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c-Kit+ progenitors generate vascular cells for tissue-engineered grafts through modulation of the Wnt/Klf4 pathway

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A B S T R A C T
The development of decellularised scaffolds for small diameter vascular grafts is hampered by their limited patency, due to the lack of luminal cell coverage by endothelial cells (EC) and to the low tone of the vessel due to absence of a contractile smooth muscle cells (SMC). In this study, we identify a population of vascular progenitor c-Kit+/Sca-1- cells available in large numbers and derived from immuno-privileged embryonic stem cells (ESCs). We also define an efficient and controlled differentiation protocol yielding fully to differentiated ECs and SMCs in sufficient numbers to allow the repopulation of a tissue engineered vascular graft. When seeded ex vivo on a decellularised vessel, c-Kit+/Sca-1-derived cells recapitulated the native vessel structure and upon in vivo implantation in the mouse, markedly reduced neo-intima formation and mortality, restoring functional vascularisation. We showed that Krüppel-like transcription factor 4 (Klf4) regulates the choice of differentiation pathway of these cells through β-catenin activation and was itself regulated by the canonical Wnt pathway activator lithium chloride. Our data show that ESC-derived c-Kit+/Sca-1-cells can be differentiated through a Klf4/β-catenin dependent pathway and are a suitable source of vascular progenitors for the creation of superior tissue-engineered vessels from decellularised scaffolds.

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1. Introduction
Therapies for coronary and peripheral vascular diseases often involve vascular grafts to restore blood perfusion. In clinical practice, the transplantation of an autologous vessel is the primary choice; however, almost 40% of patients lack autologous vessels of an adequate quality. To overcome this limitation, artificial alternatives have been devised, including synthetic expanded polytetrafluoroethylene (ePTFE) and Dacron grafts and decellularisation of natural vessels [1]. In particular, the development of decellularised vessel scaffolds allows for the creation of an ‘off-the-shelf’ material combining the characteristics of the natural vessels and as a substrate for cell adhesion and growth with low immunogenicity. Despite their promise, the application of decellularised vessel scaffolds in the clinic has been hampered by inadequate long-term patency due to thrombosis and neointimal hyperplasia [2]. We previously showed in an animal model that the patency of decellularised vessels is limited due to the lack of adequate endothelialisation [2]. The outcome of the graft was greatly improved by the local application of vascular endothelial growth factor (VEGF), which induced the local recruitment of endothelial progenitor cells to the scaffold [2]. For the same reason, the seeding of the luminal side of the scaffold with endothelial cells has been demonstrated to improve graft success rate by ensuring a non-thrombogenic surface of contact with the blood. Similarly, the seeding of smooth muscle cells (SMCs) in the media of the decellularised vessel was shown to improve the mechanical stability of the graft and its compliance [3,4]. However, the use of mature endothelial cells (ECs) and SMCs is hampered by their limited availability from an autologous source and low proliferation rate. For this reason, the exploitation of the immuno-privileged and plastic embryonic stem cells (ESCs) is
highly advantageous. Stem cells are highly proliferative and can give rise to a high number of differentiated cells including ECs and SMCs, provided the mechanisms regulating the differentiation process are well understood and controlled.

In this study, we focused on the isolation of an abundant and clinically relevant c-Kit positive/Sca-1-negative (c-Kit+/Sca-1-) vascular progenitor population from ESCs and their differentiation to both ECs and SMCs. We then applied these cells to repopulate the decellularised scaffold for vascular graft. The c-Kit antigen has previously been used to define progenitor cells in the human and mouse heart where they participate to repair after myocardial infarction [5] and form both ECs and SMCs [6,7]. Regulating the choice of differentiation pathway in progenitor cells is fundamental: (a) to obtain a highly differentiated and pure population for tissue engineering; (b) for the maintenance of the physiological state once the vessel is grafted and (c) to manipulate the progression of the in vivo re-endothelialization of the vascular graft. For vascular progenitor cells, the mechanisms have been only partially elucidated. In this paper, we focused on Krüppel-like factor 4 (Klf4), which is known to play an atheroprotective function in the vessel [8–12] and participate to anti-inflammatory [13–16] and shear stress response [17,18]. We also investigated the interaction of Klf4 with Wnt/β-catenin signalling, which plays an important role in vasculature development and endothelial function/remodeling [19–21] through the shuffling of β-catenin and activation of T cell factor (TCF) target genes [13,14,22]. Understanding the differentiation mechanisms underpinning c-Kit+/Sca-1- cell fate is crucial for their use as a cell source for vascular grafts.

