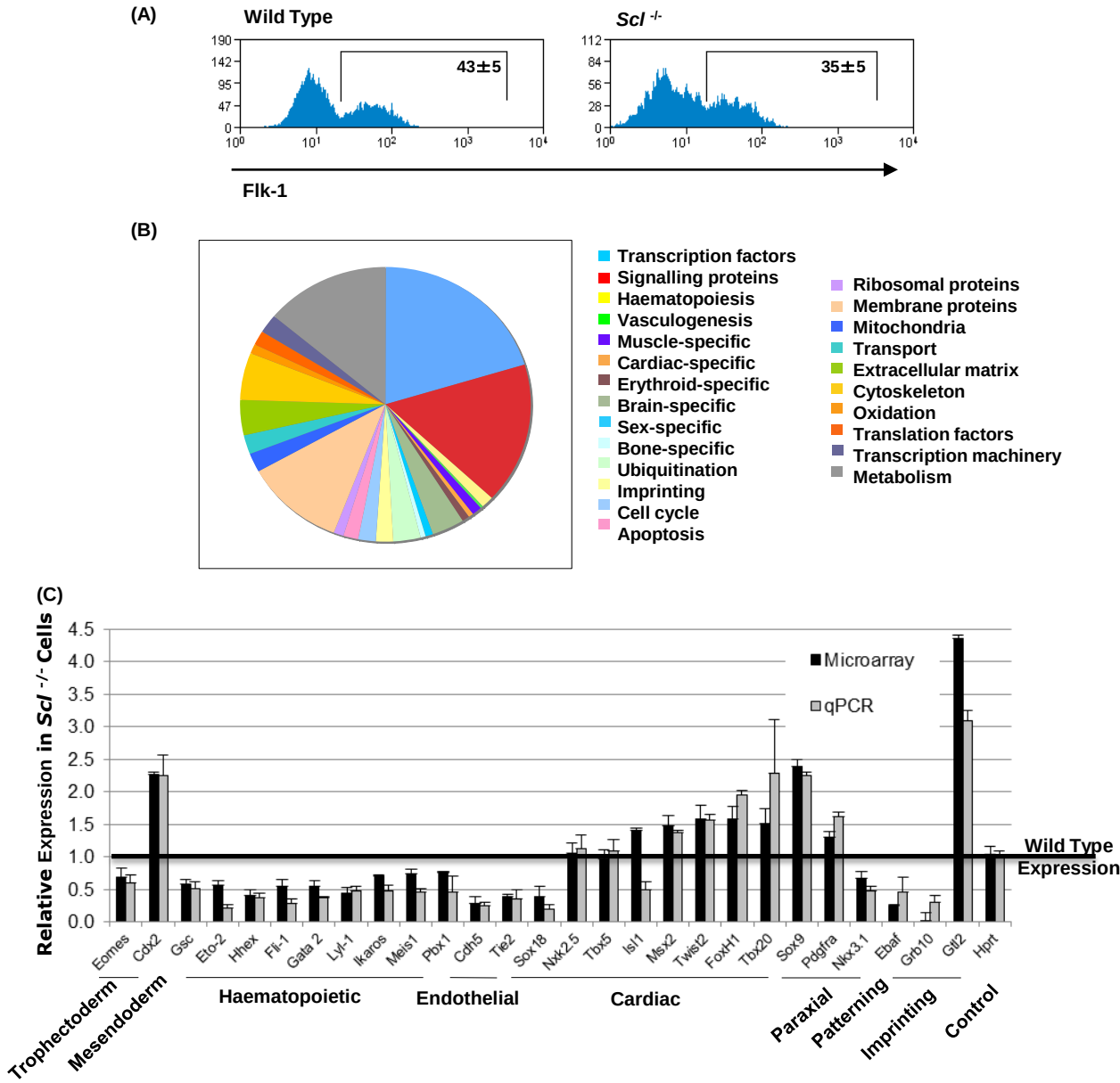


Figure 6.1a Flk-1 expressing cells were isolated from day 3.5 wild type and *Scl* $^{-/-}$ EBs (n=3 biological replicates). Their transcriptone was differentially analysed using gene expression microarrays (Illumina). (A) FACS analysis of the Flk-1^{+} cell populations isolated from wild type and *Scl* $^{-/-}$ EBs. Values show mean and standard deviation where n=3 (B) Pie chart representing the biological processes/gene function perturbed in *Scl* $^{-/-}$ Flk-1^{+} EB cells when compared to wild type controls. (C) Validation of differential gene expression using real time PCR. Graph compares data obtained from microarray data to data obtained by real time PCR analysis. All PCR reactions were run in duplicate with gene expression being normalised to *Gapdh*. Values represent gene expression in *Scl* $^{-/-}$ Flk-1^{+} cells, relative to wild type expression levels (where wild type = 1) and represent three biological repeats.

Figure 6.1b
Functional Gene Ontology
of Differentially Expressed Genes

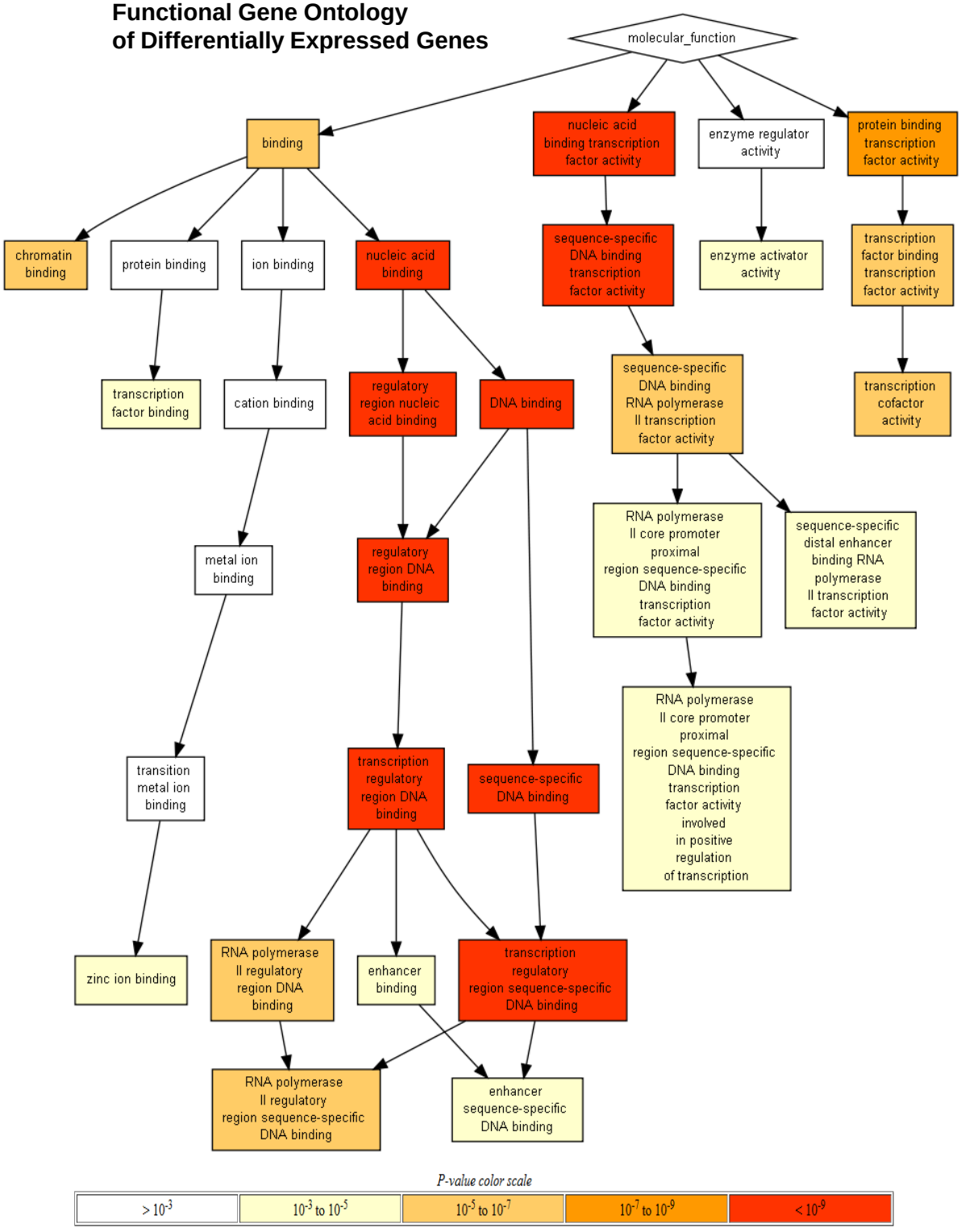


Figure 6.1b Flow diagram shows the functional ontology of genes found to be deregulated in *Scl* $^{-/-}$ Flk-1^{+} cells, compared to wild type Flk-1^{+} cells, as identified by microarray analysis (Figure 6.1a and Table 5). Deeper colouring corresponds to a smaller P-value, and indicates an over-representation of gene type (grouped by function) compared to their over genome-wide representation. Analysis was performed using the GOrilla database.

