LETTERS

Human cardiovascular progenitor cells develop from a KDR$^+$ embryonic-stem-cell-derived population

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The functional heart is comprised of distinct mesoderm-derived lineages including cardiomyocytes, endothelial cells and vascular smooth muscle cells. Studies in the mouse embryo and the mouse embryonic stem cell differentiation model have provided evidence indicating that these three lineages develop from a common Flk-1$^+$ (kinase insert domain protein receptor, also known as Kdr) cardiovascular progenitor that represents one of the earliest stages in mesoderm specification to the cardiovascular lineages[1]. To determine whether a comparable progenitor is present during human cardiogenesis, we analysed the development of the cardiovascular lineages in human embryonic stem cell differentiation cultures. Here we show that after induction with combinations of activin A, bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF, also known as FGF2), vascular endothelial growth factor (VEGF, also known as VEGFA) and dickkopf homolog 1 (DKK1) in serum-free media, human embryonic-stem-cell-derived embryoid bodies generate a KDR^{low}/C-KIT (CD117)^{neg} population that displays cardiac, endothelial and vascular smooth muscle potential *in vitro* and, after transplantation, *in vivo*. When plated in monolayer cultures, these KDR^{low}/C-KIT^{neg} cells differentiate to generate populations consisting of greater than 50% contracting cardiomyocytes. Populations derived from the KDR^{low}/C-KIT^{neg} fraction give rise to colonies that contain all three lineages when plated in methylcellulose cultures. Results from limiting dilution studies and cell-mixing experiments support the interpretation that these colonies are clones, indicating that they develop from a cardiovascular colony-forming cell. Together, these findings identify a human cardiovascular progenitor that defines one of the earliest stages of human cardiac development.

To direct the differentiation of human embryonic stem cells (ESCs) to the cardiac lineage, we designed a staged protocol that involved the formation of a primitive-streak-like population (stage 1, Fig. 1a), the induction and specification of cardiac mesoderm (stage 2) and the expansion of the cardiovascular lineages (stage 3) using combinations of factors known to have a role in these developmental steps in other systems[2–7]. Recent studies have shown that the combination of BMP4 and activin A will promote cardiac development in human ESC cultures[8]. However, the stage at which these pathways function in the establishment of this lineage was not defined. Using the protocol developed here, the combination of activin A and BMP4 at stage 1 induces a primitive-streak-like population and mesoderm, as demonstrated by the upregulation and transient expression of *T* (brachyury) and *WNT3A*—genes known to be expressed in these populations in the mouse[9,10] (Fig. 1f). At stage 2, the WNT inhibitor DKK1 is added to specify cardiac mesoderm and VEGF is included to promote the expansion and maturation of the KDR$^+$ population. bFGF is added again at day 8 of differentiation to support the continued expansion of the

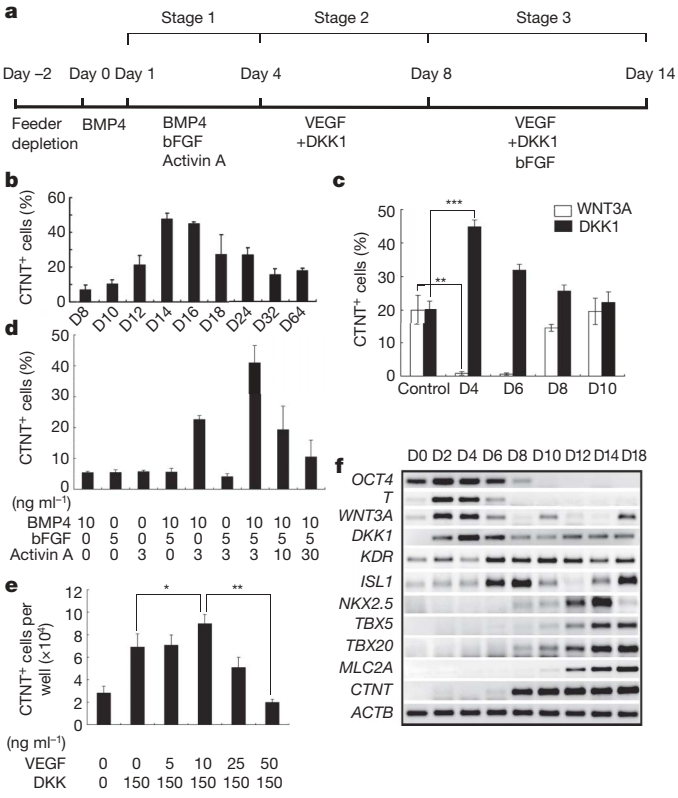

Figure 1 | Specification of the cardiac lineage from human ESCs. a, An outline of the protocol used for the differentiation of human ESCs to the cardiac lineage. **b**, Kinetics of CTNT$^+$ cell development in embryoid bodies induced with BMP4, bFGF and activin A. **c**, Frequency of CTNT$^+$ cells in day-14 embryoid bodies after manipulation of the WNT signalling pathway, as indicated. Control, cultures that did not receive WNT or DKK1. **d**, Frequency of CTNT$^+$ cells in day-14 embryoid bodies after induction with different combinations of BMP4, bFGF and activin A at stage 1. **e**, Effect of varying VEGF concentrations at stage 2 on the total number of CTNT$^+$ cells generated at day 14. **f**, Gene expression analysis of embryoid bodies at different stages of development. Where shown, bars represent standard error of the mean of three independent experiments; $*P = 0.07$, $**P < 0.01$, $***P < 0.001$. D0, D4, and so on, refer to days of culture .

