Generation of cardiomyocytes from vessel wall-resident stem cells

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Zusammenfassung

Der Myokardinfarkt (MI) ist einer der Hauptgründe für gesundheitliche Probleme und zählt zu einer der am häufigsten tödlich verlaufenden Krankheiten weltweit. Daher ist es nicht verwunderlich, dass die Regeneration von funktionellem Myokardgewebe und/oder die kardiale Reparatur durch Stammzellen eines der weltweit am meisten angestrebten Ziele darstellt. Das adulte menschliche Herz stellt aufgrund seiner äußerst eingeschränkten endogenen Regenerationskapazität, die bei weitem nicht ausreicht, das geschädigte Gewebe zu erneuern, ein ideales Zielorgan für regenerative Therapieverfahren dar. Folglich könnte die Identifizierung neuer Quellen adulter Stamm- oder Vorläuferzellen mit kardiovaskulärem Differenzierungspotential dabei helfen, verfeinerte Therapien zu entwickeln, um entweder kardiale Fehlfunktionen zu verhindern oder eine deutlich verbesserte myokardiale Reparatur zu erreichen. Die aktuelle weltweite Forschung auf diesem Gebiet fokussiert sich auf: a) induzierte pluripotente Stammzellen (iPS), b) embryonale Stammzellen (ES) und c) adulte Stammzellen, wie z. B. mesenchymale Stammzellen, kardiale Fibroblasten und Mesangioblasten sowie Myofibroblasten. Bisher haben jedoch alle Bemühungen noch zu keinem Durchbruch geführt, so dass die teilweise vielversprechenden experimentellen Ergebnisse nicht in die klinische Therapie des MI und der kardialen Defekte mittels Stammzellen transferiert werden können. Abgesehen davon, ob und wie stark so ein endogenes herzeigenes Potential wäre, würde die Identifizierung neuer endogener Stammzellen mit kardiogenem Potential, die genaue Charakterisierung ihrer Nischen und der Mechanismen ihrer Differenzierung einen Meilenstein in der kardioregenerativen Stammzelltherapie darstellen. Die Arbeitshypothese der hier vorgelegten Dissertation besagt, dass die Gefäßwand als

Nische solcher Zellen dienen könnte. Innerhalb der letzten Jahre konnte die Adventitia und die subendotheliale Zone der adulten Gefäßwand als Nische für unterschiedliche Typen von Vorläuferzellen und multipotenten Stammzellen, die sogenannten Gefäßwand-residenten Stammzellen (VW-SCs) identifiziert werden. In Anbetracht der Tatsache, dass die Blutgefäße aufgrund ihrer hohen Dichte im Herzen eine essentielle stromale Komponente des Herzgewebes darstellen, kann die mögliche klinische Relevanz von VW-SCs für das Myokardium im Moment nur erahnt werden. Ausgehend von der Annahme, dass eine Subpopulation dieser VW-SCs die Fähigkeit besitzt, sich in Kardiomyozyten-ähnliche Zellen zu differenzieren, sollte im Rahmen dieser Dissertationsarbeit das myokardiale Potential der Gefäßwand-residenten Stammzellen aus der Aorta adulter Mäuse studiert werden, indem die Zellen unter unterschiedlichen definierten Bedingungen kultiviert und dann sowohl morphologisch als auch funktionell charakterisiert werden. Erstaunlicherweise zeigten die ersten Ergebnisse die Generierung spontan schlagender Kardiomyozyten-ähnlicher Zellen, nur durch Verwendung eines speziellen Nährmediums und ohne jegliche genetische Manipulation. Die im Rahmen dieser Arbeit durchgeführten Analysen belegen zudem, dass die Kardiomyozyten-ähnlichen Zellen reproduzierbar nach ca. 9-11 Tagen in der Kultur anfangen, spontan zu schlagen. In immunzytochemischen Analysen zeigten die schlagenden Zellen ein quergestreiftes Färbemuster für α-sarkomeres Actinin. Passend dazu wiesen diese spontan schlagenden Zellen, wie reife Kardiomyozyten, Sarkomerstrukturen mit Komponenten des sarkoplasmatischen Retikulums in elektronenmikroskopischen Analysen auf. Sie zeigten dementsprechend eine mit dem spontanen Schlag assoziierte Kalzium-Oszillation. Erstaunlicherweise zeigten die hier vorgelegten

Befunde erstmalig, dass es nicht die Sca-1^+ (stem cell antigen-1) Zellen waren, denen seit Jahren eine kardiomyozytäre Kapazität zugeschrieben wird, sondern es waren die Sca-1^- Zellen der Mausaorta, die sich zu den spontan schlagenden Zellen differenzierten. Des Weiteren scheint diese Differenzierung von einer endogen generierten inflammatorischen Mikroumgebung abhängig zu sein. Die hier vorgelegten Ergebnisse legen daher den Schluss nahe, dass die VW-SCs in der vaskulären Adventitia sowohl die inflammatorische Mikroumgebung als auch die spontan schlagenden Kardiomyozyten-ähnlichen Zellen bereitstellten. So entstanden in der Kultur aortaler Zellen unter anderem auch Makrophagen, die hohe Mengen des Gefäßwachstumsfaktors VEGF (Vascular Endothelial Growth Factor) aufweisen. Wurden die Makrophagen in der Zellkultur durch Zugabe von Clodtronat-Liposomen depletiert, so wurde damit auch die Generierung spontan schlagender Zellen aus den aortalen VW-SCs unterbunden. Um zu testen, ob und inwieweit dieser Einfluss der Makrophagen auf die Entstehung spontan schlagender Zellen aus den VW-SCs auf den VEGF zurückzuführen ist, wurden kultivierte Zellen der Mausaorta mit dem VEGF-Rezeptor-2-Blocker (E7080) behandelt. Auch diese Behandlung resultierte wie bei der Depletion von Makrophagen darin, dass keine spontan schlagenden Zellen entstanden. Um die von VW-SCs generierten spontan schlagenden Zellen funktionell zu charakterisieren, wurden die kultivierten Zellen der Mausaorta mit Isoproterenol (ß-Sympathomimetikum) und Propranolol (ß-Blocker) behandelt. Eine signifikante Steigerung der Schlagfrequenz unter Isoproterenol und eine Reduzierung bei Zugabe von Propranolol unterstreichen ebenfalls die Kardiomyozyten-ähnliche Eigenschaft der spontan schlagenden Zellen. Schließlich wurden die aus der Mausaorta isolierten Zellen Fluoreszenz-markiert und dann in das kardiale Feld des

sich entwickelnden Hühnerembryos (am fünften Tag der Entwicklung) implantiert. Zwei Tage später wurden die Herzen entnommen. Immunfärbungen zeigten, dass ein Teil der implantierten Zellen auch unter diesen *in vivo*-Bedingungen für α-sarkomeres Actinin positiv wurde und somit einen kardiomyozytären Phänotyp aufwies.

Summary

Myocardial infarction (MI) is a major cause of health problems and is among the leading deadly ending diseases. Accordingly, regenerating functional myocardial tissue and/or cardiac repair by stem cells is one of the most desired aims worldwide. Indeed, the human heart serves as an ideal target for regenerative intervention, because the capacity of the adult myocardium to restore itself after injury or infarct is limited. Thus, identifying new sources of tissue resident adult stem or progenitor cells with cardiovascular potential would help to establish more sophisticated therapies in order to either prevent cardiac failure or to achieve a functional repair. Ongoing research worldwide in this field is focusing on a) induced pluripotent stem (iPS) cells, b) embryonic stem (ES) cells and c) adult stem cells (e. g. mesenchymal stem cells) as well as cardiac fibroblasts or myofibroblasts. However, thus far, these efforts did not result in therapeutic strategies that were transferable into the clinical management of MI and heart failure. Hence, identifying endogenous and more cardiac-related sources of stem cells capable of differentiating into mature cardiomyocytes would open promising new therapeutic opportunities. The working hypothesis of this thesis is that the vascular wall serves as a niche for cardiogenic stem cells. In recent years, various groups have identified different types of progenitors or mesenchymal stem cell-like cells in the adventitia and sub-endothelial zone of the adult vessel wall, the so called vessel wall-resident stem cells (VW-SCs). Considering the fact that heart muscle tissue contains blood vessels in very high density, the physiological relevance of VW-SCs for the myocardium can as yet only be assumed. The aim of the present work is to study whether a subset of VW-SCs might have the capacity to differentiate into cardiomyocyte-like cells. This assumption

was challenged using adult mouse aorta-derived cells cultivated in different media and treated with selected factors. The presented results reveal the generation of spontaneously beating cardiomyocyte-like cells using specific media conditions without any genetic manipulation. The cells reproducibly started beating at culture days 8-10. Further analyses revealed that in contrast to several publications reporting the Sca-1^{+} cells as cardiac progenitors the Sca-1^{-} fraction of aortic wall-derived VW-SCs reproducibly delivered beating cells in culture. Similar to mature cardiomyocytes the beating cells developed sarcomeric structures indicated by the typical cross striated staining pattern upon immunofluorescence analysis detecting α-sarcomeric actinin (α-SRA) and electron microscopic analysis. These analyses also showed the formation of sarcoplasmic reticulum which serves as calcium store. Correspondingly, the aortic wall-derived beating cardiomyocyte-like cells (Ao-bCMs) exhibited calcium oscillations. This differentiation seems to be dependent on an inflammatory microenvironment since depletion of VW-SC-derived macrophages by treatment with clodronate liposomes *in vitro* stopped the generation of Ao-bCMs. These locally generated F4/80^{+} macrophages exhibit high levels of VEGF (vascular endothelial growth factor). To a great majority, VW-SCs were found to be positive for VEGFR-2 and blocking this receptor also stopped the generation VW-SC-derived beating cells *in vitro.* Furthermore, the treatment of aortic wall-derived cells with the ß-receptor agonist isoproterenol or the antagonist propranolol resulted in a significant increase or decrease of beating frequency. Finally, fluorescently labeled aortic wall-derived cells were implanted into the developing chick embryo heart field where they became positive for α-SRA two days after implantation. The current data strongly suggest that VW-SCs resident in the vascular adventitia deliver both progenitors for an

inflammatory microenvironment and beating cells. The present study identifies that the Sca-1$^-$ rather than Sca-1$^+$ fraction of mouse aortic wall-derived cells harbors VW-SCs differentiating into cardiomyocyte-like cells and reveals an essential role of VW-SCs-derived inflammatory macrophages and VEGF-signaling in this process. Furthermore, this study demonstrates the cardiogenic capacity of aortic VW-SCs *in vivo* using a chimeric chick embryonic model.

1 Introduction

1.1 Stem cells

Stem cells have been one of the most attractive topics in the research for decades. Due to their potential to differentiate into different mature cell types, and thus, serve as material for replacement of damaged tissues, they represent a prominent potential for therapeutic strategies aiming on replacement or regeneration of damaged cells and tissues. Stem cells are unspecialized cells with the ability to self-renew, capable of differentiating into one or more terminally specialized cell types, and play a crucial role in homeostasis and tissue repair (Nadig, 2009). When called into action following an injury, a stem cell self-renews, undergoes cell division and gives rise to one daughter stem cell and one progenitor cell. A progenitor cell is an intermediate cell type formed before it achieves a fully differentiated state. It is regarded as committed to differentiating along a particular cellular developmental pathway of stem cells (Nadig, 2009), which is also visualized in Figure 1.