Figure 6.2
Lateral Mesoderm Gene Expression in Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs

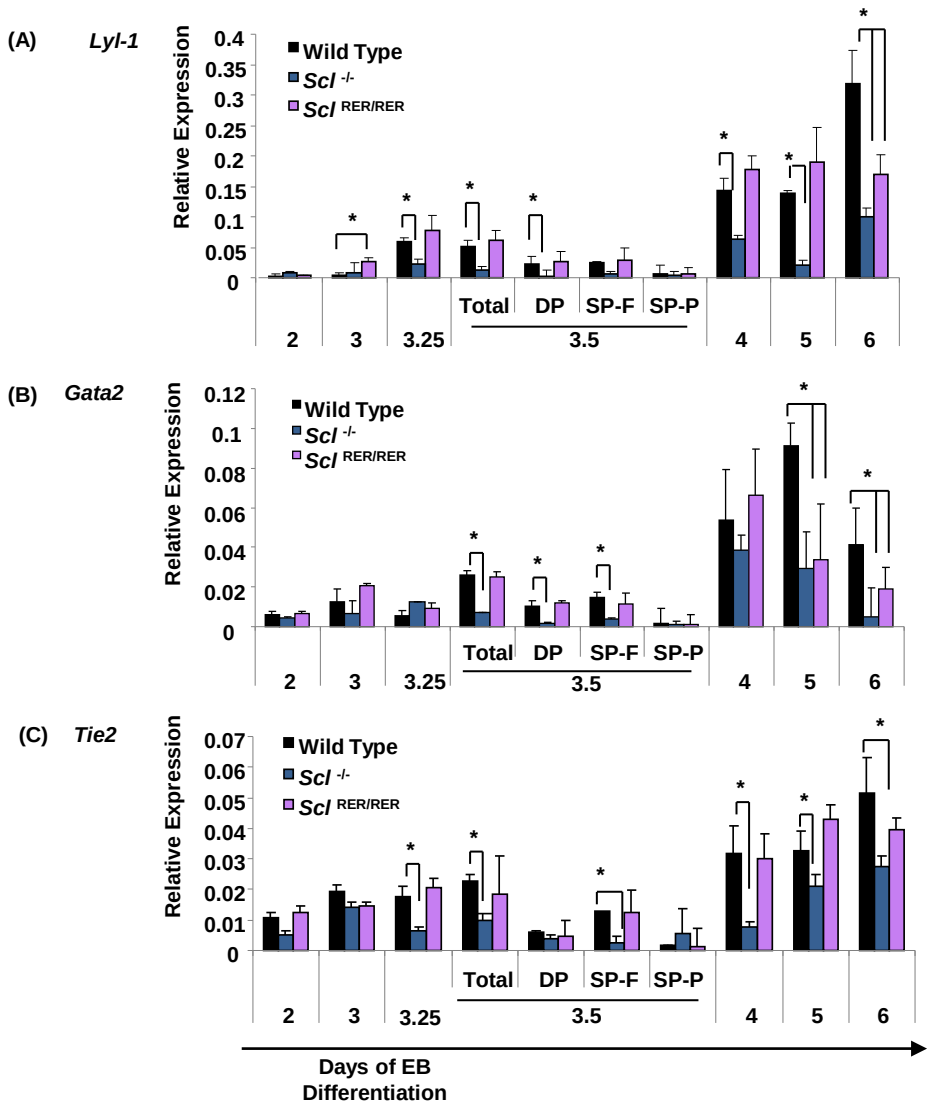


Figure 6.2 In depth expression analysis of selected lateral mesoderm genes by real time qPCR. Graphs show relative mRNA levels of (A) *Lyl-1*, (B) *Gata2* (haematopoietic genes) and (C) *Tie2* (an endothelial gene) in wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs between days 2 and 6 of differentiation. Gene expression in day 3.5 EBs is also shown in fractionated Flk-1/PDGFRα mesoderm compartments (DP; multi-potent, SP-F; lateral, SP-P; paraxial). PCR reactions were performed in duplicate, and values are normalised to *Gapdh* and *18S* expression. Graphs represent results from three independent biological repeats. * indicates P-value < 0.05.

Figure 6.3
Cardiac Gene Expression in Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs

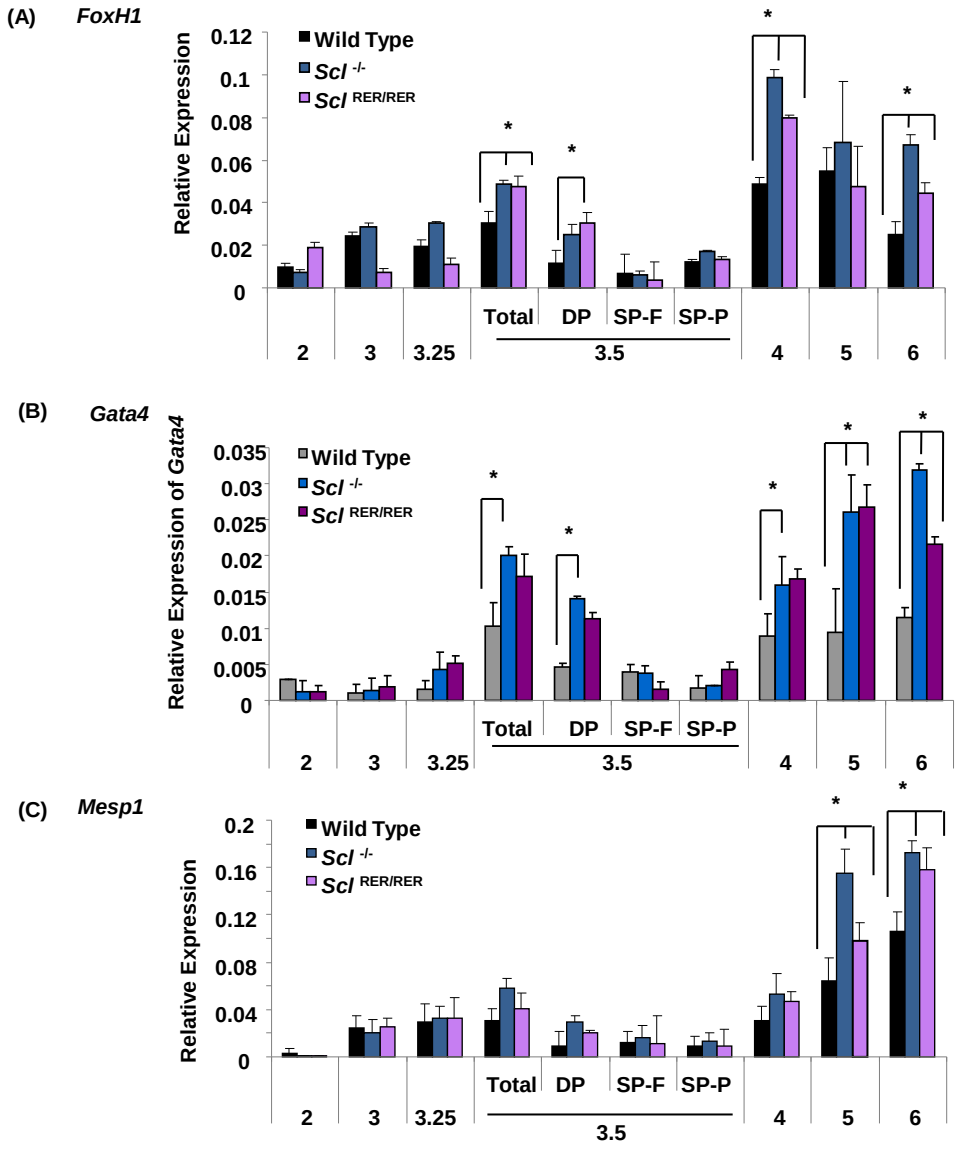


Figure 6.3 **Detailed expression analysis of selected cardiac mesoderm-associated genes was carried out by real time PCR. Graphs show relative mRNA levels of (A) *FoxH1*, (B) *Gata4* and (C) *Mesp1* in wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs between days 2 and 6 of differentiation. Gene expression in day 3.5 EBs is also shown in fractionated Flk-1/PDGFRα mesoderm compartments (DP; multi-potent, SP-F; lateral, SP-P; paraxial). PCR reactions were performed in duplicate, and values are normalised to *Gapdh* and *18S* expression. Graphs represent results from three independent biological repeats. * indicates P-value < 0.05.**