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developing cardiovascular lineages[5]. This protocol supports cardiac development, as demonstrated by the emergence of contracting embryoid bodies (Supplementary Fig. 1c) and cells that express cardiac troponin T (CTNT, also known as TNNT2; Fig. 1b), α-actinin, α/β myosin heavy chain, ANP (atrial natriuretic peptide) and connexin 43 (Supplementary Movie 1 and Supplementary Fig. 1a–b, d). The highest frequency of $CTNT^+$ cells is routinely observed between days 14 and 16 of culture (Fig. 1b and Supplementary Fig. 1b).

Because studies in other systems have shown that stage-specific inhibition of canonical WNT signalling is required for cardiac development[4,6,11], we investigated the role of this pathway in the emergence of the cardiac lineage from the human ESCs, specifically focusing on stage 2. Addition of DKK1 at day 4 of differentiation led to a more than twofold increase in the frequency of $CTNT^+$ cells (up to 40%) at day 14 (Fig. 1c). The effect of DKK1 was less pronounced if added after day 4. WNT3A had the opposite effect and completely suppressed development of $CTNT^+$ cells if added at days 4 or 6. Taken together, these findings indicate that stage-specific inhibition of the canonical WNT pathway is necessary to promote cardiac specification of the BMP4/activin-A-induced primitive streak population. To evaluate the role of BMP4, activin A and bFGF, single factors as well as different combinations were tested during the induction stage (stage 1). BMP4, bFGF or activin A alone or in combinations (BMP4 and FGF or activin A and bFGF) were poor inducers of cardiac development, as demonstrated by the low frequency (Fig. 1d) and low total number of $CTNT^+$ cells generated (Supplementary Fig. 2a, b). Although BMP4 and activin A did induce significant numbers of $CTNT^+$ cells, the combination of the three factors was the most potent and generated the highest frequency (40–50% $CTNT^+$) and largest number of cardiac cells. Cardiac development was not dependent on exogenous VEGF. However, the addition of 10 ng ml^{-1} of this factor did increase the total number of $CTNT^+$ cells generated (Fig. 1e and Supplementary Fig. 2c, d).

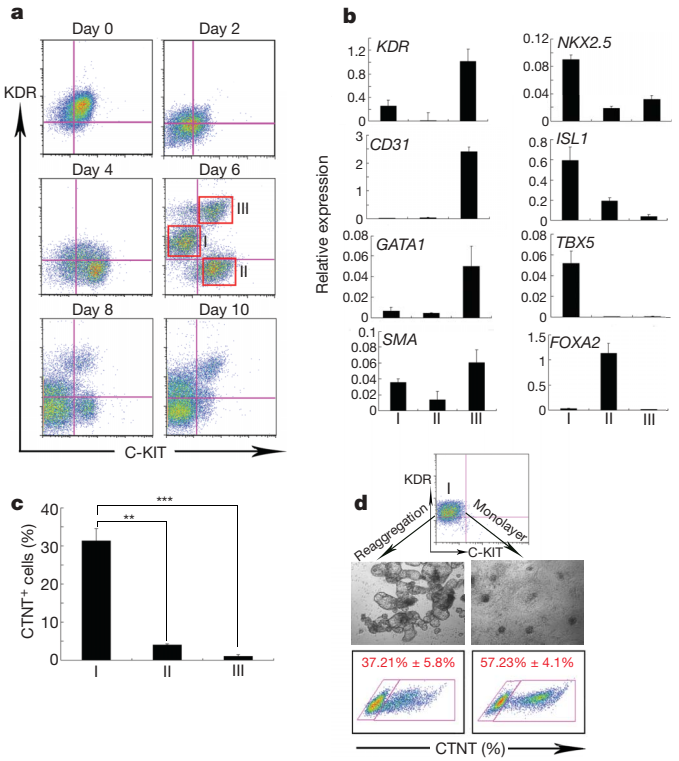

Figure 2 | Identification and characterization of the cardiovascular $KDR^{low}/C\text{-}KIT^{neg}$ embryoid body population. **a**, Flow cytometric analysis of different aged embryoid bodies, demonstrating the development of the three distinct populations ($KDR^{high}/C\text{-}KIT^+$ (III), $KDR^{low}/C\text{-}KIT^{neg}$ (I) and $KDR^{neg}/C\text{-}KIT^+$ (II)) defined by co-expression of KDR and C-KIT. **b**, Quantitative RT–PCR gene expression analysis of the $KDR^{high}/C\text{-}KIT^+$, $KDR^{low}/C\text{-}KIT^{neg}$ and $KDR^{neg}/C\text{-}KIT^+$ populations isolated from day-6 embryoid bodies. The average expression normalized to cyclophinin is shown. **c**, Frequency of $CTNT^+$ cells generated from the $KDR^{high}/C\text{-}KIT^+$, $KDR^{low}/C\text{-}KIT^{neg}$ and $KDR^{neg}/C\text{-}KIT^+$ populations cultured as monolayers in the presence of VEGF (10 ng ml^{-1}), DKK1 (150 ng ml^{-1}) and bFGF (10 ng ml^{-1}). Cells were assayed after 10 days of culture. **d**, Cardiac potential of $KDR^{low}/C\text{-}KIT^{neg}$ cells from day-6 embryoid bodies cultured as a monolayer or as aggregates in low-cluster wells with VEGF (10 ng ml^{-1}) and DKK1 (150 ng ml^{-1}). $CTNT^+$ cells were analysed after 7–10 days of culture. Where shown, bars represent standard error of the mean of three independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$.