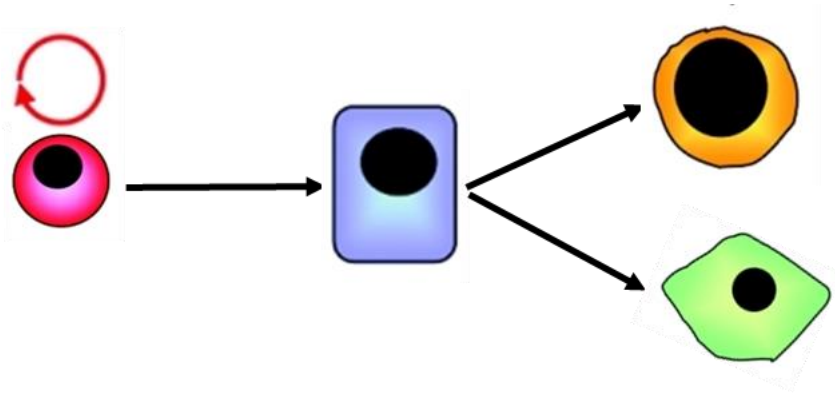

Figure 1. Stem, progenitor, and differentiated cells. Stem cells have the potential to self-renew and to divide asymmetrically in order to produce more committed progenitors, which in turn can give rise to differentiated cells. Modified image from Collas and Håkelien, 2003.

1.2 Stem cells classification

Based on their origin, stem cells are categorized into embryonic stem cells (ES cells) somatic stem cells, fetal stem cells and adult stem cells.

1.2.1 Embryonic stem cells (ES cells)

Embryonic stem cells are pluripotent cells with capacity to differentiate into every type of the three germ layers derived tissues. ES cells can be obtained from the inner cell mass of the blastocyst during the early embryonic stages (Figure 2). Although of unlimited self-renewal and pluripotentiality of ES cells, only limited advance has been achieved in technology and clinical application of human ES cells. This is due to many reasons, first of them is the controversial ethical issue about the use of human embryos for research purposes, the strong immunological problems, and the development of tumors by transplantation of ES cells in animals. These hurdles turned the attention of many researchers to adult stem cells (Rippon and Bishop, 2004).

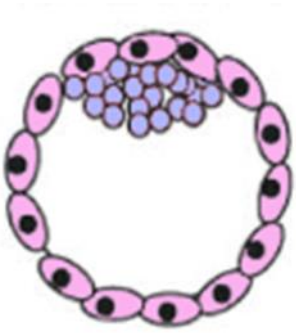

Figure 2. Embryonic stem cells (ES). ES cells are pluripotent stem cells derived from the inner cell mass of the blastocyst stage embryos. Modified image from Reinecke et al., 2008.

1.2.2 Induced pluripotent stem cells (iPS cells)

Induced pluripotent stem cells (iPS cells) are pluripotent cells with similar potential like ES cells. It was demonstrated that somatic cells can be re-programmed into pluripotent stem cells by ectopic expression of four transcription factors, namely Oct4, Klf4, Sox2, and c-Myc, using four independent retroviral vectors (Takahashi and Yamanaka, 2006) (Figure 3). However, iPS technology has still several technical issues to be addressed such as generation of iPS without tumor formation, and genetic mutations that need to be eliminated before the cells can be used in clinical applications.

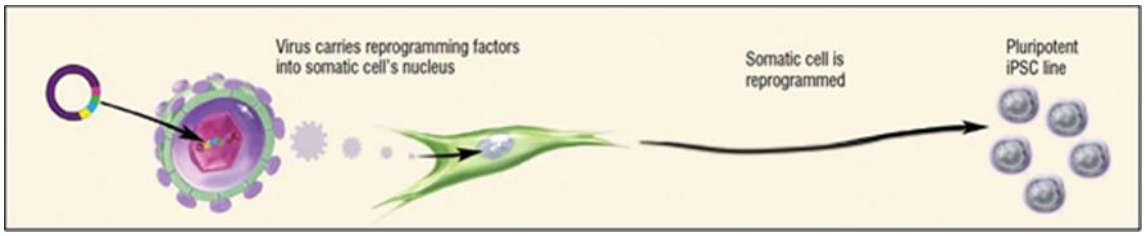

Figure 3. Generation of induced pluripotent stem cells (iPS cells). General method for creating iPS cells. Modified image from *"stemcells.nih.gov"*.

1.2.3 Fetal stem cells

Fetal stem cells can be isolated from fetal blood and bone marrow as well as from other fetal tissues, including liver and kidney. Fetal stem cells have been described to be multipotent. Both fetal hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) have advantages over their adult counterparts, including better intrinsic homing and engraftment, broader differentiation potential and lower immunogenicity (O'Donoghue and Fisk, 2004).

1.2.4 Adult stem cells

Adult stem cells are considered to be to some extent committed, but still terminally undifferentiated cells with multipotent capacity to self-renewal and differentiate into specialized cells, e.g. the HSC resident in the bone marrow or more committed progenitors. Adult stem and progenitor cells essentially contribute to maintain, to repair, and regenerate tissues they reside within. They have been isolated from different tissues and organs in adult (Blau et al., 2001) such as bone marrow (Liechty et al., 2000), brain (Altman, 1969), blood vessels (Zengin et al., 2006), heart (Orlic et al., 2001), liver (Petersen et al., 1999) and skeletal muscle (Mauro, 1961).

1.3 Stem Cells Niche

Schofield was the first person who proposed the concept of stem cell niche almost 38 years ago (Schofield, 1978). The term “niche” describes a specific anatomical microenvironment enabling to keep stem cells in a transiently silent state in order to stop their further differentiation into specialized mature cells (Hsu and Fuchs, 2012). The niche regulates stem cell self-renewal, maintenance, survival and prevents the uncontrolled proliferation of stem cells, which could lead to tumor development. The niche is composed of a complex system of cellular and molecular components which surround the stem cells and participate in governing their fate (Jones and Wagers, 2008). Adult organs and tissues including the brain, gut, bone marrow and hair follicles harbour stem cells in specialized niches, allowing for spatial and temporal regulation of the renewal process (Li and Clevers, 2010). The niche supports quiescent stem cells that upon stimulation give rise to transient amplifying progenitors that differentiate into mature, tissue-specific cell types. However, little is

known about the origin of cardiac stem cells (CSCs) or the structural organization of the CSC niche in the heart (Fioret et al., 2014).

1.4 Cardiomyocytes and stages of cardiac differentiation during embryonic development

Cardiomyocytes are the main functional cell type of the myocardial tissue. They beat continuously life long, and their beating frequency is controlled by pacemaker cells, a specialized type of cardiomyocytes. Cardiomyocytes almost need an aerobic metabolism, therefore 25% of the total cell volume is occupied by mitochondria (Dobson and Himmelreich, 2002). The contractile unit of cardiomyocytes is the sarcomere, which consists of myosin and actin filaments as well as regulatory proteins like troponins and tropomyosin. Cardiomyocytes represent 25% of the total cells in heart (Dow et al., 1981), while the rest is delivered by endothelial cells, pericytes and smooth muscle cells which construct the heart vessels as well as fibroblasts completing the cardiac stroma. There are different kinds of cardiomyocytes such as atrial, ventricular and pacemaker. Atrial myocytes are located in the myocardium of right and left atrium where blood is collected during the diastolic phase of heart action. Accordingly, ventricular myocytes build up the myocardial layer of both right and left ventricular wall, from where the blood is pushed out of the heart in the systolic phase of the heart action. Finally, pacemaker cells reside in the specialized areas of the right atrium such as sinus and atrioventricular (AV-) nodes and in a hierarchic order in the ventricular septum and the wall of right and left ventricles. Under normal conditions the sinus node generates electrical impulses which are subsequently forwarded to AV-node, then to HIS-bundle, from there to Tavara-bundles, and finally to Purkinje fibers which are located

within the whole ventricular myocardium. The cells of the myocardium form a functional syncytium within which the electrical signal is transmitted from cell to cell via gap junction channels. These electrical impulses initiate heart contraction via the so-called electro-mechanical coupling (Xin et al., 2013). For this function Ca^{2+} ions are needed in a high intracellular concentration. They are mainly released from intracellular stores and to a lesser part diffuse from the extracellular space into the cardiomyocytes via calcium channels. Correspondingly, cardiomyocytes contain a large amount of sarcoplasmic reticulum, a special form of smooth endoplasmic reticulum, of which the function is to store and release Ca^{2+} ions needed to translate the electrical signal into mechanical action (Laver, 2007).

The heart is the first organ to form during mammalian development (Buckingham et al., 2005). There are various stages of cardiac differentiation during development. As shown in Figure 4, Brachyury-positive cells characterize the first phase of cardiac differentiation. During cardiac specification, Brachyury-positive cells can give rise to cardiovascular progenitors (CVPs) which are further characterized by the expression of markers such as Insulin gene enhancer protein (Isl-1), NK2 homeobox5 (Nkx2.5) and Fetal liver kinase 1 (Flk1). Subsequently, CVPs give rise to cardiomyocytes (Moretti et al., 2006) (Figure 4).

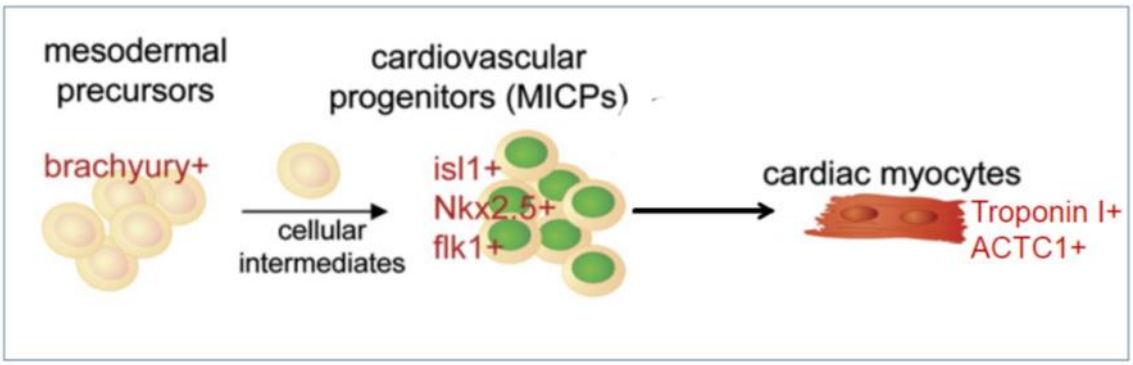

Figure 4. Differentiation of cardiovascular progenitors towards cardiomyocytes. Overview of different precursor populations arising during cardiac specification of pluripotent stem cells**.** Modified image from Moretti et al., 2006.

1.5 Different stem cell sources and cardiac stem cells (CSCs)

Despite of recent advances in medicine, cardiovascular disorders remain a major cause of mortality in the world (Bui et al., 2011) and of over 1.9 million deaths each year in the European Union (European Cardiovascular Disease Statistics 2012). As cardiomyocyte regeneration in the adult heart is limited, the identification of a putative endogenous source of cardiac stem or progenitor cells is of great interest in order to develop novel strategies for regenerative therapy (Planat-Bénard et al., 2004). In this perspective, different stem cell sources have been shown to improve cardiac function such as bone marrow (Lunde et al., 2006), peripheral blood (Zimmet et al., 2012), and adipose tissue (Bai et al., 2010). In addition to these sources, a population of resident cardiac stem cells (CSCs) has been identified in the heart (Figure 5).