Figure 6.4
***Sox9* Expression in Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs**

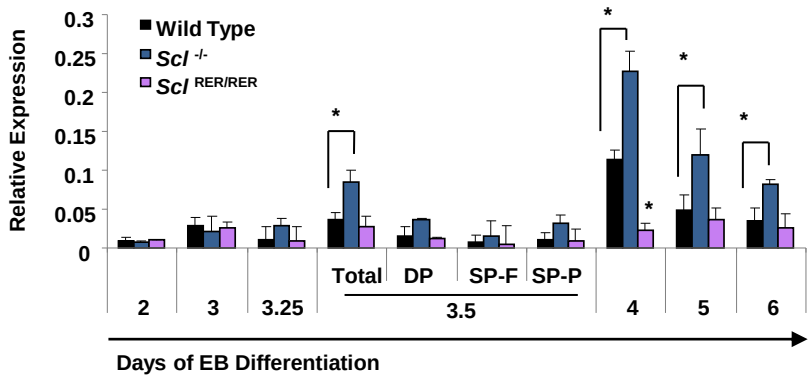


Figure 6.4 Detailed expression analysis of *Sox9* gene expression by real time PCR. Graphs show relative mRNA levels of *Sox9* (an early chondrogenic gene) in wild type, Scl $^{-/-}$ and Scl $^{RER/RER}$ EBs between days 2 and 6 of differentiation. Gene expression in day 3.5 EBs is shown in fractionated Flk-1/PDGFRα mesoderm compartments (DP; multi-potent, SP-F; lateral, SP-P; paraxial). PCR reactions were performed in duplicate, and values are normalised to *Gapdh* and *18S* expression. Graphs represent results from three independent biological repeats. * indicates P-value < 0.05.

Figure 6.5
GATA4 Expression in Wild Type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs

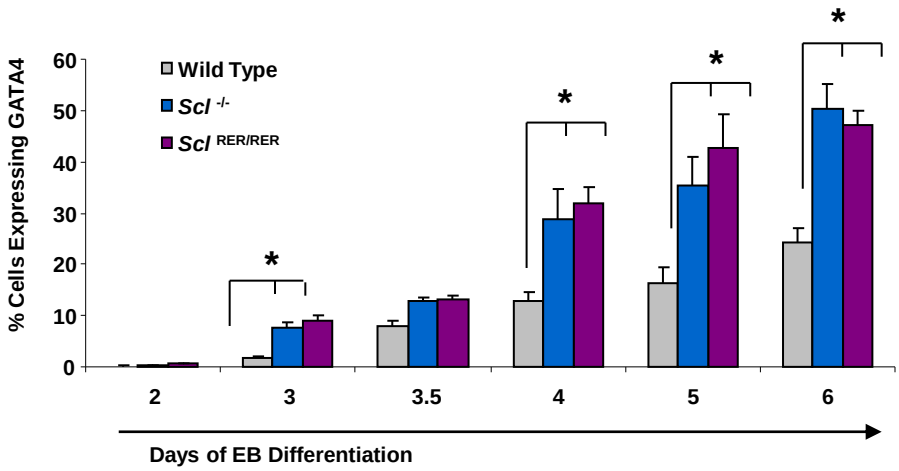


Figure 6.5 GATA4 protein expression during EB differentiation. Graph shows the percent of live cells expressing GATA4, using an internal FACS protocol, in wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs between days 2 and 6 of differentiation. Graph represents results from three independent biological repeats.* indicates where gene expression in *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ cells significantly differs from wild type expression with a P-value < 0.05.

Figure 6.6
GATA4 Expression in Differentiating EBs

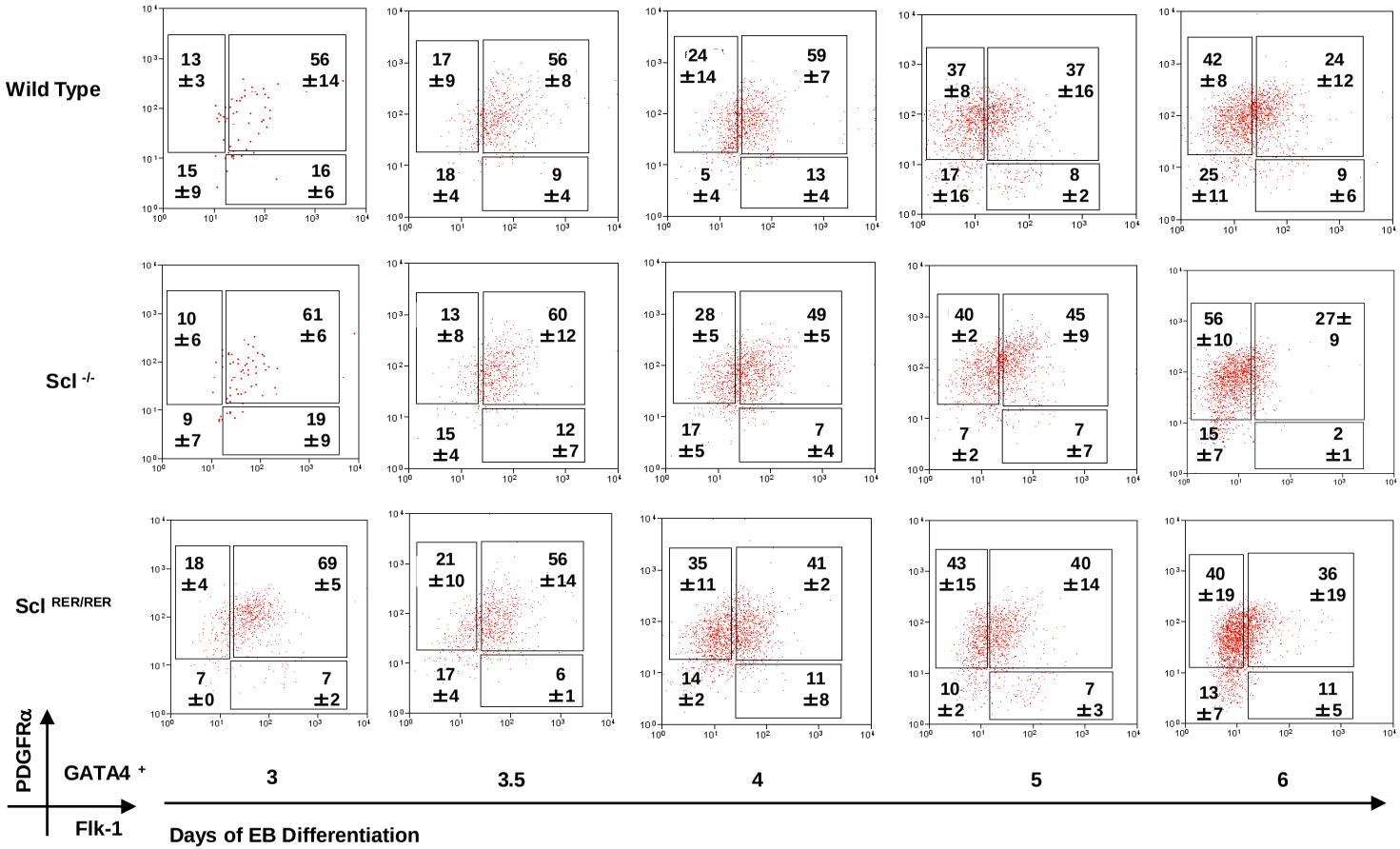


Figure 6.6 FACS showing lk-1/PDGFRα expression within GATA4⁺ populations in wild type, *Scl* $^{-/-}$ and *Scl* $^{RER/RER}$ EBs, between day 3 and 6 of differentiation. GATA4 expressing cells were isolated using intracellular FACS. Plots show back gating of GATA4⁺ cells, to determine co-expression with Flk-1 and/or PDGFRα. All values indicate the mean percentage of GATA4⁺ cells that express with Flk-1 and/or PDGFRα, ± standard deviation, where n ≥ 3. Percentage of total cells that co-express GATA4 and Flk-1/PDGFRα are shown in Table 7.