Molecular analysis of the developing embryoid bodies revealed dynamic changes in expression patterns after the establishment of the primitive-streak-like population. Together with *T* and *WNT3A*, expression of *DKK1* was upregulated early and persisted throughout the time course (Fig. 1f). KDR was expressed in undifferentiated ESCs. The levels of expression increased between days 4 and 6 and then persisted for the following 12 days. *ISL1*, a gene that marks progenitors of the secondary heart field in the early embryo[12], was expressed between days 6 and 8, preceding the expression of the cardiac transcription factor *NKX2.5* (ref. 13), which was first detected at day 8 of differentiation. Expression of two TBX transcription factors required for cardiac development, *TBX5* (ref. 14) and *TBX20* (ref. 15), as well as the contractile proteins *MLC2A* (also known as *MYL7*) and *CTNT* was upregulated between days 8 and 10, reflecting the onset of cardiac development.

Recent studies with mouse ESC differentiation cultures identified a $Flk\text{-}1^+$ cardiovascular progenitor that develops from a Flk-1 population distinct from the hemangioblast[1]. To determine whether the human cardiac lineage also develops from a KDR ($Flk\text{-}1^+$) population, we analysed developing embryoid bodies for expression of KDR and C-KIT. C-KIT was used because its expression in mouse embryoid bodies identifies the earliest hemangioblast-derived haematopoietic and vascular progenitors as well as the anterior primitive streak and the developing endoderm[16]. As shown in Fig. 2a, three distinct populations, $KDR^{high}/C\text{-}KIT^+$ (III), $KDR^{low}/C\text{-}KIT^{neg}$ (I) and $KDR^{neg}/C\text{-}KIT^+$ (II), were detected at 6 days of differentiation. Development of the three populations was dependent on induction with both BMP4 and activin A (not shown). The $KDR^{high}/C\text{-}KIT^+$ population expressed *CD31* (also known as *PECAM1*), VE-cadherin (*CDH5*) and *SMA* (smooth muscle actin), genes associated with vascular development, and *GATA1*, a gene indicative of haematopoietic commitment (Fig. 2b and Supplementary Fig. 3). Genes involved in cardiac development, including *NKX2.5*, *ISL1* and *TBX5*, were expressed at highest levels in the $KDR^{low}/C\text{-}KIT^{neg}$ fraction. This fraction also expressed *SMA*, very low levels of *GATA1*, but no detectable *CDH5* or *CD31*. The $KDR^{neg}/C\text{-}KIT^+$ cells expressed the highest levels of *OCT4*, *T*, *FOXA2* and *SOX17*, indicating the presence of residual undifferentiated ESCs and primitive-streak-like cells undergoing commitment to the endoderm lineage (Supplementary Fig. 3). *SOX1* and *PAX6* were detected at very low levels, suggesting little differentiation to the neuroectoderm lineage (Supplementary Fig. 3). Taken together, these expression patterns suggest that the $KDR^{high}/C\text{-}KIT^+$ population contains haematopoietic and vascular progenitors, that the $KDR^{low}/C\text{-}KIT^{neg}$ population contains cardiac progenitors and that the $KDR^{neg}/C\text{-}KIT^+$ population consists of undifferentiated ESCs, primitive-streak-like cells and endodermal cells.

Consistent with the above expression profile, the $KDR^{low}/C\text{-}KIT^{neg}$ population displayed the greatest cardiomyocyte potential (Fig. 2c) and readily generated $CTNT^+$ cells and populations of contracting cells when cultured either as aggregates in suspension cultures or as adherent monolayers (Fig. 2d and Supplementary Movies 2 and 3). Approximately 40% of the aggregates and more than 50% of the monolayers were $CTNT^+$ after 7–10 days of culture, reflecting efficient differentiation to the cardiac lineage (Fig. 2d). The high frequency of cardiomyocytes in the monolayer cultures

routinely led to the development of sheets of cells contracting as a synchronous mass (Supplementary Movie 3). The isolated KDR^{low}/C-KIT^{neg} cells expanded approximately 1.5-fold as aggregates (data not shown) and 3-fold in the monolayer cultures (Supplementary Fig. 4b). With this induction protocol, we estimate an output of one cardiomyocyte per four input human ESCs. Kinetic analysis of embryoid bodies generated from another human ESC line (H1) demonstrated the development of the three KDR/C-KIT populations at day 5 rather than day 6 of differentiation. Analysis of the day-5 KDR^{low}/C-KIT^{neg} population indicated that it also displays cardiac potential (Supplementary Fig. 5)