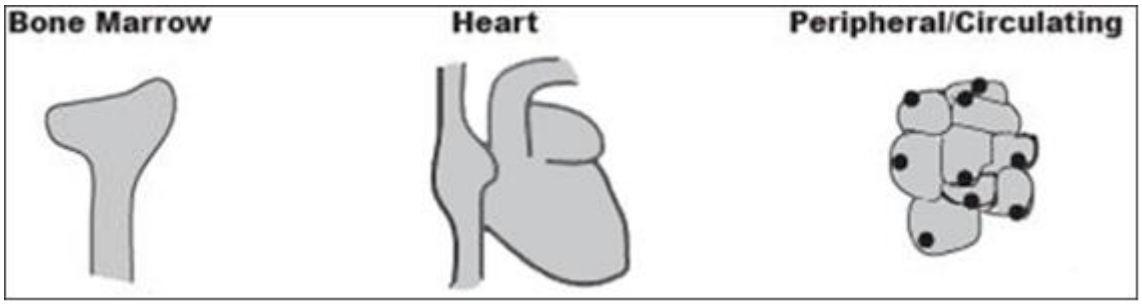

Figure 5. Various adult stem cell sources including bone marrow, heart blood circulation, and peripheral tissues are reported for potential use in cardiac repair. Modified image from Reinecke et al., 2008.

These CSCs are characterized by cell surface markers such as c-Kit$^+$ (Hosoda, 2012) and/or Sca-1$^+$ cells (Matsuura et al., 2004) or cells expressing specific transcription

factors such as Isl-1 (Di Felice and Zummo, 2013), and their physiological properties such as the ability to efflux fluorescent dye (i. e., side population) (Hou et al., 2013). Moreover, CSCs expressing the tyrosine kinase receptor c-Kit are the most extensively investigated subtype (Hosoda, 2012). The contribution of these cell types to myocardial regeneration is still a matter of conflicting debate. Some studies reported, that when transplanted, c-Kit$^+$ cells induce large scale cardiac regeneration after myocardial infarction (MI), and contribute to both generation of new myocardium and formation of new vessels, whereas others suggest a contribution to cardiac regeneration only at a small-scale (Hosoda, 2012). On the other hand, recent studies demonstrated that transplanted bone marrow-derived MSCs may stimulate endogenous c-Kit$^+$ CSCs in the infarcted pig heart (Hatzistergos et al., 2010). However, therapeutic applications of CSCs are limited due to their lesser number and low proliferation capacity (Li et al., 2014). Furthermore, these efforts were not transferable into the clinic of MI and heart failure yet. Thus, identifying endogenous sources of CSCs that are able to differentiate into mature functional cardiomyocytes would help to make a crucial step towards heart failure therapy. One promising location containing such progenitors might be the adult vascular wall which has been shown to harbor various stem or progenitor populations, e. g. for vascular cells such as endothelial and smooth muscle cells/pericytes as well as non-vascular cells such as macrophages or hematopoietic stem cells (Ergün et al., 2011), (Zengin et al., 2006).

1.6 Adult blood vessels as a source for different type of stem and progenitor cells

The cardiovascular system consists of the heart and blood vessels. The adult vascular system displays a hierarchic structure, which is composed of large, middle sized and small vessels. Functionally, the vascular system is divided into two basic parts, the macro and microcirculation. The smallest vessels, namely the capillaries together with arterioles and venules belong to the microcirculation. The capillaries are built up by two cellular layers, ECs lining the lumen and pericytes covering the endothelial cell layer from the outside. Pericytes are contractile and can modulate the capillary diameter. Both, ECs and pericytes are in tight contact with each other and share the same basal lamina (Figure 6A). Arterioles represent the arterial part of the microcirculation and their wall exhibits at least one layer of smooth muscle cells (SMCs), which are tightly organized and form a circular continuous layer around the ECs. Accordingly, venules belong to the venous part of microvessels and like arterioles they display one layer of smooth muscle cells but with a less compact and tight structure in comparison to the arterioles. Arterioles and venules might also possess a thin circularly organized adventitia containing fibroblasts. A clear three-layered vessel wall is present in large and middle-sized blood vessels such as arteries and veins. From luminal to the outside of the vessel wall these layers are named as the intima, media, and adventitia (Figure 6B).

Tunica intima: The innermost layer, which is composed of a monolayer of endothelial cells that are in direct contact with blood flow, followed by a sub-endothelial layer, which is made up of connective tissue. The inner elastic membrane (IEL) separates the tunica intima from the tunica media.

Tunica media: The intermediate layer, which is composed of smooth muscle cells (SMCs) that are embedded in an extracellular matrix of elastin, collagen, and proteoglycans. The outer elastic membrane (OEL) separates the tunica media from the tunica adventitia.

Tunica adventitia: The outer layer, which is composed of loose connective tissue containing collagen, elastic fibers, and fibroblasts. The tunica adventitia supports blood vessels to withstand the blood pressure. The vasa vasorum are found within the adventitia of big vessels, which supply oxygen and nutrition to the outer layers of the vessel wall (Figure 6C). Recently, accumulating data suggest that the adventitia serves as a niche for various stem and progenitor cells (Ergün et al., 2011).

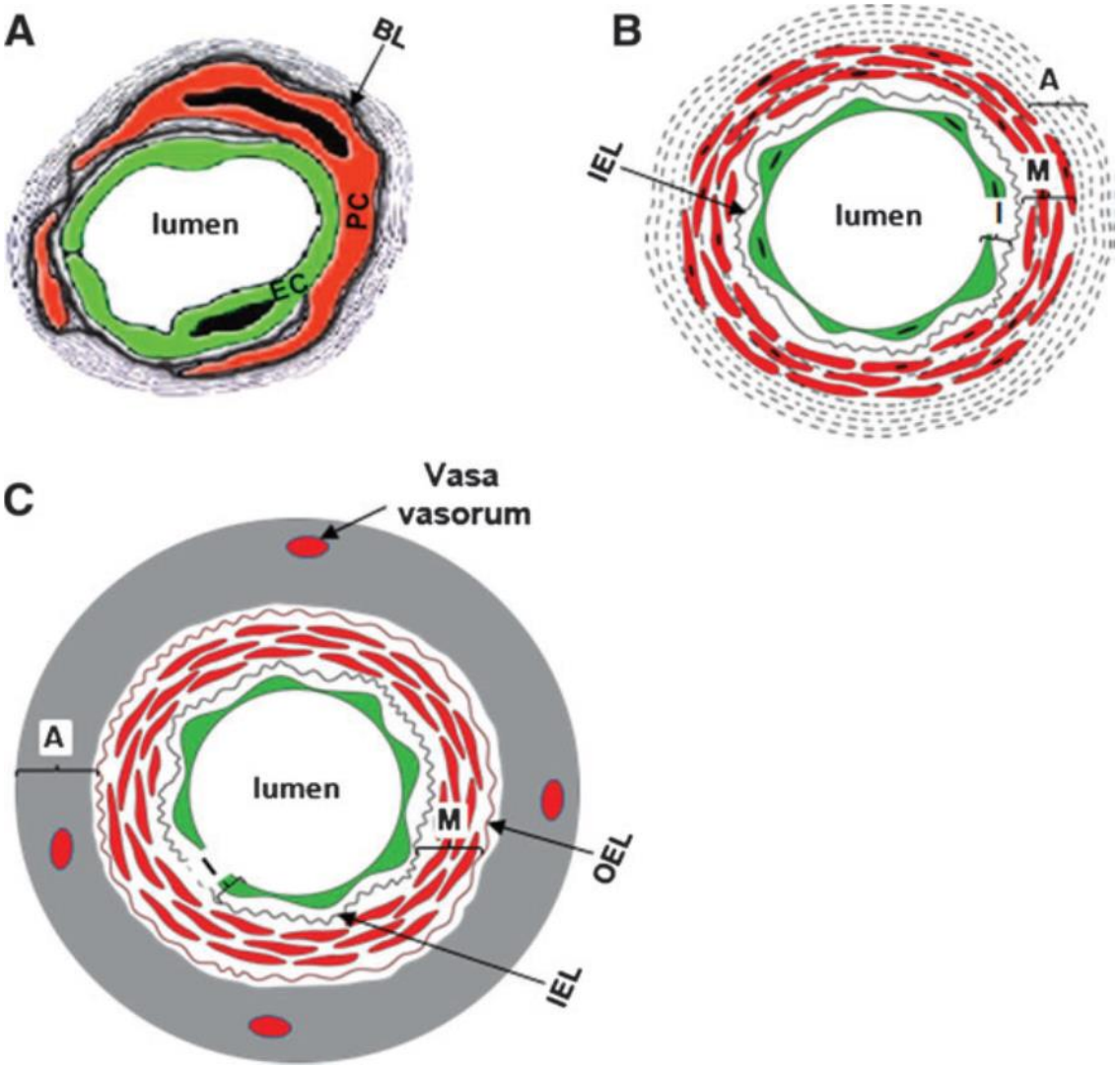

Figure 6. Basic structure of the vessel wall. A) The smallest vessels (capillaries) are made up of endothelial cells (ECs) lining the lumen and pericytes (PCs) which cover the endothelial cell layer. B) Medium-sized arteries are made of three layers such as intima (I), media (M), and the adventitia (A). The inner elastic membrane (IEL) separates intima from media. C) Large vessels are constructed with three layers: intima (I), media (M), and adventitia (A) with vasa vasorum. The outer elastic membrane (OEL) separates the tunica media from the tunica adventitia (Ergün et al., 2011).

1.6.1 Vascular wall-resident stem cells

During the last decade, various groups have reported results identifying the adult vessel wall as a reservoir of various stem and progenitor cell populations (Ingram et al., 2005), (Klein et al., 2011), (Passman et al., 2008), (Psaltis et al., 2011), (Tintut et al., 2003), (Zengin et al., 2006). To mention only a few discovery steps: In 2001, Alessandri and coworkers demonstrated the presence of $CD34^{+}CD31^{-}$ endothelial progenitor cells (angioblasts) in the wall of the embryonic aorta (Alessandri et al., 2001). Few years later a distinct circularly organized area within the human vascular adventitia was identified for the first time to contain different types of vascular and non-vascular progenitors and/or stem cells. This area was named as “vasculogenic zone” which was found to be present in the wall of human adult arteries and veins (Zengin et al., 2006) (Figure 7).

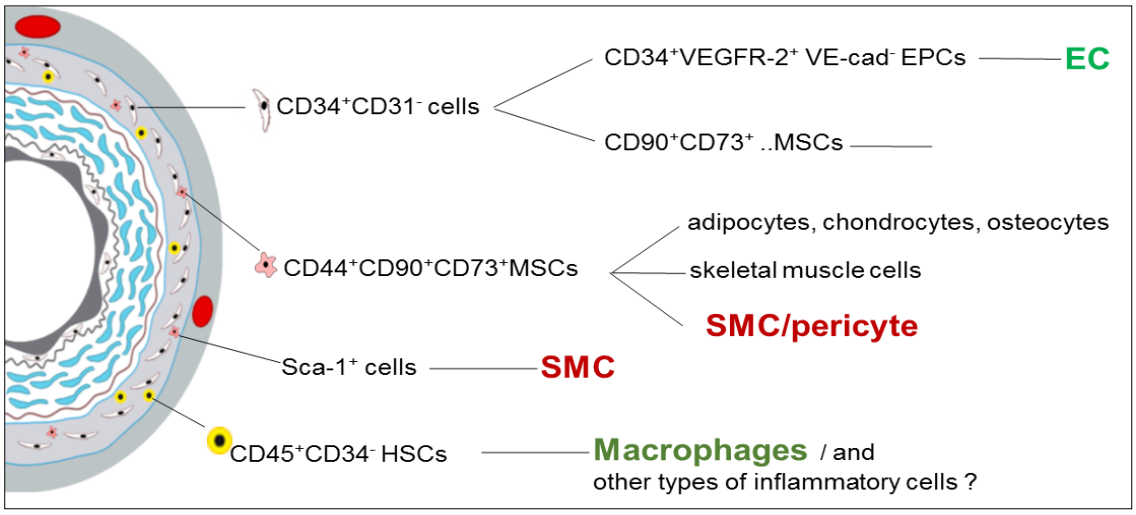

Figure 7. Distribution of various VW-SC types in the adult vascular adventitia and their derivatives. Modified image from Ergün et al., 2011.