Figure 6.7
GATA4 Expression in Day 6 SCL Over-Expressing and *Scl* $^{-/-}$ / SCL EBs

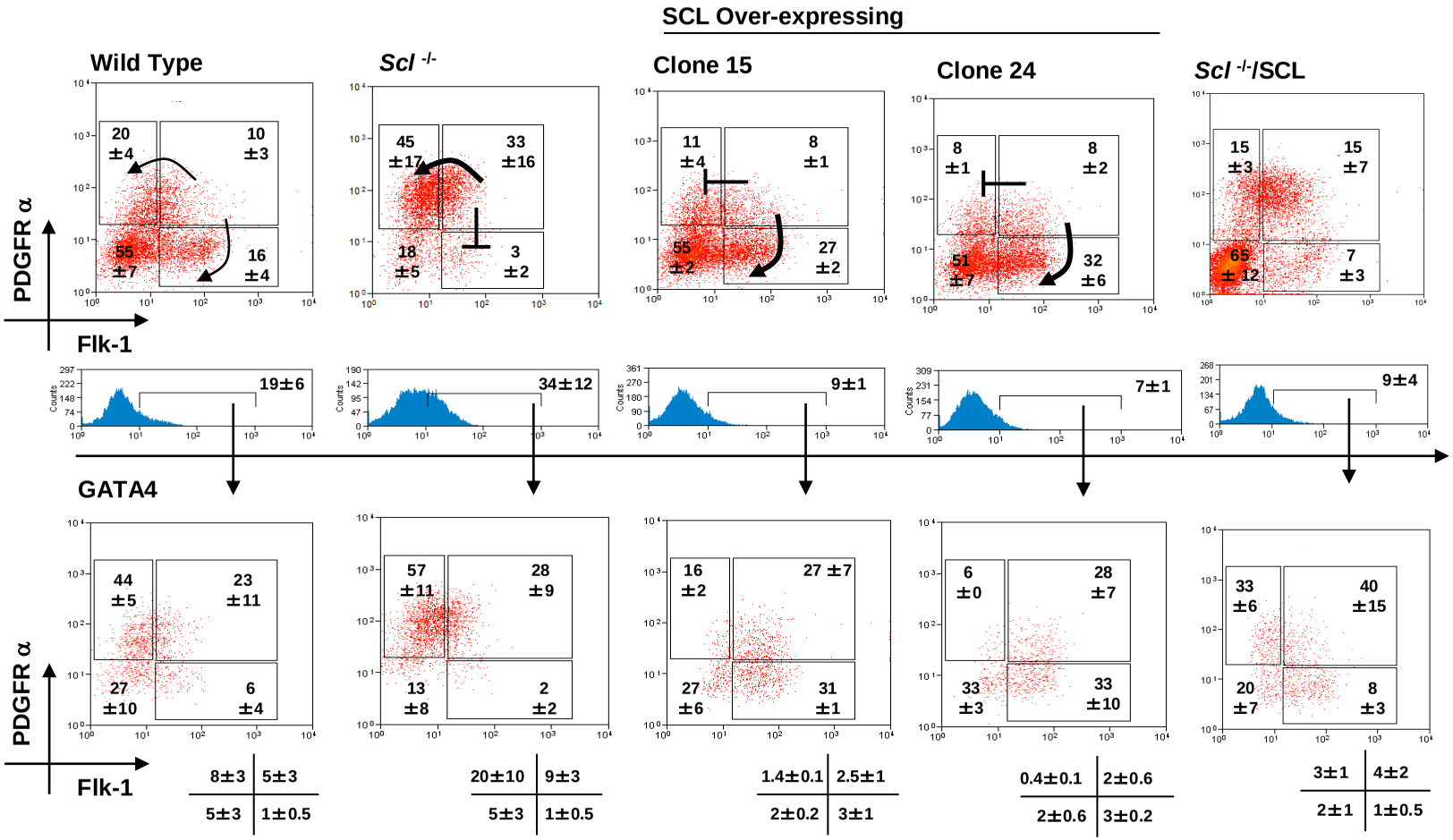


Figure 6.7 Expression of GATA4 in SCL over-expressing EBs. Top plots show the Flk-1/PDGFRα immuno-phenotype exhibited by day 6 EBs. Middle plots show the percentage of cells that express GATA4. Bottom plots show the distribution of GATA4 expressing cells in Flk-1/PDGFRα populations. Values represent the mean percent of live cells which lie within in indicated gates, ±standard deviation (where n ≥ 2). Values in quadrants below indicate the percentage of total cells that co-express GATA4 and Flk-1/PDGFRα Arrows indicate gain and loss of cell surface marker expression anticipated with differentiation.

Figure 6.8
Generation of a Gata4 Over-Expressing ES Cell Line

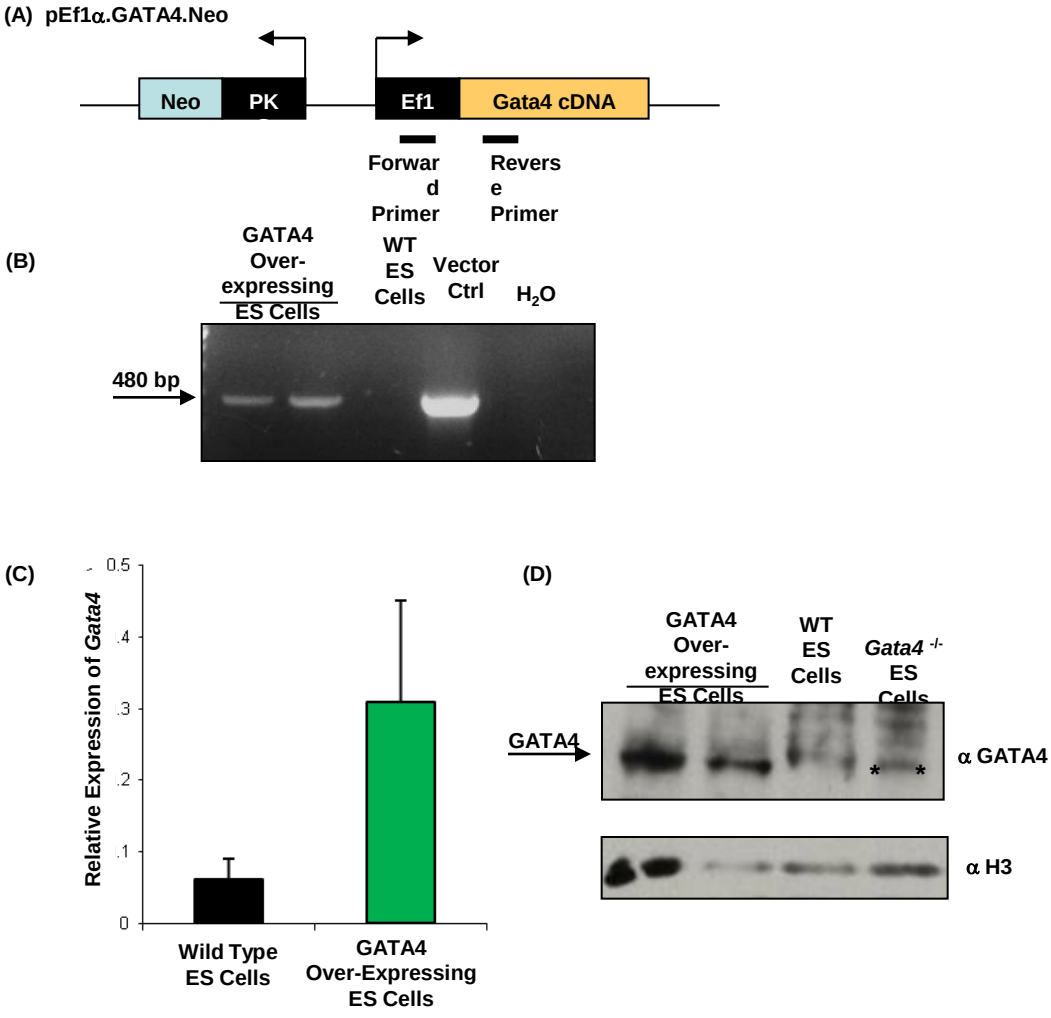


Figure 6.8 **Wild type ES cells were targeted with an expression vector (A) designed to drive ectopic GATA4 expression. (B) Targeted, neomycin resistant, ES cell populations were genotyped by PCR to confirm introduction of the Ef1a.GATA4 into the genome. Location of primers is show in (A). (C) qPCR for *Gata4* mRNA expression in wild type and GATA4 over-expressing ES cells. PCR was performed in duplicate, on two independent samples. Results were normalised to *Gapdh* expression. Error bars indicate the standard deviation. (D) Western blot confirming GATA4 protein expression in ES cells. *Gata4* $^{-/-}$ ES cells were used as negative controls. * indicates background staining.**