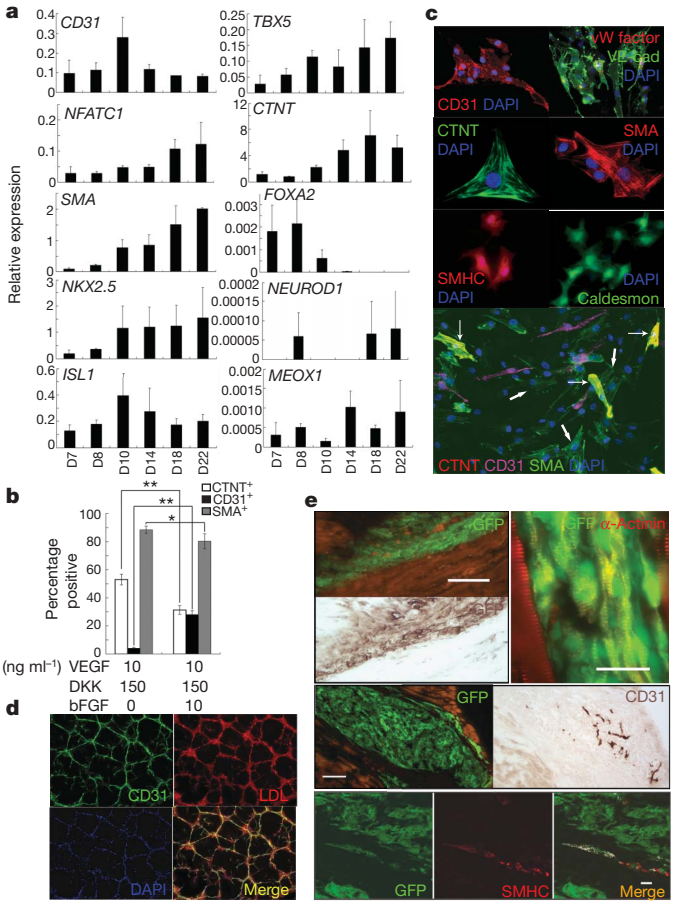

Figure 3 | Characterization of the KDR^{low}/C-KIT^{neg}-derived lineages. **a**, Quantitative RT–PCR analysis of adherent populations generated from the day-6 KDR^{low}/C-KIT^{neg} fraction cultured with VEGF (10 ng ml^{-1}), DKK1 (150 ng ml^{-1}) and bFGF (10 ng ml^{-1}). (D7 represents one day following plating.) The average expression normalized to cyclophinin is shown. **b**, Effect of bFGF on differentiation of day-6 KDR^{low}/C-KIT^{neg} cells. Cells were harvested and analysed by flow cytometry after 10–12 days of culture. **c**, Immunostaining analysis of the day-6 KDR^{low}/C-KIT^{neg}-derived population cultured in the presence of VEGF (10 ng ml^{-1}), DKK1 (150 ng ml^{-1}) and bFGF (10 ng ml^{-1}). Thin white arrows in the bottom panel indicate cardiac cells that express both CTNT and SMA; thick white arrows mark putative VSM cells that express SMA but not CTNT. Magnification; ×400 (CTNT, SMA), ×200 (all other panels). vW factor, von Willebrand factor. **d**, CD31 immunostaining and Dil-AC-LDL uptake of KDR^{low}/C-KIT^{neg}-derived cells cultured on Matrigel-coated glass coverslips. Magnification, ×50. **e**, Derivation of cardiac lineages from the KDR^{low}/C-KIT^{neg} population in the mouse heart. Top left, fluorescent micrograph of grafted heart viewed with a FITC/TRITC (fluorescein isothiocyanate/tetramethylrhodamine isothiocyanate) cube, demonstrating the presence of GFP-positive donor cells (green, upper panel). Below this is a light micrograph of immuno-histochemical staining of same section with anti-GFP antibody visualized with DAB (brown signal, lower panel). Scale bar, 100 μm. Top right, immunostaining of donor-derived GFP cells with anti-α-actinin antibody (yellow). Scale bars, 10 μm. Middle left, donor-derived GFP cells. Middle right, immunohistochemical staining of serial section with human-specific anti-pecam (CD31) antibody (brown signal). Scale bar, 100 μm. Bottom, confocal images of grafted heart stained with anti-smooth muscle MHC antibody (red). Green signal indicates GFP-expressing grafted cells in the same section. Colocalization is indicated with the white signal. Scale bar, 10 μm. Where shown, bars represent standard error of the mean of three independent experiments; $*P = 0.099$, $**P < 0.01$.

Expression analysis (quantitative PCR, qPCR) of the KDR^{low}/C-KIT^{neg}-derived adherent populations at different days after plating demonstrated the upregulation of genes associated with endothelial (*CD31*, *CDH5*), VSM (vascular smooth muscle) (calponin, *SMA*), cardiac development (*NKX2.5*, *ISL1*, *TBX5*, *TBX20*) and cardiac maturation (*CTNT*, *MLC2A*) (Fig. 3a and Supplementary Fig. 4a). Expression of *NFATC1* and neuregulin 1 (*NRG1*) suggests the presence of endocardium in the cultures[17,18]. The low levels of *NEUROD1*, *PAX6*, *SOX1*, *FOXA2*, *FOXA3*, *SOX17* and *MEOX1* expression indicate little, if any, contamination of these cultures with neuroectoderm, endoderm or somitic mesoderm (Fig. 3a and Supplementary Fig. 4a). Flow cytometric analysis of the KDR^{low}/C-KIT^{neg}-derived adherent population cultured for 10–12 days in VEGF and DKK1 revealed that almost 90% expressed SMA, 50% expressed CTNT and 4% expressed CD31 (Fig. 3b). Addition of bFGF to the cultures reduced the proportion of CTNT^{+} and SMA^{+} cells to 30% and 80%, respectively, and increased the CD31^{+} subpopulation to 30%. The addition of bFGF did not significantly influence cell numbers in the monolayer cultures (Supplementary Fig. 4b). These findings indicate that the majority of cells within the KDR^{low}/C-KIT^{neg}-derived population are of the cardiovascular lineages and that bFGF can influence the proportion of cardiomyocytes and endothelial cells that develop in this population.