The presence of this stem cell niche was confirmed by Passman and co-workers, who characterized a sonic hedgehog (Shh) signaling mechanism that supports resident Sca1^{+} vascular progenitor cells which were found to be localized exactly in the adventitial “vasculogenic zone” of mouse arterial wall (Passman et al., 2008). They further demonstrated the capacity of these progenitors to differentiate into vascular smooth muscle cells *in vitro*. More interestingly, using the genetic approach with LacZ as reporter gene, the authors could demonstrate that the Sca-1^{+} smooth muscle progenitors were already detectable in the adventitial “vascuologenic zone” of embryonic mouse vessels and continued to adult period (Figure 8).

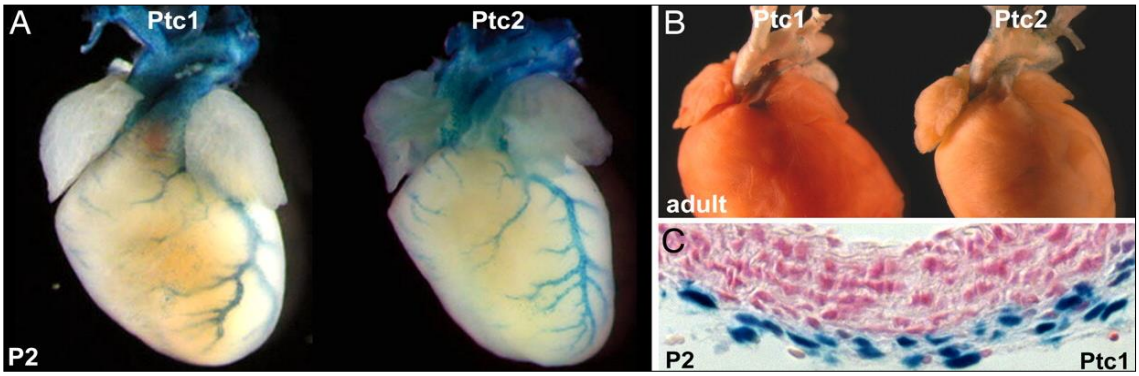

Figure 8. β-gal activity in blood vessels of patched-1 lac-Z (Ptc^{lacZ}) reporter mice at post-natal day2 (P2) and adult period (A and B). In the post-natal period, Sca-1^{+} smooth muscle cell progenitors remained in the adventitial “vasculogenic zone” (C, blue staining) of the coronary arteries, whereas low levels were detected in coronary arteries and large vessels of adult mice (B) (Passman et al., 2008).

Hence, VW-SCs reside in distinct zones of the vessel wall such as the sub-endothelial space within the vascular intima and the “vasculogenic zone” within the vascular adventitia (Tilki et al., 2009). In addition, in a recent study it was reported that CD44^{+} cells from hiTA wall have clonal capacity and differentiate into pericytes

and smooth muscle cells in both *in vitro* and *in vivo* conditions (Klein et al., 2011) (Figure 9).

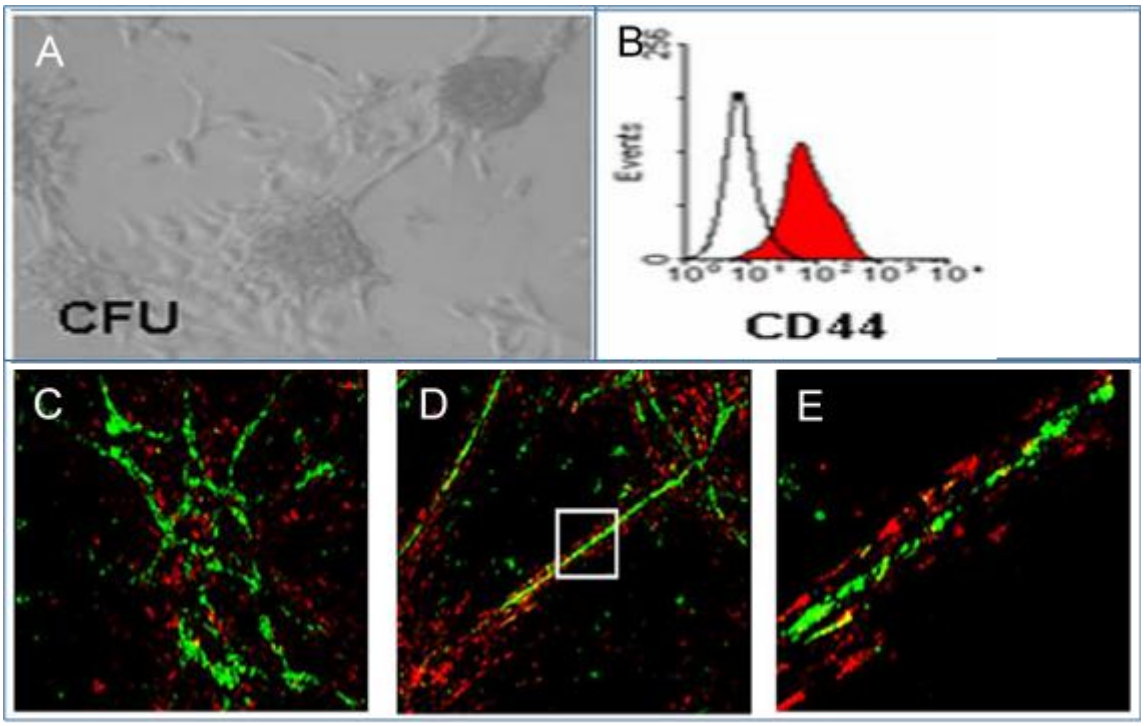

Figure 9. Clonal capacity of CD44$^+$ VW-SCs from human internal thoracic artery wall (A-B). Co-cultivation of CD44$^+$ VW-SCs (C-E, red) with HUVECs (human umbilical vein endothelial cells, C-E, green) in matrigel showed pericyte-like coverage of CD44$^+$ VW-SCs with tube like structures of HUVECs (C-E) (Klein et al., 2011).

In summary, the published data convincingly underline that the vascular wall contains different types of multipotent stem cells and progenitors with the capacity to differentiate not only into vascular cells but also into non-vascular cell types, e. g. macrophages. CD44$^+$ mesenchymal stem cell (MSC)-like multipotent vascular wall-resident stem cells (VW-MPSCs) were described to be expandable and give rise to different mature cell types such as osteocytes, chondrocytes and adipocytes, but also vascular smooth muscle cells. Detailed analyses revealed that not only the wall

of large and middle-sized blood vessels such as arteries and veins but also that of micro vessels such as arterioles, venules and capillaries within organ parenchyma contains VW-SCs (vascular wall-resident stem cells) as detectable by immunostaining for CD34 and CD44 (unpublished data, communicated by Prof. S. Ergün).

Interestingly, the localization pattern seems to be very similar in human, mouse, and rat tissues suggesting that the adventitial vascular stem cell niche represents an overall biological principle. In human, the total length of the vascular system is estimated to be at about 80,000 – 100,000 km. Assuming that all parts of the adult vascular system harbor VW-SCs, one can imagine the huge stem cell potential that exists along the blood vessels in the whole body. Since myocardium belongs to the highest vascularized tissues, stem cells resident in the wall of coronary vessels might serve as a source for cardiac regeneration. Until now, this potential has not been sufficiently studied.

1.6.1.1 Vessel wall-resident cardiac progenitors?

Mature cardiomyocytes exhibit only a low proliferation rate and little is known about the role of endogenous progenitors with cardiac differentiation capacity. Numerous populations of putative adult endogenous cardiomyocyte progenitors were reported in different organs during the past decade based on cell surface marker, and cardiac protein expression as well as on testing of some functional properties *in vitro*. In some studies these cells were also used for the delivery into recipient hearts. (Beltrami et al., 2003), (Chong et al., 2014), (Malliaras et al., 2014), (Messina et al., 2004), (Oh et al., 2003), (Ott et al., 2007), (Tamura et al., 2011). Moreover, vascular progenitor cells (VPCs) have been observed in the walls of coronary arteries based

on expression of VEGFR-2 (KDR or Flk1) and c-Kit in the human heart. Interestingly, VPCs were self-renewing, clonogenic, and differentiated into ECs and SMCs and partly into cardiomyocytes, as shown by FACS analysis under *in vitro* conditions (Bearzi et al., 2009). In addition, others have also reported that endothelial-derived cardiac progenitor cells are localized in coronary vessels and serve as a cardiac stem cell (CSC) niche in the adult heart (Figure 10). However, functional properties of these progenitors have not been assessed in the study (Fioret et al., 2014).

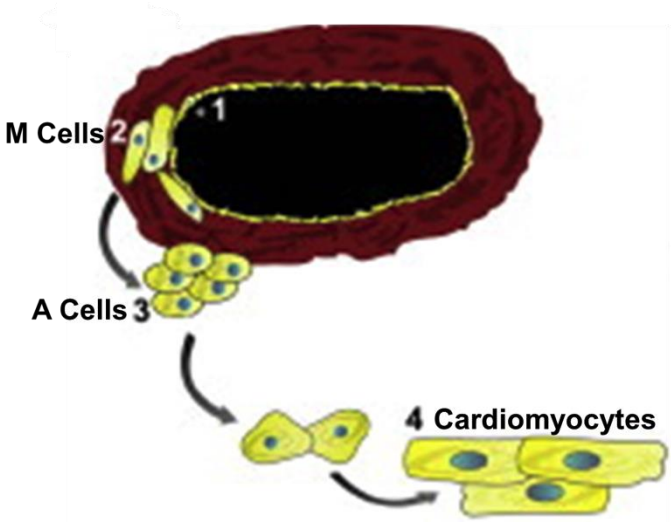

Figure 10. Endothelial-derived cardiac progenitor cells are localized in the arterial coronary walls. Endothelial cells (1) gave rise to quiescent cells (M cells) in the media (2) and proliferating cells (A cells) in the adventitia (3). Further, these proliferating A cells differentiate into cardiomyocytes (4) (Fioret et al., 2014).

1.6.1.2 Vascular wall-resident endothelial progenitor cells (VW-EPCs)

Endothelial progenitor cells (EPCs) are defined as cells with capacity to differentiate into mature endothelial cells (ECs) and form capillary-like structures *in vitro* and capillaries *in vivo*. The EPCs are adherent to matrix molecules and form colonies (Yoder, 2012). Although, several markers are considered as typical for EPCs such as

CD34, AC133, KDR (VEGFR-2 or Flk1 in mice), Tie-2 and UEA-1 (ulex europaeus agglutinin 1 lectin) as well as acLDL uptake, there is still no single marker to distinguish between circulating mature ECs or EPCs and hematopoietic stem cells (HSCs) (Ergün et al., 2011). However, morphologically, the one approach currently is to define endothelial lineage in tubulogenesis assay. Tubular network formation in matrigel is a main feature of endothelial progenitors (Leeper et al., 2010). EPCs were discovered in 1997 by identifying KDR/Flk1-positive cells circulating in the peripheral blood, which were shown to contribute to the formation of new vessels (Asahara and Isner, 2002). Later it was shown that a subset of mononuclear cells circulating in the peripheral blood has the capacity to differentiate into EPCs *in vitro* and contribute to tumor vessel formation *in vivo* when they were implanted subcutaneously into mice together with tumor cells (Gehling et al., 2000). For several years the main sources for EPCs have been considered to be bone marrow (BM) and peripheral blood and thus, EPCs were called bone marrow-endothelial progenitor cells (BM-EPCs) or circulating-endothelial progenitor cells (C-EPCs), respectively (Aicher et al., 2007), (Asahara and Isner, 2002), (Nolan et al., 2007). However, data published in the last decade demonstrate the presence of EPCs within the adult vascular wall which consequently were termed as vascular wall resident endothelial progenitor cells (VW-EPCs) (Pasquinelli et al., 2007), (Tavian et al., 2005), (Zengin et al., 2006). Moreover, immunostaining analyses identified a high number of $CD34^{+}VEGFR\text{-}2^{+}Tie\text{-}2^{+}CD31^{-}VE\text{-}cadherin^{-}$ VW-EPCs within the adventitia of blood vessels, the so-called vasculogenic zone as described above (Zengin et al., 2006). VW-EPCs are attractive for several reasons: these cells could be easily obtained from small pieces of superficial veins or arteries such as the internal thoracic artery, and be used for

cellular therapy or can be targeted by drugs applied intravenously, and finally, they can be used for personalized tissue engineering strategies. To this end it is still necessary to perform further studies in order to expand these cells and to better understand the molecular and cellular mechanisms that regulate the differentiation of VW-SCs under normal and pathological conditions such hypoxia, inflammation and tumor formation (Ergün et al., 2011).