2. Material and methods

For expanded Materials and methods, refer to the Supplemental Methods provided.

2.1. Cell culture, isolation and differentiation

Mouse ESCs (ES-D3 cell line, American Type Culture Collection [ATCC]) and isolated c-Kit+/Sca1-cells were cultured as previously reported [23]. Differentiation was induced by plating c-Kit+/Sca1-cells on collagen IV-coated flasks in presence of differentiation medium (DM; α-MEM containing 10% FBS, 0.2 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin; Invitrogen) containing either VEGF (50 ng/ml, Peprotech) or platelet-derived growth factor (PDGF, 20 ng/ml, Sigma) for up to 21 days. The endothelial differentiation protocol was improved with the application of shear stress between Day 3 and 5 using an orbital shaker.

2.2. Decellularised vessel preparation and seeding

The preparation of the decellularised vessels was performed as previously described [2] treating the descending aorta with 0.075% SDS for 2 h. c-Kit+ ECs were seeded using a bioreactor where the grafts were fixed between two needles and the bioreactor was set up in a standard incubator at 37°C. Scaffolds were preconditioned for 2 h with the indicated coating. 2 × 107 c-Kit+ EC were then injected and allowed to seed for 12 h at a continuous rotational movement before initial flow was set up. For double seeded scaffolds, a second seeding step was performed with 1 × 107 c-Kit+ derived SMCs. After seeding, medium flow rate was adjusted stepwise to reach a shear stress of 30 dyn/cm². Vessels were harvested on Day 5 and then used for further analysis ex vivo or immediately grafted to animals. Decellularised vessels were used as a control.

Picrosirius collagen and Miller’s elastin staining was performed to assess the collagen content in the tissue-engineered vessels.

2.3. Decellularised and tissue-engineered vessel grafting to the right carotid artery

Mice were anesthetised and the middle portion of the carotid artery was ligated and dissected between the two ties. The graft was interposed as previously described using a two-cuffs based system [2]. A total of 56, 28 and 13 mice were transplanted respectively with non-seeded, c-Kit+ EC seeded, and c-Kit+ ECs+ SMCs seeded grafts, respectively. Survival was monitored for up to 56 days and tissues for patency monitoring were collected at 2, 4 and 8 weeks.

Mice underwent magnetic resonance imaging (MRI) on a 7T Agilent MRI scanner tuned to 400 MHz 1H frequency.

2.4. Lesion measurement

For en face staining, the tissue-engineered vessels were cut longitudinally and mounted with the lumen facing up before incubation with Alexa Fluor 488 phallolidin and/or 4′,6-Diamidino-2-phenylindole (DAPI). Lesion measurement was performed by staining cross-sections obtained at the centre of the graft with hematoxylin and eosin (H&E stain).

2.5. Statistical analysis

Data were analysed with GraphPad Prism 5.02 program using ANOVA and two-tailed student’s t-test for two-groups or pair-wise comparisons. The results were expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Isolated c-Kit+/Sca-1- cells represent a novel population of ESC-derived vascular progenitor cells

ESCs cultured on collagen IV became adherent and elongated (Fig. 1ai) and contained high numbers of c-Kit+/Sca-1- cells (about 50% of the total, Fig. 1aii). c-Kit+/Sca-1- cells were isolated by flow cytometry (FACS) and stimulated with VEGF in combination with shear stress or with PDGF-BB (Fig. 1aiii–iv). In the presence of VEGF, the cells assumed cobblestone-like features (Fig. 1aiii) and expressed EC markers CD31, Flk-1 and VE-cadherin at both mRNA (Fig. 1bi) and protein level (Fig. 1bii). Flow cytometry analysis showed that under these culture conditions, the population expressing endothelial markers accounted to about the 80% of the cells (Fig. 1bii). Functional differentiation was demonstrated by Matrigel assay and Ac-LDL uptake (Fig. 1biv and Supplemental Fig. 1). When cultured with PDGF-BB, the cells grew in size, assuming a polygonal shape (Fig. 1biv), and started overexpressing SMC specific genes (Fig. 1ci) and proteins (Fig. 1cii–iii). Image analysis indicated a differentiation efficiency nearing 100% (Supplemental Fig. 2).