Immunostaining analysis of the KDR^{low}/C-KIT^{neg}-derived population demonstrated the presence of CD31^{+}, VE-cadherin^{+} and von Willebrand factor^{+} endothelial cells, of CTNT^{+} cardiomyocytes, and of SMA^{+}, SMHC^{+} (also known as MYH11^{+}) and caldesmon^{+} cells, indicative of VSM development (Fig. 3c). The immature cardiomyocytes within the population expressed both CTNT and SMA (lower panel of Fig. 3c, thin arrows), whereas the VSM cells expressed only SMA (thick arrows). KDR^{low}/C-KIT^{neg}-derived cells that were expanded in the presence of VEGF and bFGF formed a lattice, indicative of the formation of tube-like structures when cultured on Matrigel-coated coverslips. The cells within these structures expressed CD31 and displayed the capacity to take up Dil-AC-LDL (1,1′-dioctadecyl-1,3,3,3′,3′-tetramethylindocarbocyanine perchlorate acetylated low-density lipoprotein), confirming their endothelial phenotype (Fig. 3d). The findings from the immunostaining analysis are consistent with those from the flow cytometric studies, and demonstrate that the KDR^{low}/C-KIT^{neg}-derived population consists of cells of the cardiac, endothelial and vascular smooth muscle lineages.

KDR^{low}/C-KIT^{neg}-derived cells generated from a green fluorescent protein (GFP)-expressing hES2 cell line (GFP–hES2) were transplanted into the hearts of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice to document their developmental potential *in vivo*. Histological analyses revealed the presence of GFP^{+} donor cells detected by epifluorescence and by staining with an anti-GFP antibody (Fig. 3e, top left panel). GFP^{+} populations co-expressing α-actinin (Fig. 3e, top right panel), CD31 (Fig. 3e, middle panel) or SMHC (Fig. 3e, lower panel) were detected in the grafts, indicating differentiation to the cardiac, endothelial and vascular smooth muscle lineages *in vivo*, respectively. Teratomas were not observed in any of the transplanted animals. KDR^{low}/C-KIT^{neg}-derived cells were also transplanted directly into infarcted hearts of SCID beige mice. When analysed two weeks later, animals transplanted with the KDR^{low}/C-KIT^{neg}-derived cardiovascular population had a 31% higher ejection fraction than those injected with media alone (56% ± 3.6% versus 39% ± 4.8% (mean ± s.e.m, $P = 0.008$)). These findings are consistent with previous reports[8,19,20] in demonstrating that transplantation of human ESC-derived

cardiomyocytes leads to improvement in cardiac function in rodent models of myocardial infarction. Although such cell transplantation does improve function, it is important to stress that the mechanisms mediating this effect are currently not known[21].

To establish the lineage relationship between these three cell types, we adapted the methylcellulose colony assay used to identify the cardiovascular progenitor in mouse ESCs cultures[1]. When plated in methylcellulose, KDR^{low}/C-KIT^{neg}-derived cells generated small compact colonies within 4 days of culture (Fig. 4a, light). PCR analysis of individual 4-day-old colonies demonstrated co-expression of