1.6.1.3 Vessel wall-resident macrophage progenitors

Macrophages belong to the innate immune system. With their capacity of phagocytosis of cells or microbes that do not carry receptors on their surface which are specific of healthy body cells they engulf and digest cellular debris and foreign substances (Aderem and Underhill, 1999), (Herwald and Egesten, 2013). Macrophages are present in all tissues in varying numbers. They are derived from monocytes which circulate in the peripheral blood and become macrophages after transmigrating through the vessel wall and infiltrating the target tissues. Recent evidence suggests that macrophages and macrophage-associated processes are involved in several types of heart failure such as dilated cardiomyopathy (DCM), but also play a role in the regeneration of the myocardium. There is growing evidence that macrophage infiltration does not only represent an inflammatory response, but that it is also instrumental for potential repair of the heart tissue. It has been shown that proinflammatory cytokines induce dedifferentiation of cardiomyocytes as judged by morphological reconstruction and up-regulation of the stem cell marker c-Kit (Kubin et al., 2011). However, a prolonged activation and a high number of infiltrating macrophages has been observed in the adult heart, e. g. after MI might counteract the endogenous regenerative process (Santini and Rosenthal, 2012), (Epelman et

al., 2014). The regulative mechanisms controlling macrophage-induced regeneration in the neonatal heart resulting in active regeneration are largely unknown. Intriguingly, besides stem cells differentiating into vascular cells and leading to new vessel formation, VW-SCs also give rise to macrophages independently from circulating monocytes coming from the bone marrow (Zengin et al., 2006), (Psaltis et al., 2012), (Psaltis et al., 2014) as exemplarily shown in Figure 11.

These data raise the possibility that the activation and differentiation of VW-SCs themselves, e. g. by secretion of factors acting on adventitial stem cells in a paracrine manner, are influenced by VW-SC-derived inflammatory cells such as macrophages. Recent studies report that inflammatory pathways induce endogenous reprogramming of somatic cells (Lee et al., 2012) and/or dedifferentiation of mature cells enabling them to either transdifferentiate into other cell types, and/or to contribute to tissue repair by proliferation (Kubin et al., 2011), (Masaki et al., 2013).

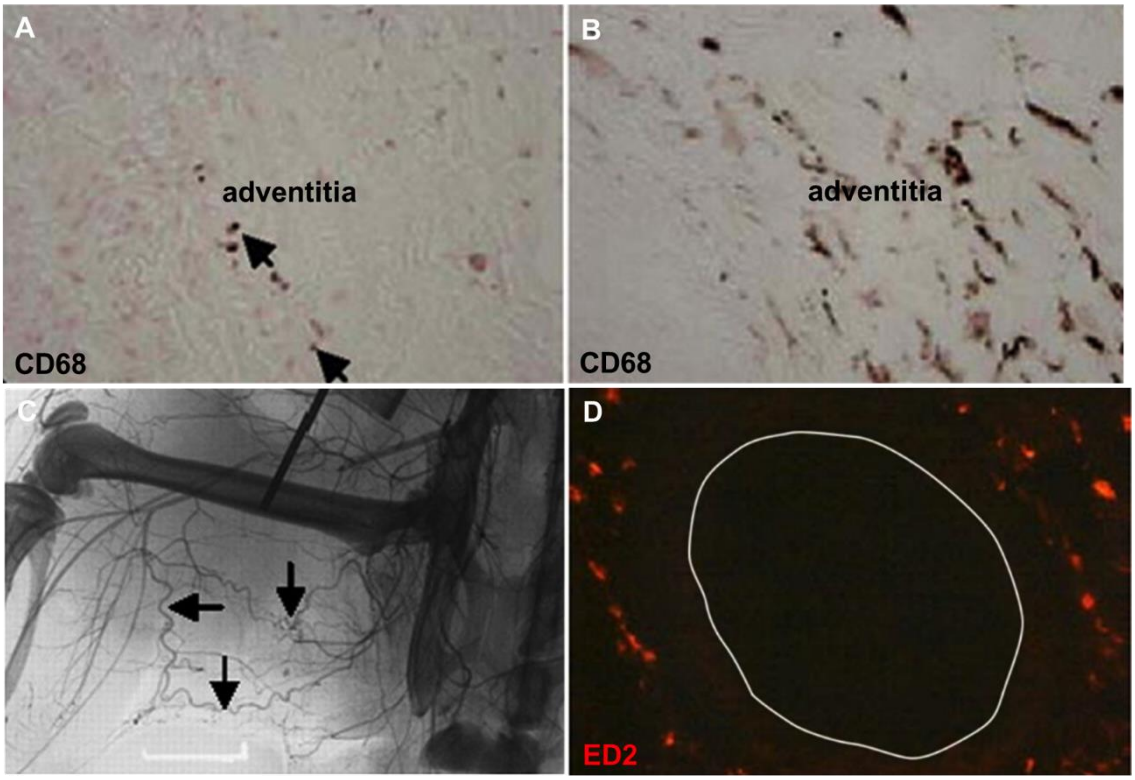

Figure 11. Vascular adventitia-derived macrophages. Generation and localization of few CD68$^+$ (dark staining) macrophages in the adventitia of untreated hITA (A), whereas higher accumulation of CD68$^+$ cells in the hITA-adventitia after ring assay (B). These results were confirmed by *in vivo* studies, in which rat femoral artery ligation and bone marrow cell depletion leads to induced growth of collateral arteries (arrows, C) that revealed a higher accumulation of ED2-positive macrophages (red, D) in the collateral arterial adventitia. Intima marked by white line (Zengin et al., 2006).

1.6.1.4 Mesoangioblasts

Mesoangioblasts are progenitor cells which have been shown to be associated to developing vessels and were originally isolated from the dorsal embryonic aorta (De Angelis et al., 1999), (Minasi et al., 2002). They give rise to vascular cells as well as other mesodermal cell types including skeletal and cardiac muscle cells during embryonic and fetal development (Motoike et al., 2003). The term mesoangioblast is introduced in analogy to the term hemangioblast which has been recognized as the

embryonic ancestor stem cell type delivering both, angioblasts or ECs and HCs (Majesky, 2007). While the exact niche of mesoangioblasts within the vessel wall is still not described, except in studies suggesting their origin from endothelial cells the mesoangioblast might share some features with VW-SCs and finally support the concept that vessel walls provide a niche for a variety of stem cell populations (Zengin et al., 2006), (Klein et al., 2010), (Ergün et al., 2011), (Chong et al., 2014). As already mentioned above, mesoangioblasts were originally isolated from the dorsal embryonic aorta and were shown to be clonogenic, self-renewing, and multipotent (Minasi et al., 2002). Later, mesoangioblasts have been demonstrated in juvenile mouse aorta and heart (Galvez et al., 2008). However, the existence and the potential niche as well as the differentiation fate of mesoangioblasts in adult tissues is largely unknown (Chong et al., 2014), (Tavian et al., 2005). Although, the possibility that mesoangioblasts and HSCs might arise from a common ancestor has been raised, this also remains speculative at the current time (Chong et al., 2014), (Minasi et al., 2002). Moreover, in culture, freshly established mesoangioblast clones consistently express markers of early endothelial lineage commitment such as CD34, VEGFR-2 (Flk1), vascular endothelial (VE)-cadherin, and c-Kit (Minasi et al., 2002), (Cossu and Bianco, 2003). Other studies also confirmed the existence of endothelial progenitor (EPC)-like $CD34^{+}CD31^{-}$ cells in the outer stromal layer of the human embryonic aorta and adult human arteries (Alessandri et al., 2001), (Zengin et al., 2006). Besides, mesoangioblasts appear devoid of hematopoietic activity (Cossu and Bianco, 2003). These findings suggest that mesoangioblasts probably share endothelial progenitor markers. Mesoangioblasts were also isolated from cardiac tissue and were shown to express Sca-1, c-Kit, NG2, CD31, CD34 while negative for

CD45, and they were also reported to express cardiac transcription factors and to differentiate into cardiomyocytes when co-cultured with neonatal rat cardiomyocytes (Galvez et al., 2008), (Barbuti et al., 2010). However, the origin and *in vivo* lineage of descendants of cardiac mesoangioblasts remain to be determined (Chong et al., 2014). Although, mesoangioblasts have been shown to express stem cell markers such as CD34, Flk1, Sca-1, Thy1, and c-Kit (Sampaolesi et al., 2003), more investigation is required to clarify unique markers for mesoangioblasts in adult tissues such as vessel-wall in order to better distinguish them from VW-SCs and to follow their differentiation fate.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and consumables

Chemicals	Supplier
3,3'-diaminobenzidine DAB	Sigma-Aldrich (USA)
Glucose Oxidase	Sigma-Aldrich (USA)
BSA	AppliChem (Germany)
Cell culture flasks, plates, and tubes	Cell Star (Germany)
DMSO	Roth (Germany)
Collagenase type 2	Worthington, Lakewood (USA)
FCS	PAA Laboratories (Germany)
HCL	Roth (Germany)
Hydrogen peroxide	Sigma (USA)
L-Glutamine	Gibco BRL (Germany)
Nuclear Fast Red	Merck (Darmstadt)
Paraformaldehyde	Sigma-Aldrich (USA)
PBS	Sigma-Aldrich (USA)
Penicillin-Streptomycin	Gibco BRL (Germany)
Trypsin/EDTA (1x)	Gibco BRL (Germany)
Normal donkey serum	Sigma-Aldrich (USA)
Normal rabbit serum	Sigma-Aldrich (USA)
Normal goat serum	Sigma-Aldrich (USA)

Ammoniumchlorid	Fluka (USA)
Nickel sulfat	Merck (Germany)
Glucose	AppliChem (Germany)
Rabbit anti-goat biotin	Vector Laboratories (USA)
Goat anti-mouse biotin	Vector Laboratories (USA)
Goat anti-rabbit biotin	Vector Laboratories (USA)
Goat anti-rat biotin	Vector Laboratories (USA)
Depex Mountant	Serva (Germany)
Nuclear fast red solution	Merck (Germany)
DAPI	Roche (Germany)
VECTASTAIN ABC kit	Vector Laboratories (USA)
Poly-L-ornithine hydrobromide	Sigma-Aldrich (USA)
Bovine fibronectin protein	Life technologies (USA)
Tissue tec	Sakura (The Netherlands)
Cell strainer (70µm)	BD Biosciences (USA)
PAP rabbit	Jackson Immuno Research (USA)
PAP mouse	Jackson Immuno Research (USA)
PAP goat	Jackson Immuno Research (USA)