3.2. c-Kit+ derived vascular cells repopulated the decellularised graft and improved in vivo outcome

When suitable patient-derived vessels are unavailable for vessel graft procedures, decellularised vessels offer an attractive alternative. Our lab has developed an efficient mouse model mimicking the clinical situation whereby the aorta is harvested and decellularised ex vivo before being grafted in the mouse carotid artery, thus simulating the patient situation during vessel surgery [2]. In this study, we aimed to exploit the potential of c-Kit+ derived cells for pre-clinical application by seeding them on the decellularised scaffold and implanting it in the mouse model of vessel graft. We
tested a set of conditions to determine the most efficient seeding protocol leading to a homogenous luminal attachment of ECs on the graft. Application of shear force was initiated at a rate of 3 dyn/cm² and then gradually increased to reach the physiological shear stress of 30 dyn/cm². The protocol proved crucial for the cell attachment to the lumen; the best coverage was obtained when the flow was...
increased hourly over the first 24 h (Fig. 2a and b). Next, the decellularised vessel was coated with gelatin or fibronectin and results showed that cell attachment was vastly improved after coating, upon the application of the shear (Fig. 2c). Under these conditions, c-Kit+ ECs efficiently attached to the luminal surface of the vessel, producing a continuous monolayer of CD31+ ECs (Fig. 2d) that responded to the application of physiological shear stress by aligning to the direction of the flow and becoming elongated (Fig. 2e). Collagen and elastin staining showed that cell seeding improved the extracellular matrix content (Fig. 2f). When decellularised vessels seeded with c-Kit+ ECs were implanted in a model of vascular graft, we observed increased residual lumen area (Fig. 2g) and reduced neointimal formation (Fig. 2h). Overall, 100% of the seeded grafts were patent at 4 weeks, compared with 60% of the control grafts (Supplemental Table 1). As reported in Supplemental Table 2, the major complication observed in mice grafted with decellularised vessel was thrombus formation, which was completely abolished by graft seeding. To further improve the design of the graft and to ameliorate the performance in terms of graft rupture (accounting for 28.6% of the graft failures in c-Kit-EC seeded vessels, Supplemental Table 2), we seeded the decellularised vessels with a combination of c-Kit+ derived SMCs. Seeding of the graft with ECs only or ECs+ SMCs significantly increased the survival of the mice (Fig. 2i) by reducing graft occlusion (Fig. 2j). Survival rate was increased to over 80% in the double seeded vessels (Fig. 2i). MRI confirmed the superior performance of ECs+ SMCs seeded vessels (Fig. 2k).

Together, these data indicate that the application of progenitor-derived vascular cells for repopulation of vascular grafts is feasible and that seeding dramatically reduces lumen occlusion and ultimately mortality.

3.3. Klf4 induces EC differentiation and inhibits SMC differentiation

Data presented so far demonstrated that c-Kit-derived vascular progenitor cells can give rise to both ECs and SMCs and that their application to tissue-engineered vascular grafts can significantly improve in vivo performance and survival. Next we studied the underlying molecular mechanisms determining the choice of differentiation pathway of vascular progenitor cells. The understanding of the regulation of progenitor cell differentiation is fundamental for the development of strategies aimed at the reduction of graft complications due to the over-proliferation of SMC and the lack of EC coverage.

We established that Krüppel-like transcription factor 4 (Klf4) was regulated upon differentiation. In particular, Klf4 expression was strongly induced during EC differentiation and downregulated during SMC differentiation (Fig. 3a). Upon adenoviral overexpression of Klf4 (Supplemental Fig. 3), we observed increased EC commitment of the c-Kit+ derived vascular progenitors as shown by increased tube-formation capacity (Fig. 3b). Klf4 overexpression led to increased expression of EC markers CD31, VE-cadherin and eNOS at both RNA and protein level, and a concomitant reduction in SMC markers (Fig. 3c and d). Additionally, lentiviral knockdown of Klf4 (Supplemental Fig. 3) resulted in increased expression of SMC and the inhibition of EC markers at both mRNA and protein levels (Fig. 3e and f). These results highlight the central role of Klf4 in the differentiation of the determination fate in c-Kit-derived vascular progenitor cells.