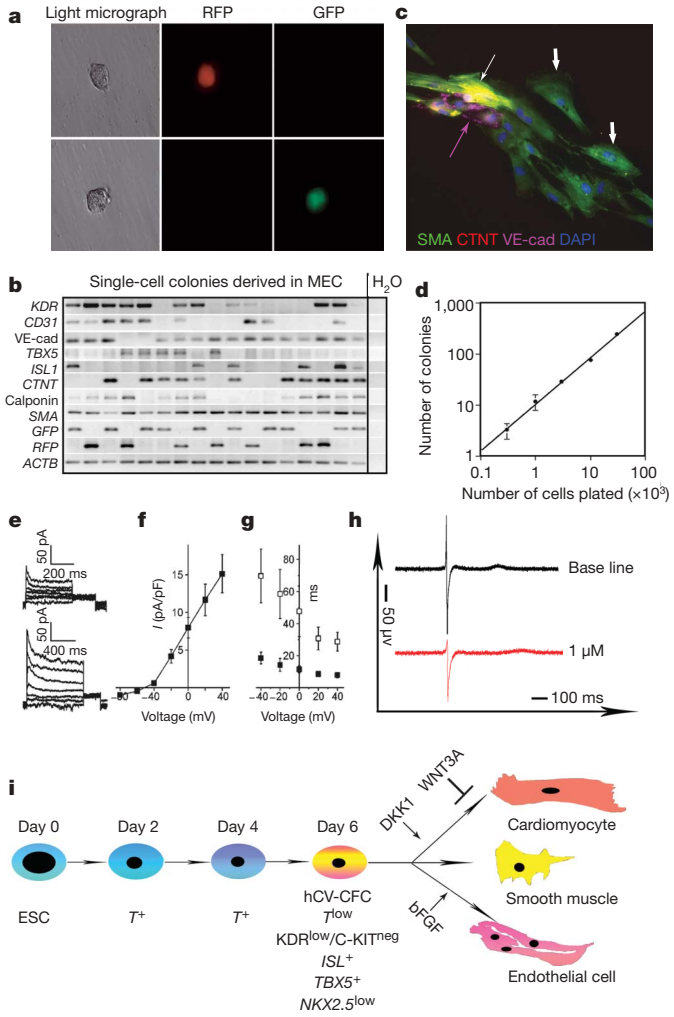

Figure 4 | Identification and characterization of human cardiovascular progenitors. **a**, Four-day-old cardiovascular colonies from mixed cultures showing of either RFP or GFP expression. **b**, Expression analysis of 4-day-old cardiovascular colonies isolated from the mixed RFP/GFP cultures. **c**, Immunostaining of cells grown from a single colony. The thin white arrow indicates cardiac cells that express both CTNT and SMA; the thick white arrows mark putative VSM cells that express SMA but no CTNT. The thin purple arrow identifies VE-cadherin$^+$ endothelial cells. Magnification, ×200. **d**, Cell-dose response showing the relationship between the number of KDR^{low}/C-KIT^{neg}-derived cells plated and the number of cardiovascular colonies that develop. Error bars, s.e.m. **e**, Exemplar traces showing whole-cell voltage-clamp recordings of transient outward K^+ current (I_{to}) natively expressed in KDR^{low}/C-KIT^{neg}-dervied cardiomyocytes. **f**, Mean current density–voltage relationship for cells as in **e**. From a batch of ten cells, eight showed the I_{to} current; the mean ± s.e.m. current densities were plotted using traces from these eight cells. **g**, Mean time-to-peak current (solid squares) and inactivation τ (open squares) for cells as in **f** ($n = 8$). **h**, Extracellular electrical activity recorded without (base line) or with 1 μM quinidine (lower red line) from cells cultured in a MEA (multi-channel system) chamber. **i**, Model depicting development of cardiovascular progenitors (hCV-CFC) in human ESC cultures.

markers indicative of cardiac (*CTNT*), endothelial (*CD31* and/or *CDH5*) and VSM (*SMA* and/or calponin) development (Fig. 4b and Supplementary Fig. 4c). When maintained in culture for a further 6 days, a portion of these colonies generated contracting cardiomyocytes (Supplementary Movie 4). *ISL1* and *TBX5* were typically not expressed in the same colonies, suggesting that their expression may define colonies that contain distinct cardiac subpopulations from different heart fields. Immunostaining of adherent populations from individual colonies confirmed the presence of the cardiac, endothelial and VSM lineages (Fig. 4c).

Two different approaches were used to determine if the cardiovascular colonies are clonal. First, KDR^{low}/C-KIT^{neg}-derived cells from the GFP-hES2 cell line and from a human ESC line expressing red fluorescent protein (RFP) (HES2.R26)[22] were mixed in the methylcellulose assay. Colonies that developed expressed either GFP or RFP, but not both (Fig. 4a, b), consistent with the interpretation that they arise from a single cell and not from cell aggregation. In the second approach, we carried out a cell-dose response experiment. The relationship between the number of colonies that developed and the number of cells plated was linear, with a slope approaching one, further supporting the notion that the colonies are derived from a single cell (Fig. 4d). Taken together, these findings strongly suggest that these colonies represent clones of cardiovascular cells derived from a cardiovascular colony-forming cell (hCV-CFC).

The functional potential of KDR^{low}/C-KIT^{neg}-derived cardiomyocytes was evaluated with whole-cell current and field potential measurements. In whole-cell voltage-clamp analysis, 80% of cells studied expressed a predominant voltage-gated, transient outward potassium current (Fig. 4e, f). The voltage dependence, density and gating kinetics of this current (Fig. 4f, g) resembled those of the I_{to} (transient outward K^+ current) potassium current found in human atrial and ventricular myocytes[23,24]. Field potential recordings using microelectrodes revealed that the KDR^{low}/C-KIT^{neg}-derived cardiac cells were electrically coupled to one another. In addition, as expected, the Vaughan Williams class-Ia-agent quinidine decreased the measured T-wave amplitude and increased the QT interval[25] (Fig. 4h).

Recent studies in the mouse have provided evidence that the main lineages of the heart develop from a common cardiovascular progenitor[1,26,27]. The identification of a hCV-CFC in this study suggests that cardiovascular development in humans is similar to this and that the cardiac, endothelial and vascular smooth muscle lineages are derived from a common progenitor (model, Fig. 4i). A recent study has reported the identification of a C-KIT$^+$KDR$^-$ cardiac stem cell in the adult human heart that also displays the capacity to generate myocytes, smooth muscle cells and endothelial cells[28]. The differences in surface markers between the two populations may reflect the fact that one represents an early embryonic stage of development whereas the other is derived from the adult heart.

In mouse ESC cultures, the cardiovascular progenitor develops from a Flk-1 population that is distinct from the Flk-1 population containing the hemangioblast. Although we have not measured the temporal development of the two KDR populations in this study, we have previously demonstrated that the first haematopoietic progenitors to develop in human embryoid bodies are present in a KDR$^+$/C-KIT$^+$ fraction, comparable to the KDR^{high}/C-KIT$^+$ fraction in this study[29]. The finding that the cardiovascular progenitors are detected in a distinct KDR^{low}/C-KIT^{neg} fraction demonstrates a similar segregation of early haematopoietic and cardiac potential in the human system. The identification of the KDR^{low}/C-KIT^{neg} population that contains cardiovascular progenitors provides a unique opportunity to investigate the mechanisms that regulate the onset of human cardiac development as well as those that control their specification to the cardiac and vascular lineages. Access to this population also provides an enriched source of progenitors for engineering cardiovascular tissue *in vitro* and for transplantation

to large animal models that may accurately reflect human cardiac function.