2.1.2 Kits used and the suppliers name

Product	Supplier
mRNA isolation kit	Quiagen (Germany)
QuantiTect Reverse Transcription Kit	Quiagen (Germany)

2.1.3 Cytokines used in the study

Cytokine	Source
E7080	Selleckchem (USA)
VEGF	Sigma-Aldrich (USA)
Clodoronate	Clodronate.Liposomes.com (Netherlands)
PBS liposomes	Clodronate.Liposomes.com (Netherlands)

2.1.4 Stock solutions and buffers

Phosphate Buffer (PB) (0.1M PB, pH 7.4)

KH2HPO4 ……..... 4.9 g

Na2HPO4×2H2O ……..… 29.2 g

Deionized H_2O …… 2000 ml

Adjust the 7.4 pH with HCL /NAOH titration

Citrate Buffer (CB) (10mM, pH 6)

0.1 M Citric acid ……. 18 ml

0.1 M Trisodiumcitrat ……. 82 ml

Deionized H_2O …….. 900 ml

Adjust the 6.0 pH with HCL /NAOH titration

Tris-EDTA Buffer (TEB) (pH 9.0)

10 mM Tris Base ……..…..... 1.2 g

1 mM EDTA ……..…….. 0.37 g

Deionized H_2O ……..... 1000 ml

Adjust the 9.0 pH with HCL /NAOH titration

Ammonium chloride solution

Ammonium chloride …… 18 g

Deionized H_2O ……..…. 100 ml

Nickel sulfate solution

Nickel sulfate ……..…..... 1,3 g

Deionized H_2O ……..…. 100 ml

Nuclear Fast Red

Aluminium sulfate×18 H2O ……..... 50 g

Nuclear Fast Red ……..... 1 g

Deionized H_2O ……..….. 1000 ml

Dissolve with heating, cooling, then filter it.

10% Glucose

D-Glucose-Monohydrate ..... 10 g

Deionized H2O …….. 100 ml

2.1.5 Instruments and suppliers

Instruments	Suppliers
Autoclave	Thermo Scientific (USA)
Centrifuge 5430R	Eppendorf (Germany)
Cell spin II centrifuge	Tharmac (Germany)
FACS CantoII	Beck Dickinson (USA)
Vortex	Süd-Laborbedarf (Germany)
Mini MACS	Miltenyi Biotec (Germany)
5% CO_2 incubator	Binder (USA)
Light Microscope	Carl Zeiss (Germany)
pH Meter	inolab (Germany)
Balance AB104	Mettler Toledo (Switzerland)
Leica SM 2000R Microtome	Leica Microsystems (Germany)
Leica Embedding TP1020 Station	Leica Microsystems (Germany)
Leica CM3050S Cryotome	Leica Microsystems (Germany)
Stereomicroscope Stemi 2000	ZEISS (Jena)
Keyence BZ-9000 Fluorescence Microscope	Keyence (Japan)
Laser confocal microscope (Nikon Eclipse Ti inverted microscope)	Nikon Instruments (Germany)
Real time PCR 7300	Applied Biosystems (Germany)

2.1.6 Primary antibodies

Antibody	Source	Supplier	Catalog Number
Monoclonal Anti-CD34	Rat	Santa Cruz Biotechnology	Sc-18917
Polyclonal Anti-Sca1	Goat	R&D systems	AF1226
Polyclonal Anti-cKit	Rabbit	Biorab	Orb38329
Polyclonal Anti- CD31	Rabbit	Santa Cruz Biotechnology	SC-28188
Monoclonal Rat anti-CD44 antibody	Rat	Biolegend	103001
Monoclonal Anti-Troponin I	Mouse	Millipore	MAB1691
Monoclonal Anti-CD 34	Rat	Abcam	Ab8158
Polyclonal Anti- Connexin 43	Rabbit	Abcam	Ab11370
Monoclonal Anti-Sarcomeric α-Actinin	Mouse	Abcam	Ab9465
Polyclonal Anti-Brachyury	Goat	R and D Systems	Af2085
Polyclonal Anti-Flk1	Goat	Santa Cruz Biotechnology	Sc-48161
Polyclonal Anti-Nkx2.5	Goat	Santa Cruz Biotechnology	Sc-8697
Polyclonal Anti- Isl-1	Rabbit	Biorbyt	Orb100390
Polyclonal Anti-α-smooth muscle Actin	Rabbit	Abcam	ab5694
Monoclonal Anti- F4/80	Rat	Abcam	Ab6640
Monoclonal Anti -MYH7	Mouse	Santa Cruz Biotechnology	Sc-53090
Monoclonal Anti-Ly6c	Rat	Abcam	Ab15627

2.1.7 Secondary antibodies

Antibody	Source	Working Dilution	Supplier
Goat Anti-Mouse IgG, Cy3	Goat	1:800	Jackson immuno research (USA)
Goat Anti-Mouse IgG, Cy5	Goat	1:400	Jackson immuno research (USA)
Goat Anti-Rat IgG, Cy3	Goat	1:800	Jackson immuno research (USA)
Goat Anti-Rat IgG, Cy5	Goat	1:400	Jackson immuno research (USA)
Goat Anti-Rabbit IgG, Cy3	Goat	1:800	Jackson immuno research (USA)
Goat Anti-Rabbit IgG, Cy5	Goat	1:400	Jackson immuno research (USA)
Donkey Anti-Goat IgG, Cy3	Donkey	1:800	Jackson immuno research (USA)
Donkey Anti-Mouse IgG, Cy5	Donkey	1:400	Jackson immuno research (USA)

2.1.8 Medias used in the study

Media	Source
Dulbecco's modification of Eagle's medium	Gibco BRL (Germany)
Modified Endothelial Cell Growth Medium	Promocell (Germany)
RPMI medium	Gibco BRL (Germany)

2.2 Methods

2.2.1 Isolation, purification and differentiation of AoCs

Thoracic aorta of adult C57BL/6 mice (2-4 months old) was dissected after PBS injection into the left ventricle. Subsequently, the thoracic aorta was enzymatically dispersed into a single cell suspension using 0.2% type 2-collagenase (Worthington, Lakewood, USA) at 37°C for 90 minutes. After washing in PBS which contained 5% fetal calf serum (FCS), cellular suspensions were passed through 70 µm pore size filters. Enrichment of Sca-1^+ and Sca-1^- AoCs was achieved by Magnetic Cell Sorting (MACS) (Miltenyi Biotec, Germany) using Sca-1 microbeads according to the manufacturer's instructions. Total aortic wall-derived cells as well as sorted Sca-1^+ and Sca-1^- aortic wall-derived cells were cultured in modified endothelial cell growth medium (mECGM) (PromoCell, Germany) for 10 days. The medium was changed every day. Cells were maintained at 37 °C in humid air with 5% CO_2 (Figure 12).

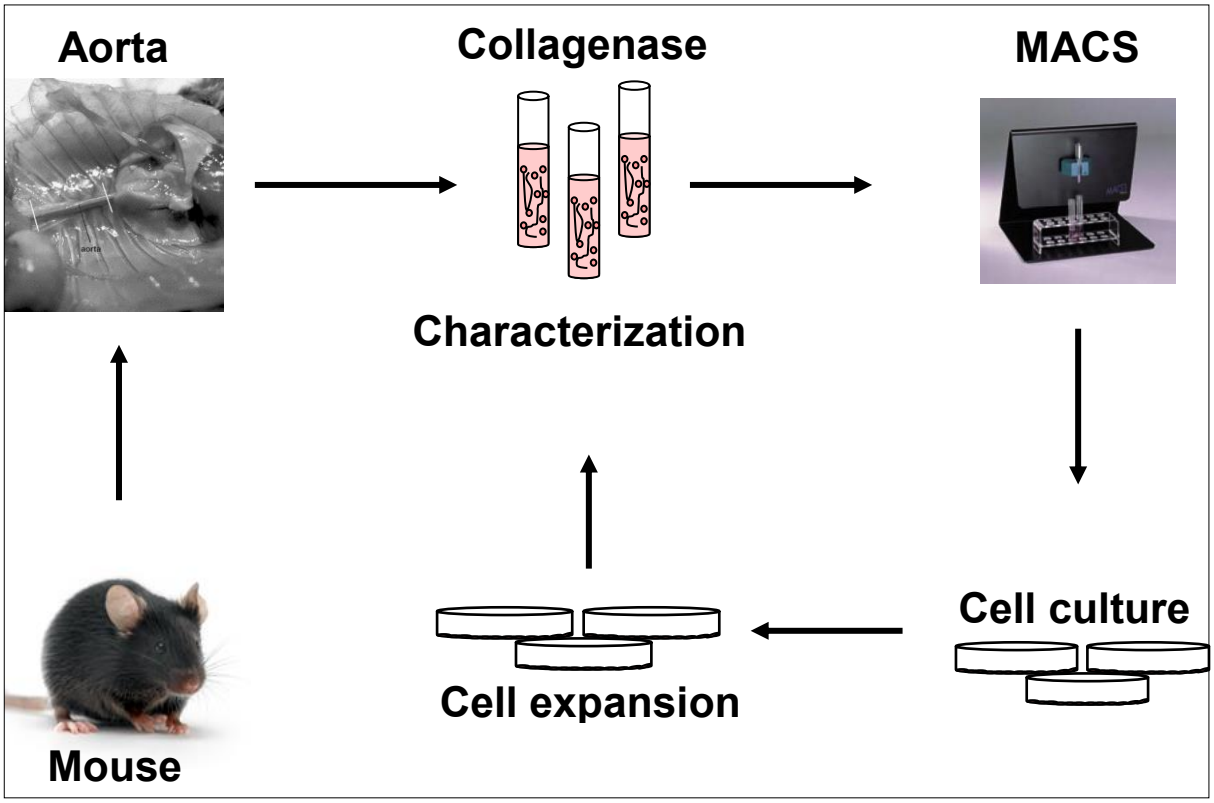

Figure 12. Isolation, purification and differentiation of AoCs. The thoracic aorta was isolated from adult mice and enzymatically dispersed into a single cell suspension using 0.2% type 2-collagenase. Subsequently, cellular suspensions were enriched for Sca-1^{+} and Sca-1^{-} AoCs using Magnetic Cell Sorting (MACS). Further, both sorted cell fractions were plated in cell culture and expanded in mECGM for 10 days and cellular and functional characterization was performed.

2.2.2 Fibronectin coating on glass coverslips

Poly-L-ornithine hydrobromide (Sigma Aldrich, USA) was dissolved in water to reach a final concentration of 15 µg/ml. Next, the solution was sterilized by filtration. This solution was coated on glass coverslips for 2 hours at 37°C or overnight at 4°C. After that, three times washed with DPBS. Subsequently, fibronectin (Life Technologies, USA) was diluted in sterile PBS to reach a final concentration of 1µg/ml. Fibronectin

solution was coated over Poly-L-ornithine hydrobromide coated glass coverslips and stored at 4°C.