3.4. Klf4 and canonical Wnt pathway interact to determine c-Kit+ cell differentiation fate

The canonical Wnt pathway has been implicated in the fate decision of stem cell-derived vascular cells [17,18,21,24]. Klf4 has also been shown to interact with β-catenin in the nucleus in the context of tumour growth [25,26]. In our study, we found that Klf4 overexpression led to a nuclear accumulation of β-catenin as shown by confocal imaging and western blot analysis (Fig. 4a and b); this effect was reversed when Klf4 expression was inhibited (Fig. 4c). The reported downstream effectors of the Wnt pathway Axin2, ID2 and Cyclin D1 were altered by Klf4 overexpression and knockdown (Supplemental Fig. 4).

Treatment of cells with lithium chloride (LiCl), a Wnt pathway and β-catenin activator, resulted in an increase of Klf4 expression and EC markers and a reduction of SMC markers expression (Fig. 4c), mimicking the effects of the exogenous upregulation of Klf4 described above. Conversely, when β-catenin expression was knocked down, EC differentiation was impaired: EC marker expression was reduced alongside increased levels of SMC markers (Fig. 4d and e).

To confirm the importance of Klf4 in the activation of the Wnt pathway, we established that the activity of the β-catenin-dependent TCF promoter was ablated by knockdown of Klf4, as shown by TOP-flash luciferase assay (Supplemental Fig. 5). Interestingly, LiCl-dependent EC marker upregulation was reduced in Klf4-deficient cells (Fig. 4f). Conversely, β-catenin knockdown blunted the Klf4-induced upregulation of CD31 expression and downregulation of SMA (Fig. 4g). Our results reveal an interesting relationship between Klf4 expression and β-catenin activation, whereby LiCl induction of EC differentiation is mediated by Klf4 and Klf4 requires β-catenin to upregulate EC markers. This observation is in line with a previously reported mechanism showing that canonical Wnt activation directly increases Klf4 expression [16], indicating that Klf4 might partially mediate β-catenin activation. However, at this stage further studies are required to elucidate in the exact mechanism.

4. Discussion

Acute coronary obstruction requires arterial bypass surgery where an autologous vessel is grafted to the narrowed artery [15,16,27]. When autologous grafts are unavailable, the decellularisation of native vessels can potentially provide alternative material endowed with comparable characteristics [28–32]. However, the delayed endothelialisation dramatically increases the potential for neointimal formation [28–12], which creates the need for a suitable source of transplantable ECs. Stem cell-derived vascular cells allow the generation of a greater number of ECs compared to mature sources that could be exploited in the clinical practice owing to their privileged immunogenic profile [19–21,33]. In this study, we provide evidence that ESC-derived c-Kit+/Sca1- cells are a suitable source of ECs and SMCs and can therefore be considered bona fide vascular progenitor cells. In particular, we demonstrated the clinical relevance of ESC-derived cells in tissue engineering by seeding them on a decellularised scaffold using a bioreactor and applying it to a model of vascular graft. The optimised graft preparation and seeding allowed the creation of an improved cellularised graft displaying a continuous luminal layer of endothelial cells and a robust SMC layer. This improved survival and patency and reduced graft failure by avoiding thrombus, neointima development, and graft rupture [32,34,35]. Previously, our group has shown that decellularised vessels seeded with endothelial and smooth cells derived from adult reprogrammed cells displayed increased patency and survival, compared to their non-seeded counterparts [4]. In the present study, we explored the application of embryonic derived c-Kit+ cells as a cell source of endothelial and smooth muscle cells. This approach has the potential advantage to provide a relatively easier protocol to obtain larger number of vascular cells with high purity. Additionally, the implantation of
Fig. 2. Ex vivo seeding of decellularised vessel scaffolds and in vivo grafting. Two protocols were followed to increase the shear from 3 to 30 dyn/cm² in the tissue engineered grafts after luminal seeding of ECs: ‘daily step-wise’ (protocol A) or ‘hourly step-wise’ (protocol B) (a); cell retention was quantified in (b). Effect of PBS or gelatin or fibronectin coating on cell retention in static (empty bars) and shear (grey bars) was quantified in (c). c-Kit ECs formed a homogeneous CD31+ luminal monolayer on the decellularised vessel (d) and elongate in response to shear (e). Picosirius staining for collagen and Miller’s elastin staining of decellularised vessels showed improved matrix deposition after cell seeding (f). In vivo implantation of vessels seeded with c-Kit+ ECs showed improved lumen patency (g) and reduced neointima formation (h). Both the seeding with ECs and with the combination of ECs and SMCs (ECs+SMCs) increased the survival of grafted animals (i) and reduced vessel obstruction (j). MRI detection of the blood flow in the normal carotid (k, i), an occluded carotid grafted with a decellularised vessel (k, ii) and a patent carotid grafted with ECs+SMCs seeded scaffolds at Day 2 (k, iii), and 2 weeks (k, iv). Red arrows indicate the position of the grafts. Scale bars: 50 μm (d, f, l) and 100 μm (e). *P < 0.05; **P < 0.01; ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the vascular grafts prepared using c-Kit+ progenitors-derived EC and SMC determined better survival rate (over 80%) compared to the adult cell-derived grafts (60%). Furthermore, the use of an abundant population of mouse progenitor cells characterised by the expression of the c-Kit antigen makes our findings easily translatable to their human counterpart. The c-Kit antigen is expressed in several progenitor populations in adult human tissues including the heart where they act as a reservoir of cardiomyocytes, ECs and SMCs[5,36].