Figure 6.9
Cardiac Potential of GATA4 Over-expressing and *Gata4* $^{-/-}$ EBs

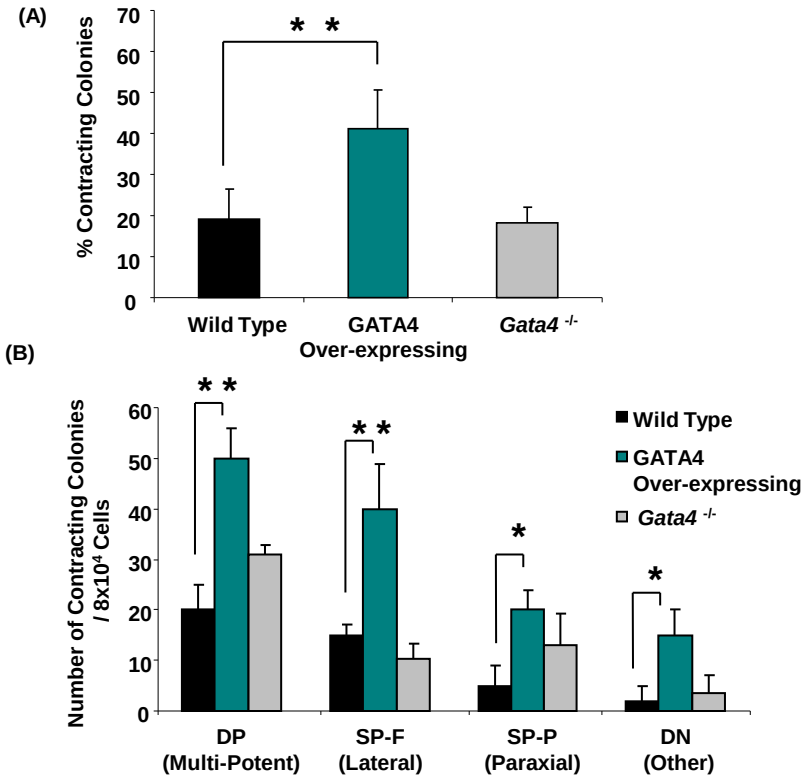


Figure 6.9 Cardiac potential of GATA4 over-expressing and *Gata4* $^{-/-}$ EBs was assessed by the formation of spontaneously contracting colonies after plating EBs onto gelatin under serum-free conditions. (A) Graph shows the percent of contracting colonies, from Day 4 EBs re-plated under cardiac conditions for 6 days (10 days total differentiation). (B) Number of contracting colonies generated by re-aggregating 4x10^5 cells, sorted from day 3.5 EB according to their Flk-1/PDGFRα immuno-phenotype, after 6 days in cardiac culture. All graphs represent at least three independent experiments. Error bars represent standard deviation of results. Error bars represent the standard deviation, * indicated a P-value < 0.05 and * * indicates a P-value < 0.01.

Figure 6.10
Haematopoietic Potential of GATA4 Over-expressing and *Gata4* $^{-/-}$ EBs

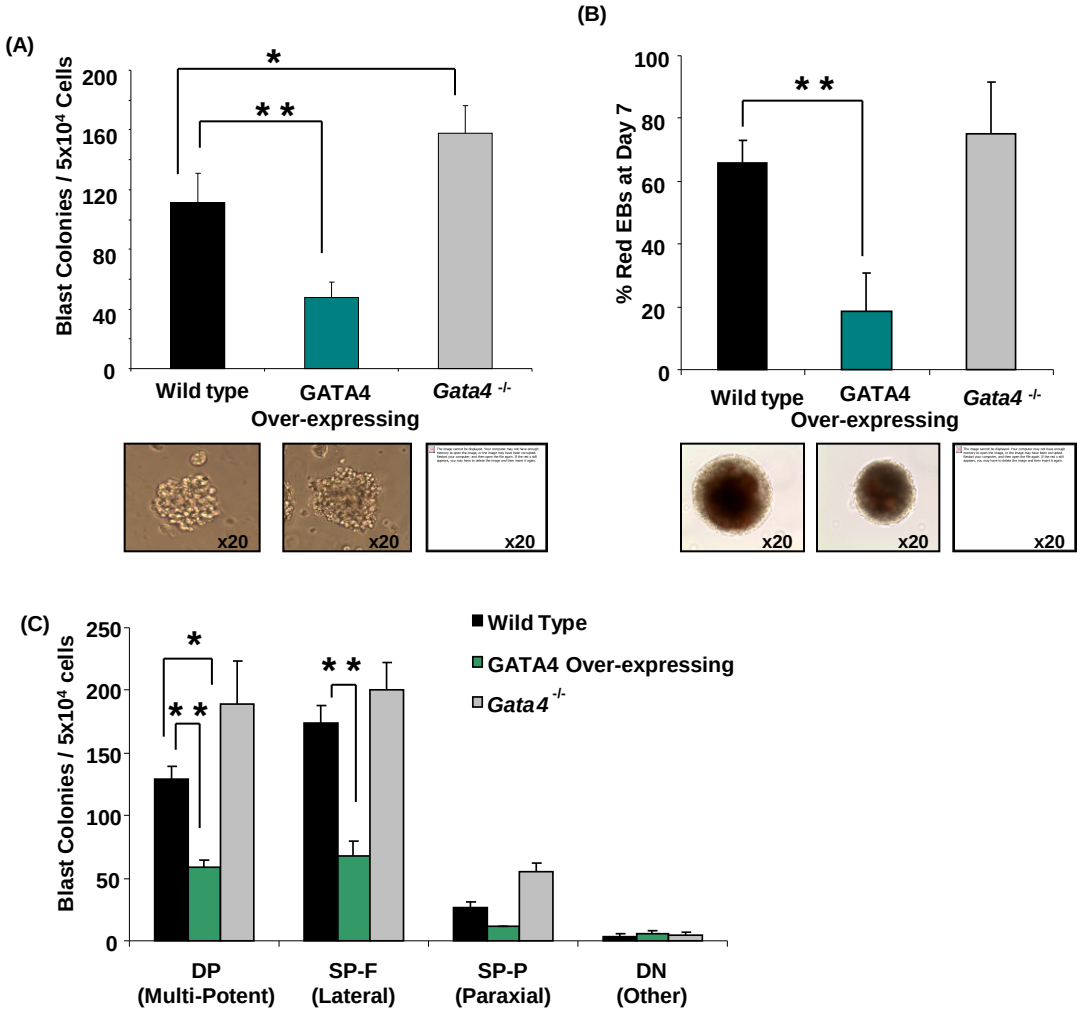


Figure 6.10 **Haematopoietic potential of GATA4 over-expressing and *Gata4* $^{-/-}$ EBs was measured by ability to generate blast colonies (A & C) and produce hemoglobin (B). (A) Number of blast colonies generated from re-plating day 3.5 EB cells in the presence of VEGF. (B) Percentage of day 7 EBs containing visible red cells. (A-B) Morphology of blast colonies and EBs is shown in photos beneath graphs. (C) Number of blast colonies generated from re-plating day 3.5 Flk-1/PDFGRα fractionated EB cells. All graphs represent results from three independent experiments. Error bars represent the standard deviation, * indicates a P-value < 0.05 and ** indicates a P-value <0.01.**