2.2.3 Immunohistochemistry

Paraffin embedded tissue sections were deparaffinised with xylene for 20 minutes and rehydrated using descending ethanol concentrations (100%, 96%, 80%, 70%). To recover tissue antigenicity, the sections were incubated for 20 minutes at 95ºC in target retrieval buffers such as citrate buffer (CB) (10mM PB, pH 6) or Tris-EDTA buffer (TEB) (pH 9.0). Next, sections were incubated with blocking solution. For intracellular staining, cells were permeabilized in blocking solution containing 0.1% Triton X-100 for 60 minutes. Afterwards, sections were incubated with the primary antibodies at 4°C overnight. The following antibodies were used: Anti-CD31 (Santa Cruz; 1:50), anti-CD34 (Abcam; 1:50), anti-Flk1 (Santa Cruz; 1:50), anti-CD44 (R&D systems; 1:100), anti-Ly6c (Abcam; 1:50), anti-Isl-1 (Biorbyt; 1:50), anti-c-Kit (Abcam; 1:100), anti-Sca-1 (R&D systems; 1:100). The sections were then exposed to the corresponding biotin-conjugated secondary antibodies (1:250) at room temperature for 1 hour and then processed with corresponding peroxidase anti-peroxidase (PAP) (Jackson Immuno Research; 1:200) and subsequently incubated with avidin-biotin-peroxidase complex (Vector Laboratories; 1:250) for 30 minutes. Finally, sections were treated with Ni-enhanced DAB staining solution (67,5 ml PB Buffer, 1350 µl Nickelsulfat, 1350 µl 10% Glucose, 150 µl Ammonium Chloride [18 mg], 1500 µl DAB [22,5 mg], 225 µl glucose oxidase [1,2 mg/ml]) for 10-30 minutes in dark at room temperature (Klein et al., 2011), (Shu et al., 1988). Then the DAB reaction was stopped by washing the slides with PBS and distilled water and counterstained with Nuclear Fast Red solution (Merck, Germany) for 3 minutes, dehydrated through

increased ethanol concentrations (70%, 80%, 96%, 100%) and finally permanent mounted with depex mountant (Serva, Germany). Negative controls were performed by omitting the primary antibodies. Sections were analyzed and the staining was documented using a Keyence microscope equipped with a digital camera (Keyence BZ-9000, Japan).

2.2.4 Immunocytochemistry

Freshly isolated and/or cultured AoCs were fixed with 4% phosphate-buffered paraformaldehyde solution at room temperature for 30 minutes and permeabilized using 0.3% Triton X-100 for 10 minutes. The cells were then blocked with 5% serum from the species of the secondary antibodies at room temperature for 2 hours. Subsequently, cells were incubated with specific primary and secondary antibodies at room temperature for 1 hour. The following primary antibodies were used: Anti-α-SRA (Abcam; 1:100), anti-F4/80 (Abcam; 1:50), anti-Connexin 43 (Abcam; 1:100), anti-CD31 (Santa Cruz; 1:50), anti-CD34 (Abcam; 1:50), anti-Flk1 (Santa Cruz; 1:50), anti-CD44 (R&D systems; 1:100), anti-Isl-1 (Biorbyt; 1:50), anti-c-Kit (Abcam; 1:100), anti-Sca-1 (R&D systems; 1:100). DAPI was used as a nuclear counterstain. The samples were mounted and imaged using a fluorescence (Keyence BZ-9000, Japan) or laser confocal microscope (Nikon Eclipse Ti inverted microscope, Germany).

2.2.5 Flow Cytometry Analyses

Flow cytometry was used to characterize the immunophenotype of freshly isolated AoCs. First, to discriminate viable cells from apoptotic cells, added 1 µl of the fixable viability stain 450 (BD Biosciences, USA) to cell suspension for 15 minutes and vortexed immediately. After that, the cells were fixed with 4% paraformaldehyde in

PBS. After two washes with PBS, to detect surface antigens, cells were blocked with 5% serum from the species of the secondary antibodies (blocking buffer) at room temperature for 1 hour. Whereas to detect cytoplasmic or nuclear antigens, cells were blocked with 5% serum containing 0.1% Triton X-100 (blocking buffer) for 1 hour. Subsequently, cells were incubated with primary antibodies in blocking buffer at room temperature for 1 hour. The following primary antibodies were used such as anti-CD34 (Abcam; 1:50), anti-Flk1 (Santa Cruz; 1:50), anti-CD44 (R&D systems; 1:100), anti-Isl-1 (Biorbyt; 1:20), anti-c-Kit (Abcam; 1:100), anti-Sca-1 (R&D systems; 1:100) and anti-Ly6c (Abcam; 1:50). After three washes with PBS, cells were incubated with specific secondary antibodies in PBS at room temperature for 45 minutes. Unstained AoCs were used as negative control to subtract auto fluorescence. The samples were measured with BD FACSCanto II flow cytometry (BD Biosciences, USA) and the data were analyzed by FACSDiva software (version 6.1.3) (BD Biosciences, USA).

2.2.6 Gene expression analyses by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cell pellets of AoCs on culture days 0 (freshly isolated) and 10 as well as from adult mouse heart tissue.

2.2.6.1 Isolation of Total RNA

Total RNA was extracted from cell pellets of AoCs using Rneasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. First, lysis buffer was prepared by adding 10 µl β-mercaptoethanol to 990 ul RLT buffer (Qiagen, Germany). After that, 350 µl of lysis buffer was added to cell pellet in 15 ml falcon tube. Then, cell suspension was mixed with 1 ml syringe containing 20 G needle and

added 350 µl 70% ethanol to sample in the 15 ml falcon tube. Next, 700 µl of the sample was transferred into mini spin column and kept for centrifugation at 10,000 rpm for 15 seconds, and then discarded flow through. After that, 700 µl of RWI (Qiagen, Germany) (wash buffer) was added to the sample in spin column and centrifuged at 10,000 rpm for 15 seconds, and then discarded flow through. Next, 500 µl of RPE (Qiagen, Germany) was added to the sample in spin column and centrifuged at 10,000 rpm for 15 seconds, and then discarded flow through. After that 500 µl of RPE was added again to the sample in spin column and centrifuged at 10,000 rpm for 2 minutes, and then discarded flow through. Next, empty column was centrifuged with new 2 ml collection tube to remove extra buffers at 13000 rpm for 1 minute. Finally, column was placed in 1.5 ml sterile Eppendorf tube and added 25 µl RNAse free water and kept for centrifugation 10,000 rpm for 1 minute. After that, the RNA was stored at -80°C.

2.2.6.2 Determination of RNA Quality and Concentration

The quality and concentration of RNA was determined by NanoDrop (Infinite® 200 PRO NanoQuant, Switzerland). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA. A ratio of ~ 2.0 was accepted pure for RNA. If the ratio is lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. For nucleic acid quantification, a modification of the Beer-Lambert equation is used to calculate sample concentration ($C = (A * \varepsilon)/b$), where C is nucleic acid concentration in ng/µl, A is absorbance in AU, ε is the wavelength dependent extinction coefficient in ng-cm/µl and b is the path length in cm.

2.2.6.3 cDNA synthesis

50 ng of RNA was converted into cDNA using Quanti Tect Reverse Transcription kit (Qiagen, Germany). First, template RNA was thawed on ice. Whereas, gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water was thawed at room temperature (15–25°C). Next, the genomic DNA elimination reaction was prepared on ice according to Table 1.

Table 1. Genomic DNA elimination reaction components

Components	Volume (µL)
gDNA wipeout buffer (7x)	2
RNA	Variable (10pg-1µg)
RNAse free water	Variable
Mix1	14

Next, 14 µL of genomic DNA elimination reaction components (Mix1) contents were incubated for 2 min at 42°C and then placed immediately on ice. After that, reverse-transcription master mix was prepared on the ice according to Table 2.

Table 2. Reverse-transcription reaction components

Components	Volume (µL)
Quant script reverse transcriptase	1
Quant script RT buffer (5x)	4
RT-Primer Mix	1
Mix2	6

Finally, genomic DNA elimination reaction components (Mix1) from table 1 (14 µl) to reverse transcription reaction components (Mix 2) from table 2 (6 µl) were mixed to prepare a final volume of 20 µl and were incubated for 15 minutes at 42°C and 3 minutes at 95°C using thermocycler. The cDNA was stored at -80°C.

2.2.6.4 Real-time PCR (qRT-PCR)

Real time PCR (qRT-PCR) was used to amplify cDNA products to study and compare gene expression between groups. In the real-time quantitative TaqMan® assay, a fluorogenic non-expandable probe (TaqMan probe) has been used. The probe has a fluorescent reporter dye attached to its 5' end and a quencher dye at its 3' terminus. If the target sequence is present, the fluorogenic probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of the Taq polymerase enzyme during the extension phase of the PCR. During the extension phase, the 5' endonuclease activity of the Taq DNA polymerase cleaves the probe which separates reporter and quencher dyes and fluorescence is detected (Arya et al., 2005). Real-time PCR record fluorescence and report the results as a Ct (cycle threshold). The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold. This threshold is a fluorescence value slightly above the background fluorescence measured before exponential growth starts. Calculated each component needed for each gene and each reaction using the following Table 3.

Table 3. qRT-PCR reaction Mix (For 1x reaction)

Components	Volume (µL)
2x universal master mix (UMM)	12.50
TaqMan Gene Expression Assay (Primer and Probe)	1.25
cDNA (200ng)	4.0
Water	9.25
Total volume	25.0

First water was added to a 96 well PCR plate. After that, 2x universal master mix was added into 96 well PCR plate. Then primer and probes for the TaqMan® Gene Expression Assay were added into 96 well PCR plate. The following pre-designed TaqMan® gene expression assays (Life Technologies, USA) were used: 18s rRNA (Mm03928990_g1), Isl-1 (Mm00517585_m1), troponin I (Mm00437164_m1), HCN4 (Mm01176086_m1), ACTC1 (Mm01333821_m1) and MEF2C (Mm01340842_m1). Finally, added cDNA into 96 well PCR plate. The mix was spun down by centrifugation at 10,000 RPM for 1 minute. The PCR conditions are given in Table 4.

Table 4. qPCR conditions

Step	UNG incubation	AmpliTaq Gold activation	PCR	
	HOLD	HOLD	Cycle(40 cycles)	
			Denature	Anneal
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	25 µl			

Next, 96 well PCR plate with reaction contents were loaded on the qPCR machine (Applied Biosystems) to run the reaction and acquire the data. Relative changes in the gene expression profile were normalized to 18S rRNA gene expression levels. The quantification was performed according to the manufacturer's instructions (Applied Biosystems) as described in the following website http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf, [delta][delta]C_t=[delta]$C_{t,sample}$-[delta]$C_{t,reference}$Fold change = 2[delta][delta]C_t.

2.2.7 Transmission electron microscopy (TEM)

The transmission electron microscopy (TEM) operates on the same basic principles as the light microscopy, but uses electrons instead of light. TEM uses electrons as

"light source" and their much lower wavelength makes it possible to get a resolution a thousand times better than with a light microscopy.

Cells were collected using sterile cell scraper after washing with PBS. After that, cells were fixed in 4.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 (PB). After washing with 0.1 M PB, specimens were fixed with 1% osmiumtetroxide in PB for 1 hour and washed with water. Specimens were then dehydrated in ascending concentrations of ethanol including en-bloc contrasting using 2% uranylacetate in 70% ethanol for 1 hour. Subsequently, they were embedded in Epon812 and used for preparation of ultrathin sections, which were post-stained with 2% uranylacetate and 0.2% lead citrate. The sections were observed using a LEO AB 912 transmission electron microscope (Zeiss NTS, Oberkochen, Germany).

2.2.8 Patch clamp Electrophysiology

The electric activity of cells is controlled by transmembrane pores called ionic channels. With the patch-clamp technology the functions of the ion channels in cell membranes can be studied. Erwin Neher and Bert Sakmann developed the patch clamp in the year 1976, and for this work they received Nobel Prize in physiology of medicine in 1991.

Whole-cell recordings were performed at room temperature in a bath solution consisting of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM glucose, pH 7.4. Patch pipettes were pulled from borosilicate glass capillaries (Kimble Products, England), and heat-polished to give input resistances of 3-7 MΩ (whole-cell). The pipette recording solution contained 140 mM KCl, 2 mM $MgCl_2$, 0.01 mM $CaCl_2$ mM ethylenebis(oxyethylenenitrilo) tetraacetate (EGTA), 1 mM Na_2ATP, 0.1 mM cyclic AMP, 0.1 mM ATP, and 5 mM HEPES (pH 7.3).