A thorough understanding of the molecular mechanisms implicated in the differentiation of stem cells into the vascular cell lines used for the creation of the grafts will help the development of strategies to improve the long-term performance of vascular grafts. In this study, we present a novel mechanism involving the expression of Klf4 in differentiating c-Kit+ cells. VEGF treatment induced and PDGF decreased Klf4 expression (Fig. 3a, vs. Day 0, dotted line). Overexpression of Klf4 (AdKlf4) increased EC differentiation and reduced SMC markers as shown by tube-formation (Fig. 3b), PCR (Fig. 3c) and western blot (Fig. 3d), as compared to control virus (AdCTL). Klf4 knockdown (ShKlf4) reduced endothelial marker expression and increased levels of SMC markers at RNA (Fig. 3e) and protein level (Fig. 3f) *P < 0.05, **P < 0.001, ***P < 0.0001 vs. control.
Klf4 and canonical Wnt pathway are interconnected in the determination of c-Kit+ cells differentiation. Confocal imaging showing the membrane localisation of β-catenin in control cells (AdCTL) and nuclear accumulation in Klf4 overexpressing cells (AdKlf4, a). Western blot confirmed β-catenin activation by AdKlf4 (b) and inhibition by ShKlf4 (c). LiCl (β-catenin activator) induced Klf4 and EC marker expression and reduced the SMC marker expression in a concentration-dependent manner (c: vs. NaCl, dotted line). Knockdown of β-catenin (Shβcat) impaired VEGF-dependent EC differentiation and increased SMC markers (d, vs. control virus: dotted line and e). LiCl-induced increase of EC markers was inhibited by Klf4 knockdown (f, ShKlf4). Klf4-induced EC marker upregulation and SMC marker downregulation is ablated by β-catenin knockdown (g). *P < 0.05; **P < 0.01. Scale bars: 10 μm (a). *P < 0.05; **P < 0.01 and ***P < 0.001.
transcription factor Klf4 and the canonical Wnt pathway that could be exploited in future to improve the levels of endothelialisation of vascular grafts and control SMC overgrowth leading to neointimal growth. In particular, we described the central role of the transcription factor Klf4 in driving differentiation preferentially towards the EC lineage and therefore reducing SMC differentiation. This was in line with previous findings showing that Klf4 is upregulated by shear stress [18], a typical EC differentiation stimulus, and that Klf4 regulates eNOS [10,14,31,37] and VE-cadherin expression [16] and inhibits SMC maturation [38]. Furthermore, a recent publication showed that Klf4 played a fundamental role in the maintenance of the vascular phenotype of endothelial progenitor cells [39]. Interestingly, the Klf4-induced canonical Wnt pathway activation and effect on differentiation was mimicked by LiCl, a Wnt activator. These results are in line with previous studies describing the importance of Wnt in the development of the embryonic vasculature [20,21]. These in vitro studies create the foundation for future investigations on the application of Klf4 and β-catenin overexpression/stimulation in the context of vascular graft in vivo in order to ameliorate the graft endothelialization, as previously demonstrated for VEGF activation [2].

The data presented described for the first time the vasculogenic potential of Gata4+ ESC-derived endothelial cells as a source of vascular cells for the creation of alternative grafts. We provided a novel insight into the mechanism regulating their differentiation, thus providing the translational potential for this stem cell therapy.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.04.055.

References