Figure 6.11
SCL Expression in GATA4 Over-expressing & *Gata4* $^{-/-}$ EBs

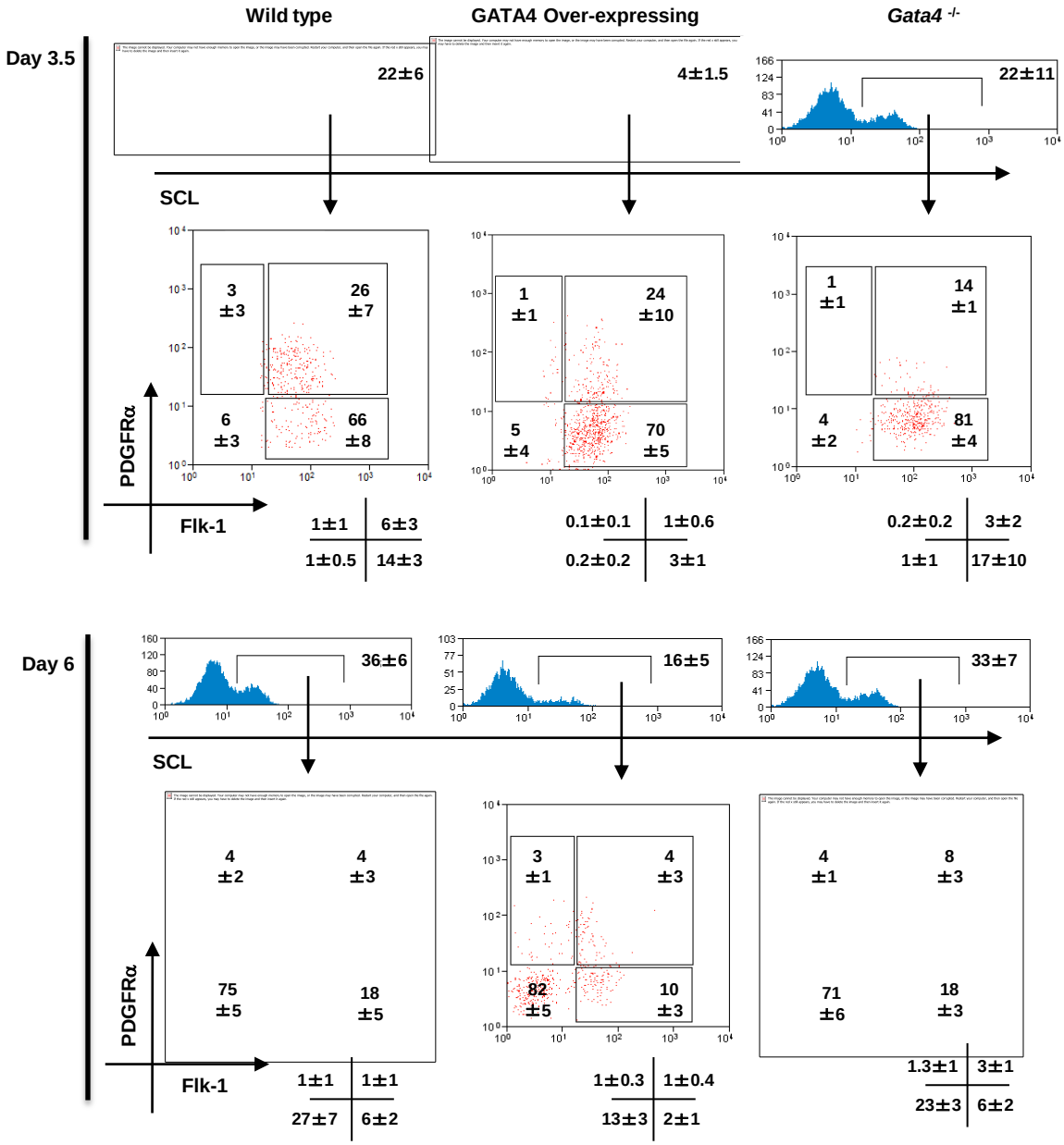


Figure 6.11 Expression of Flk-1, PDGFRα and SCL in GATA4 over-expressing and *Gata4* $^{-/-}$ EBs after 3.5 and 6 days of differentiation. Blue histograms show the percentage of EB cells that express SCL. Red dot plots below show co-expression of SCL^{+} cells with Flk-1 and PDGFRα. Values represent the mean percentage of EB cells that lie within the indicated gates, ± standard deviation, where n≥3. Percentage of total cells to co-express SCL with Flk-1 and/or PDGFRα is indicated in the quadrants below.

Figure 6.12
SCL does not directly bind the *Gata4* Proximal Promoter (-6Kb to +4Kb)

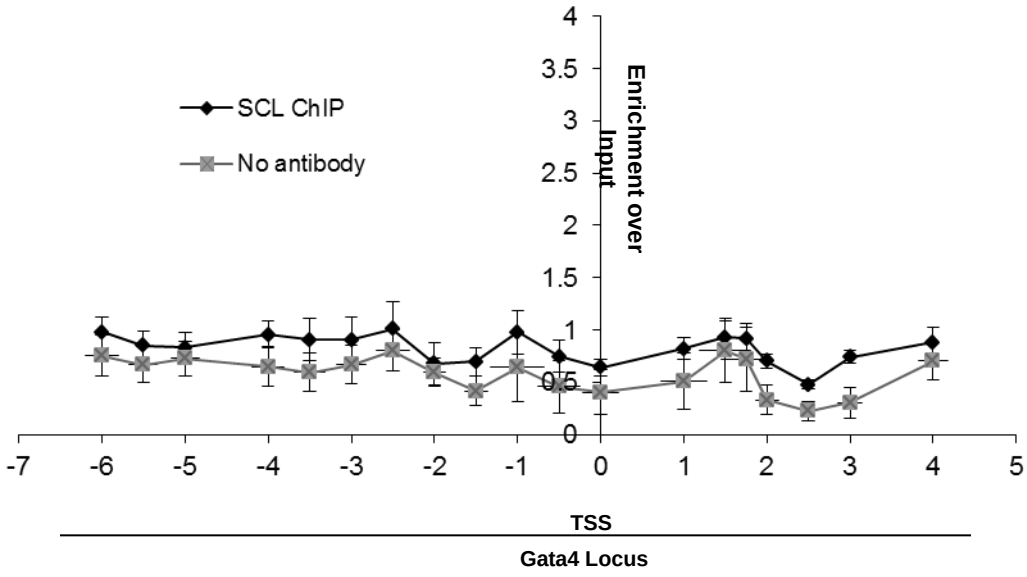


Figure 6.12 qPCR performed on ChIP material (from Flk-1$^+$ cells from day 3.5 EBs, precipitated with an anti-SCL antibody) using primers around the TSS (6kb upstream to 4 Kb downstream) of the *Gata4* locus. Results represents PCR results from two ChIP experiments, with PCR reactions performed in duplicate.

Figure 6.13a
Genome-wide Mapping of Regions Bound by SCL in Day 3.5 Flk-1^{+} EB Cells