METHODS SUMMARY

Maintenance and differentiation of human ESCs. The different human ESC lines were maintained as described[29]. For differentiation to the cardiac lineage, the following cytokines were used: days 0–1, BMP4 (0.5 ng ml^{-1}); days 1–4, BMP4 (10 ng ml^{-1}), bFGF (5 ng ml^{-1}) and activin A (3 ng ml^{-1}); days 4–8, DKK1 (150 ng ml^{-1}) and VEGF (10 ng ml^{-1}); after day 8, VEGF (10 ng ml^{-1}), DKK1 (150 ng ml^{-1}) and bFGF (5 ng ml^{-1}). Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for the first 10–12 days and were then transferred into a 5% CO_2/air environment.

***In vitro* analysis of the KDR^{low}/C-KIT^{neg} population.** Isolated KDR^{low}/C-KIT^{neg} cells were cultured as a monolayer on a gelatin-coated surface or as aggregates in low-cluster wells in Stempro34 medium supplemented with VEGF (10 ng ml^{-1}) and DKK1 (150 ng ml^{-1}). Where indicated, bFGF (10 ng ml^{-1}) was added to the cultures to promote endothelial development. For analysis of the endothelial lineage, KDR^{low}/C-KIT^{neg} cells were cultured in Stempro34 medium supplemented with VEGF (25 ng ml^{-1}) and bFGF (25 ng ml^{-1}) for 5–7 days and then seeded onto Matrigel-coated glass coverslips for an additional 24 h.

***In vivo* analyses of KDR^{low}/C-KIT^{neg}-derived populations.** KDR^{low}/C-KIT^{neg} cells (100,000) derived from GFP–human ESCs were injected into the left ventricular wall of NOD/SCID-γ mice. Hearts were harvested 2–11 weeks post surgery. Immunohistochemistry was carried out with anti-GFP antibody (Chemicon; Vector ABC and DAB kits), α-actinin antibody (Sigma), CD31 antibody (Dako) and SM-MHC antibody (Biomedical Technologies). Confocal images were analysed for colocalization using ImageJ and Pierre Bourdoncle's plugin with default settings. Myocardial infarction was induced in SCID beige mice. After 10–20 min, the mice were injected with 500,000 KDR^{low}/C-KIT^{neg}-derived cells ($n = 9$) or an equivalent volume of basic media ($n = 12$). Two weeks later, assessment of ventricular function was performed using 9.4 tesla magnetic resonance imaging.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Maintenance of human ESCs. H1 ESCs (National Institutes of Health, NIH code WA01) from the WiCell Research Institute and hES2 ESCs (NIH code ES02) from ES Cell International (ESI) were maintained as described[29]. The AAVS1 (adeno-associated virus integration site 1)-targeted hES2 cell line was generated by co-infection of parental hES2 cells with 10^6 viral particles of both AAV2-TRUF11 (CAG-GFP-TK-neo) and wild-type AAV2. After G418 selection to deplete the cells not infected by AAV2-TRUF11, GFP-positive cells were sorted and subclones were isolated. Targeted integration of the transgenes was confirmed by ligation-mediated PCR. Wild-type AAV sequences were not detected in GFP-positive clones. Generation of the RFP-expressing hES2 cell line was previously described[22].

Differentiation of human ESCs. Embryoid bodies for differentiation were generated as described previously[29]. In brief, embryoid bodies were formed by plating small aggregates of human ESCs in 2 ml basic media (StemPro34, Invitrogen, containing 2 mM glutamine, 4×10^{-4} M monothioglycerol (MTG), 50 μg ml^{-1} ascorbic acid, Sigma, and 0.5 ng ml^{-1} BMP4). The following concentrations of factors were used for primitive-streak formation and for mesoderm induction and cardiac specification: BMP4, 10 ng ml^{-1}; human bFGF, 5 ng ml^{-1}; activin A, 3 ng ml^{-1}; human DKK1, 150 ng ml^{-1}; and human VEGF, 10 ng ml. The factors were added with the following sequence: days 1–4, BMP4, bFGF and activin A; days 4–8, VEGF and DKK1; after day 8, VEGF, DKK1 and bFGF. All factors were purchased from R&D Systems. Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for the first 10–12 days and then transferred to a 5% CO_2/air environment.

Flow cytometry. Embryoid bodies were harvested and dissociated to single cells with trypsin (0.25% trypsin-EDTA). For intracellular FACS, cells were fixed and stained with primary and secondary antibodies in PBS plus 0.5% saponin (Sigma). Analyses were carried out using a Facscalibur flow cytometer (Becton Dickinson). Cells were sorted from day-6 embryoid bodies using a MoFlo (Dako Cytomation) cell sorter. Data were analysed using the FlowJo (Treestar) software. Anti-KDR-PE and anti-C-KIT-APC (allophycocyanin) were purchased from R&D Systems.