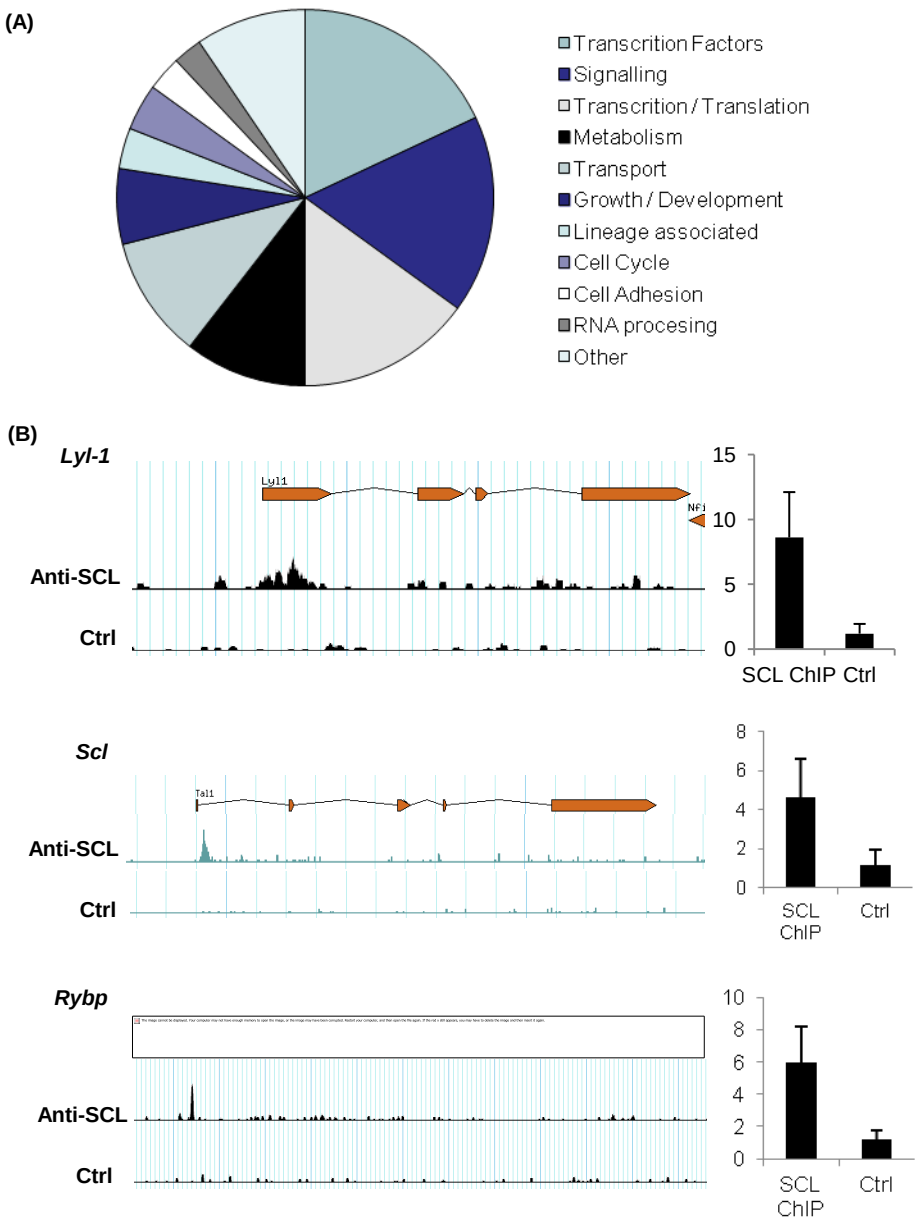
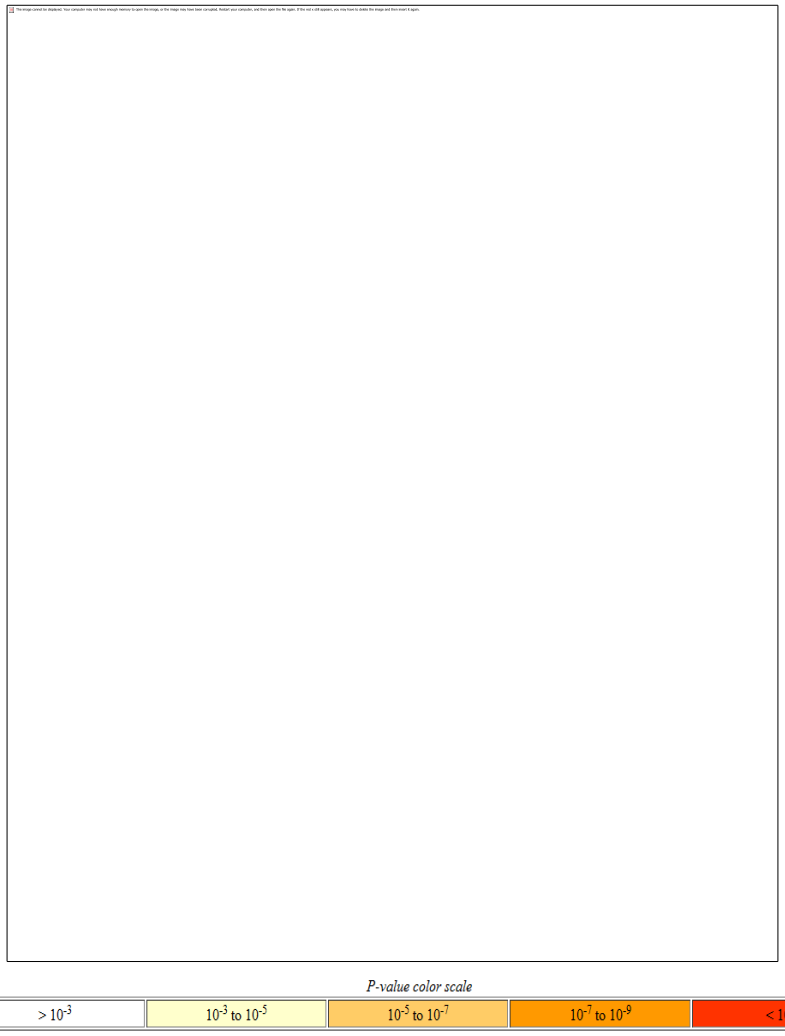


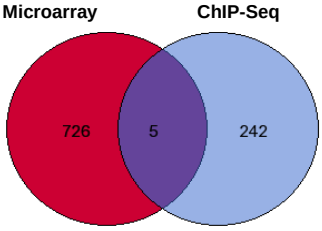
Figure 6.13a. Genome-wide ChIP-sequencing was performed on Flk-1+ cells derived from day 3.5 wild type EBs. Uniquely mapped reads were mapped onto the mouse genome, and significant peaks were defined as being >15 fold enriched over control samples. (A) Pie chart representing the biological processes/gene function of associated genes (based on the distance of a peak to Exon1 of a gene). (B) Validation of ChIP-sequencing results by real-time qPCR. Traces compare sequencing results for anti-SCL and no antibody ChIP at *Lyl-1*, *Scl* and *Rybp* loci. Graphs show enrichment of DNA sequencing in material from anti-SCL and no antibody ChIP experiments, relative to input material. Graphs represent results from three ChIP experiments, with each PCR reaction performed in duplicate.

Figure 6.13b
Gene Ontology of Regions Bound by SCL in Day 3.5 Flk-1^{+} EB Cells

(A)



(B)



5 Overlapping genes:
Axin2
CXCR4
Eomes
Tbx20

Figure 6.13b. (A) Flow diagram showing the functional ontology of genes associated with genomic regions bound by SCL in day 3.5 $^{-/-}$ Flk-1^{+} cells (Table 5), highlighting the over-representation genes (grouped by function) compared to their representation throughout the genome. Deeper colouring indicates a smaller P-value, and significant over-representation. Analysis was performed using the GOrilla. (B) Venn diagram indicating the overlap of genes identified by microarray and ChIP-Seq experiments. Comparison was performed using GeneVenn.