***In vitro* analysis of the KDR^{low}/C-KIT^{neg} population.** For cardiac differentiation, isolated KDR^{low}/C-KIT^{neg} cells were cultured as a monolayer on a gelatin-coated surface or as aggregates in low-cluster wells in Stempro34 medium supplemented with VEGF (10 ng ml^{-1}) and DKK1 (150 ng ml^{-1}). Cells were seeded at a concentration of 40,000–50,000 per well in a 96-well plate. To promote endothelial development, bFGF (10 ng ml^{-1}) was added. For analysis of the endothelial lineage, KDR^{low}/C-KIT^{neg} cells were cultured in Stempro34 medium with VEGF (25 ng ml^{-1}) and bFGF (25 ng ml^{-1}) for 5–7 days. To induce the formation of tube-like structures, the cells were transferred and cultured on Matrigel-coated glass coverslips for 24 h.

Immunofluorescence. Dissociated cells were cultured on glass cover slips for 2 days, fixed with 4% PFA for 20 min, and then stained. Cells were incubated with the primary antibody for 2 h at 37 °C, washed three times and then incubated with a secondary antibody for an additional 1 h. After staining, the cells were washed three times, and fluorescence was visualized with a Leica DMRA2 fluorescence microscope (Leica) and recorded using a digital Hamamatsu CCD camera. The following antibodies were used for staining: anti-human CD31 and anti-human VE-cadherin from R&D Systems, anti-mouse troponin T and anti-human smooth muscle actin from Lab Vision, anti-human ANP, anti-connexin 43 and anti-human α/β MHC from Chemicon, anti-human α-actinin from Sigma, and anti-human smooth muscle myosin heavy chain (SMHC), anti-human caldesmon and anti-von Willebrand factor from DakoCytomation. The Cy2-, Cy3- and Cy5-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

Colony assays. KDR^{low}/C-KIT^{neg} cells isolated from day-6 embryoid bodies were aggregated in the presence of VEGF (25 ng ml^{-1}), bFGF (10 ng ml^{-1}) and DKK1 (150 ng ml^{-1}) for 2–3 days. The aggregates were dissociated with trypsin and the cells were cultured in methylcellulose containing VEGF (25 ng ml^{-1}), bFGF (25 ng ml^{-1}) and DKK1 (150 ng ml^{-1}) in a 5% CO_2/5% O_2/90% N_2 environment. Colonies were scored after 4–6 days of culture.

RT–PCR. For expression studies, individual colonies were isolated from the methylcellulose cultures and analysed using a modified version of the protocol described[30]. The amplified complementary DNA was then subjected to normal PCR. Real-time quantitative PCR was performed on a MasterCycler EP RealPlex (Eppendorf). Experiments were done in triplicate using Platinum SYBR GreenER qPCR SuperMix (Invitrogen). All primers are described in Supplementary Table 1. All annealing reactions were carried out at 60 °C.

***In vivo* analyses of KDR^{low}/C-KIT^{neg}-derived populations.** Day-6 GFP$^+$ KDR^{low}/C-KIT^{neg} cells cultured in the presence of VEGF (10 ng ml^{-1}), bFGF (10 ng ml^{-1}) and DKK1 (150 ng ml^{-1}) for 5–10 days were injected (100,000 per recipient) directly into the left ventricular wall of NOD/SCID-gamma mice in an open-chest procedure. Hearts were harvested 2–11 weeks post surgery, fixed in 1% PFA in cacodylate buffer and sectioned at 10 μm ($n = 15$). Immuno-histochemistry was done with GFP (Chemicon, AB3080; Vector ABC and DAB kits), α-actinin (Sigma, A781), CD31 (Dako, M0823), and smooth muscle MHC antibodies (Biomedical Technologies, BT-562). Confocal images were analysed for colocalization using ImageJ and Pierre Bourdoncle's plugin with default settings. For evaluation in the infarct model, myocardial infarction was induced in SCID beige mice by means of direct coronary ligation. 1-20 min later the mice were injected with 500,000 KDR^{low}/C-KIT^{neg}-derived cells ($n = 9$) or an equivalent volume of media ($n = 12$). Two weeks later, assessment of ventricular function was performed using 9.4 Tesla magnetic resonance imaging.

Patch clamp. Whole-cell patch-clamp recordings were performed using an IX50 inverted microscope (Olympus), a Multiclamp 700A amplifier, a Digidata 1300 analogue/digital converter and a PC with pClamp9.1 software (Axon Instruments). The bath solution contained (in mM): NaCl 136, KCl 4, $CaCl_2$ 1, $MgCl_2$ 2, $CoCl_2$ 5, HEPES 10, glucose 10 and tetrodotoxin (TTX) 0.02 (pH 7.4). Pipettes were of 3–5 MΩ resistance when filled with intracellular solution containing (in mM): KCl 135, EGTA 10, HEPES 10 and glucose 5 (pH 7.2). Cells were stepped from a holding potential of −80 mV to test potentials from −80 mV to +40 mV in 20 mV increments, before a −30 mV tail pulse (durations as in Fig. 4f). Data were analysed using pClamp9.1 software (Axon Instruments). Current amplitudes were normalized to cell size (whole-cell membrane capacitance). Inactivation τ values were calculated using a single exponential fit of current decay.

Field potential recording. KDR^{low}/C-KIT^{neg} cells were cultured in a MEA (Multi Channel Systems) chamber in StemPro34 with 10 ng ml^{-1} VEGF and 150 ng ml^{-1} DKK1 for 2–4 weeks. Two days before measuring recordings, the media was changed to DMEM (Mediatech) with 15% FBS. Extracellular electrical activity was simultaneously recorded from 60 channels and analysed with the software MC Rack (Multi Channel Systems).

Data analysis. Data are shown as mean ± standard error of three independent experiments. Statistic analysis was performed with the Student's *t* test.

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