Figure 6.14
Optimisation of α-SCL ChIP on Day 3.5 Flk-1$^+$ EB Cells

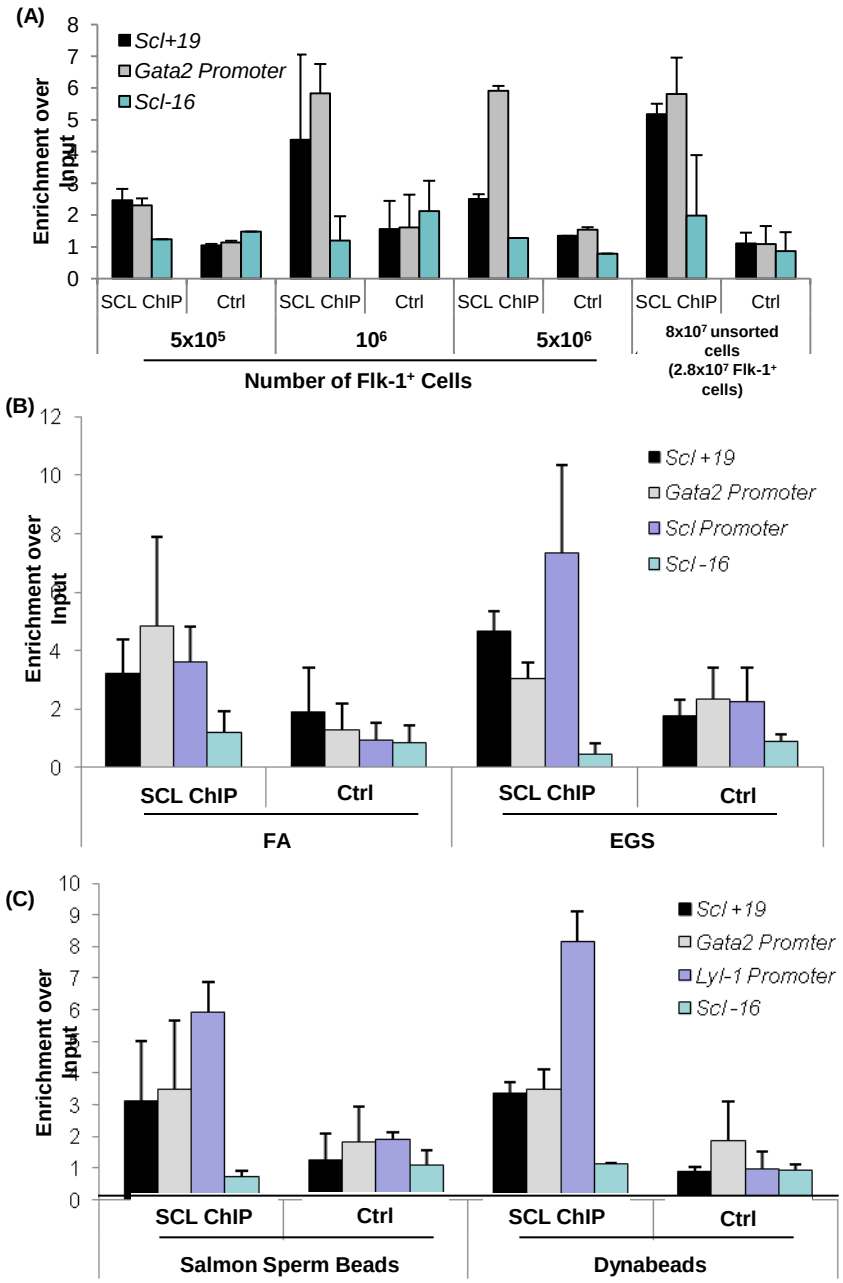


Figure 6.14 ChIP was performed on Flk-1$^+$ cells from day 3.5 wild type EBs, using an anti-SCL antibody. qPCR was used to calculate fold enrichment of specific DNA regions relative to input material. (A) ChIP using increasing amounts of starting material. (B) ChIP performed with different cross-linking reagents (FA and EGS). (C) ChIP performed using Salmon Sperm DNA-coated beads or Dynabeads to pull down SCL-DNA complexes. Results represent at least two ChIP experiments, with PCR reactions performed in duplicate. *Scl*-16 was used as a negative control point.

Figure 7.1
Model of Mesoderm Development during EB Differentiation

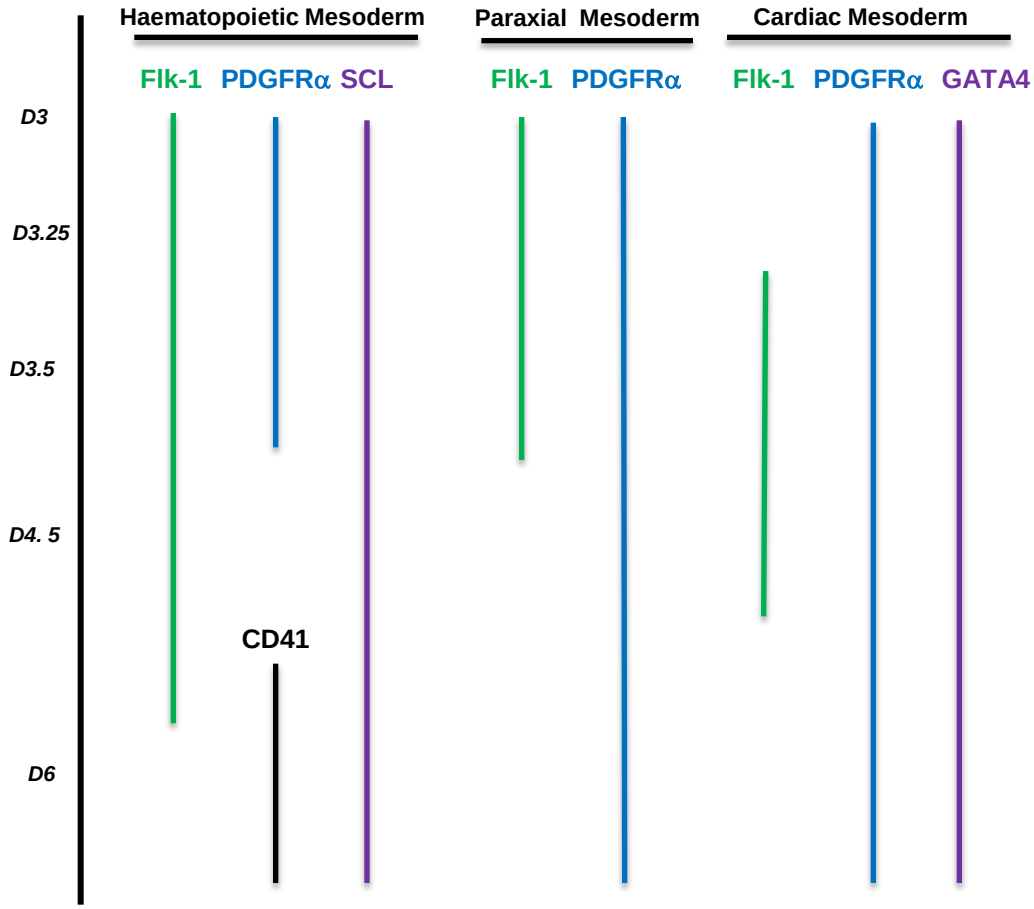


Figure 7.1 Early mesoderm populations can be fractionated according to haematopoietic, paraxial and cardiac lineage potential by the temporal expression of the cell surface proteins Flk-1, PDGFRα and CD41, and expression of the transcription factors SCL and GATA4.

Figure 7.2
Cross-Antagonism between Haematopoietic and Cardiac Lineages During Mouse Development

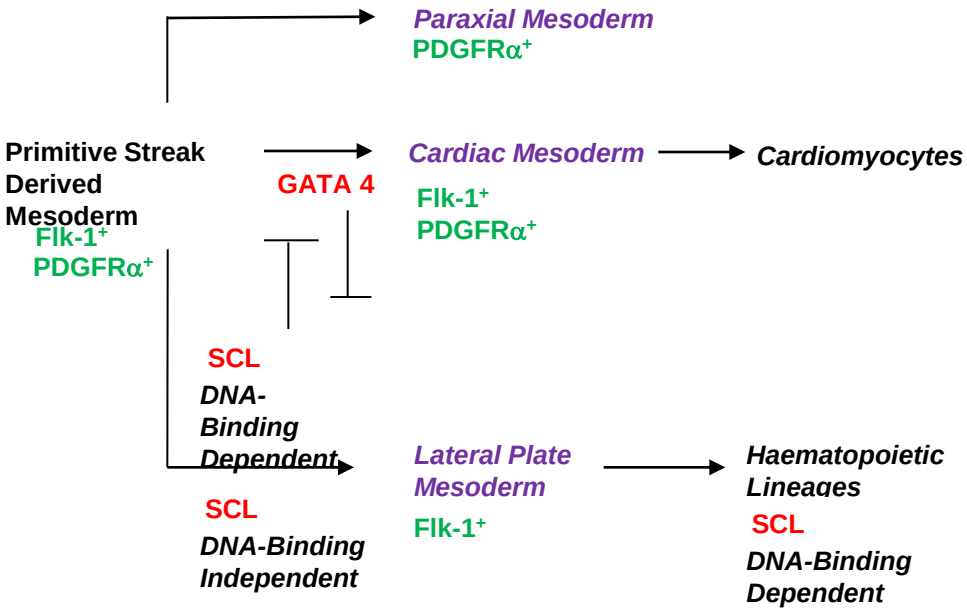


Figure 7.2 Model for reciprocal cross antagonism between blood and cardiac lineage development in the mouse. Cell surface markers to fractionate early mesoderm populations are indicated in green. Requirement for key transcription factors, SCL and GATA4, is shown in red.