Currents were recorded with an EPC9 (Heka) patch clamp amplifier and low pass-filtered at 1-2 kHz. Stimulation and data acquisition were controlled by the PULSE/PULSEFIT software package (Heka) on a Macintosh computer, and data analysis was performed with IGOR software (WaveMetrics, Lake Oswego, USA).

2.2.9 Calcium imaging

A large number of chemical indicators that are fluorescent probes have been developed to measure intracellular levels of Ca^{2+}. The green-fluorescent calcium indicator Fluo-4 is an improved version of the calcium indicator Fluo-3. In this study, intracellular Ca^{2+} was monitored using the fluorescent probe Fluo-4AM (acetoxymethyl ester of Fluo-4). Fluo-4AM is a membrane-permeable dye. After it is taken up by cells, the ester is hydrolyzed by an esterase in the cytoplasm to release the free acid (hydrolyzed) from the dye (Fluo-4), which is sensitive to calcium. Fluo-4 excited at 488 nm, provides brighter emission signals in response to Ca^{2+}-binding (Guatimosim et al., 2011).

Calcium imaging was performed in cell cultures loaded with 5 μM of the calcium indicator dye Fluo-4 AM (Life technologies, USA). Briefly, the cells were cultured in a 48-well plate and incubated with 1 ml of loading dye solution containing Fluo-4 AM in the dark at room temperature for 20 minutes. Movies were recorded using a Keyence microscope equipped with a digital camera (Keyence BZ-9000, Japan).

2.2.10 Pharmacological response of the AobCMs

The adrenergic receptors of the myocardium play an important role in the regulation of heart function. The adrenergic receptors belong to the family of G-protein coupled receptors. Three subtypes have been distinguished (β1, β2, and β3 adrenoceptors) (Wallukat, 2002).

For pharmacological treatment of the AobCMs, the baseline contraction rate was recorded before addition of adrenergic reagents. Chronotropic responses were recorded after addition of isoproterenol (6μM), a β-adrenergic agonist (Sigma-Aldrich, USA), alone or preceded by propranolol (30μM), a non-selective β-antagonist (Sigma-Aldrich, USA). Movies were recorded using a Keyence microscope equipped with a digital camera (Keyence BZ-9000, Japan).

2.2.11 Video recording

Beating cell movies were recorded with an inverted-type phase contrast live microscope (Keyence BZ-9000, Japan).

2.2.12 Macrophage depletion in AoCs

AoCs were maintained under standard cell culture conditions until day 4. After that AoCs were treated with liposomes containing either clodronate (0.45 mg/ml) or PBS (0.45 mg/ml) as a control (Clodronate.Liposomes.com, Haarlem, Netherlands) on culture days 5, 7, and 9. Subsequently, cultures were maintained under standard cell culture conditions until day 17. Immunofluorescence stainings for F4/80 were performed to evaluate the efficiency of macrophage depletion. To evaluate the effect of macrophages on the generation of Ao-bCMs, immunofluorescence stainings for α-SRA were performed and movies were recorded using a Keyence microscope (Keyence BZ-9000, Japan).

2.2.13 VEGF treatment of AoCs

AoCs were maintained under standard cell culture conditions until day 3. After that, AoCs were treated with VEGF (50 ng/ml) or the VEGFR-2 inhibitor E7080 (10 µM) from culture days 4 to 11. Lenvatinib (E7080) was obtained from Selleckchem (Boston, USA). The medium was changed every day. To evaluate the effect of VEGF treatment on the generation of Ao-bCMs, double immunofluorescence stainings were performed for CD34 and α-SRA, and movies were recorded using a Keyence microscope (Keyence BZ-9000, Japan).

2.2.14 Chick/mouse chimeric assay

Fresh fertilized eggs (VALO BioMedia GmbH, Germany) were placed in a forced-draft incubator (BSS160, Ehret, Germany) at 37.5°C with 60% humidity. After incubation for 3 days, cracked and opened the eggs, and transferred the entire content of each egg into the petri dish (100x20 mm; Greiner Bio-One GmbH, Germany) and placed them into CO_2 incubator (BINDER GmbH, Tuttlingen, Germany) at 37.5°C with about 60% humidity. After incubation for another 2 days, the embryos were used for transplantation.

A total of 1-2 x 10^3 AoCs labelled with CFDA dye (in 10 µl PBS) were injected intracardially into chick embryos at development stage 16-17 (Yamashita et al., 2000). PBS as a control was injected intracardially into chick embryos. Embryos were sacrificed 2-4 days after injection and fixed with 4% paraformaldehyde at 4°C for 1-2 hours. Afterwards, embryos were washed in PBS and transferred first to 10% then to 20% sucrose in PBS at 4°C. Finally, embryos were frozen in Tissue-Tek

(Sakura, Netherlands), cryosectioned at 6-7 μm and used for immunostaining of Flk1, Sca-1, Connexin 43, and α-SRA.

2.2.15 Generation of Flk1::myr-mCherry transgenic (Tg) mice

The mCherry fluorescent protein fused to a myristoylation motif (myr) and then SV40 polyadenylation site was cloned between Flk1 regulatory elements. The myristoylation motif enables for membrane localization of the fluorescent protein (Figure 13).

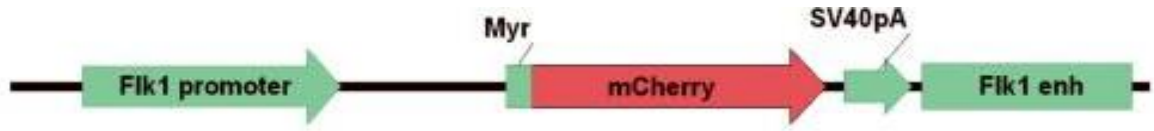

Figure 13. The Restriction map diagram for Flk1::myrm-Cherry construct that was used for establishment of Tg mouse line (Larina et al., 2009).

2.2.16 Statistics

Paired groups were assessed for normal distribution (ND) using the STATISTICA 08 quantile plot test. Error bars indicate standard deviations and the statistics were performed using either a paired Student's *t*-test (ND) or a Wilcoxon test in the case of a non-ND.

3 Results

3.1 Morphological characterization of AoCs and Ao-bCMs

Freshly isolated mouse aortic wall-derived cells (AoCs) were cultured in a modified endothelial cell growth medium (mECGM), and the formation of colony-like structures was observed on culture days 6-11. Surprisingly, spontaneously beating cells appeared within these colonies on culture days 8-10 (Figure 14 a-b, and Movie 1). Then, AoCs were cultured in DMEM, RPMI, and mECGM. However, beating cells, which were termed Ao-bCMs (aortic wall-derived beating cardiomyocytes), were found only in mECGM after 10 days of culture. Next, the Sca-1 microbeads were applied to the AoCs. Sca-1 has been suggested as a marker for cardiac progenitors and Sca-1^{+} progenitors were described within the vascular adventitia including the one of adult mouse heart vessels (Matsuura et al., 2004), (Psaltis et al., 2012). After culturing the Sca-1^{-} and Sca-1^{+} fraction of AoCs in mECGM for 10 days, cell colonies containing Ao-bCMs were only found in the Sca-1^{-} fraction (Figure 14c-f and Movie 2).

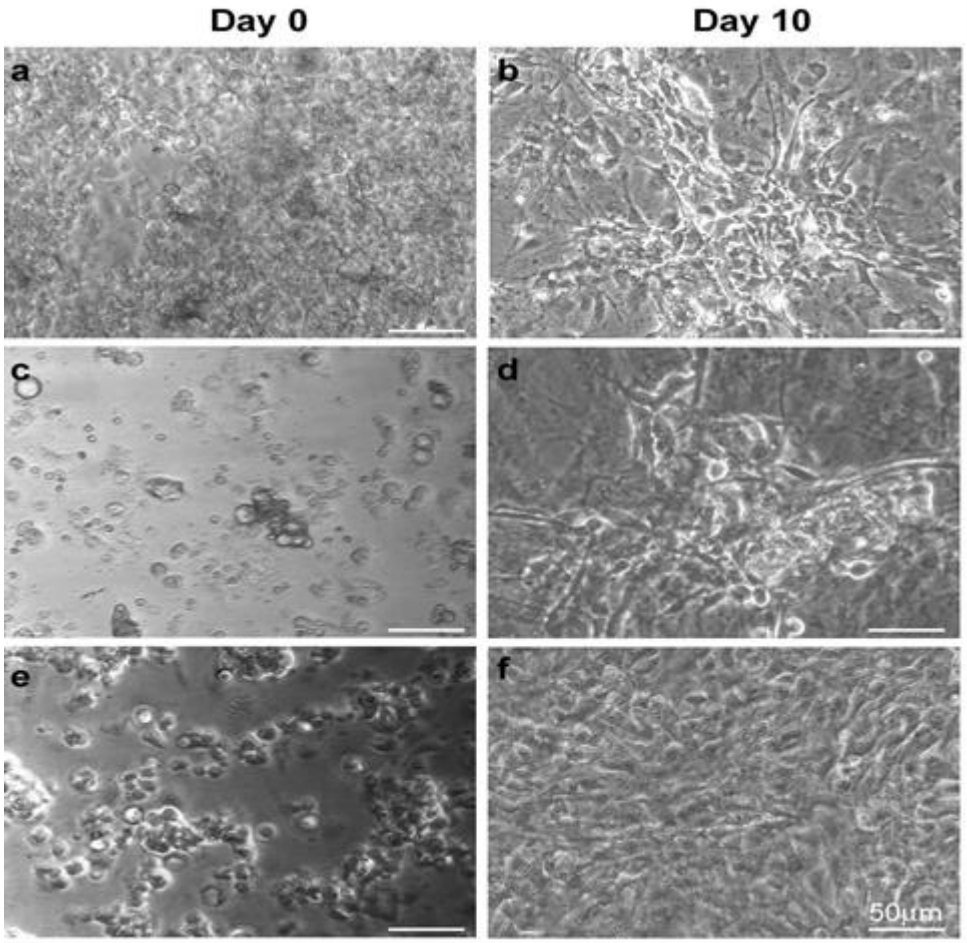

Figure 14. Morphological characterization of AoCs and Ao-bCMs. a, b) Phase contrast images of total AoCs on culture days 0 and 10. Spindle-shaped colonies containing beating cells (see Movie 1). c, d) Sca-1$^-$ AoCs: irregular morphology on day 0 and spindle-shaped colonies containing beating cells on culture day 10 (see Movie 2). e, f) Sca-1$^+$ AoCs: round shaped cells on culture day 0, absence of colony formation and beating cells on culture day 10.

3.2 Localization of Ao-bCM progenitors within the vessel wall

Next, in order to identify the exact localization of potential Ao-bCM progenitors within the vessel wall immunostainings for CD34, CD31, CD44, Flk1, Sca-1, c-Kit, and Isl-1 were performed on tissue sections of adult mouse aorta. As expected, CD31 staining was only detectable in mature endothelial cells lining the aortic lumen (Figure 15).

Besides confirming the previously shown localization of $CD34^+$, $CD44^+$, $Sca\text{-}1^+$, and $Flk1^+$ cells within the adult vascular adventitia (Klein et al., 2013), (Passman et al., 2008), (Zengin et al., 2006), (Psaltis et al., 2014) (Figure 15), $c\text{-}Kit^+$ cells were found within the aortic adventitia in a considerable number. In addition, few single cells expressing Isl-1 and Ly6c (Figure 15), which serves as monocyte progenitor marker, were also detected.

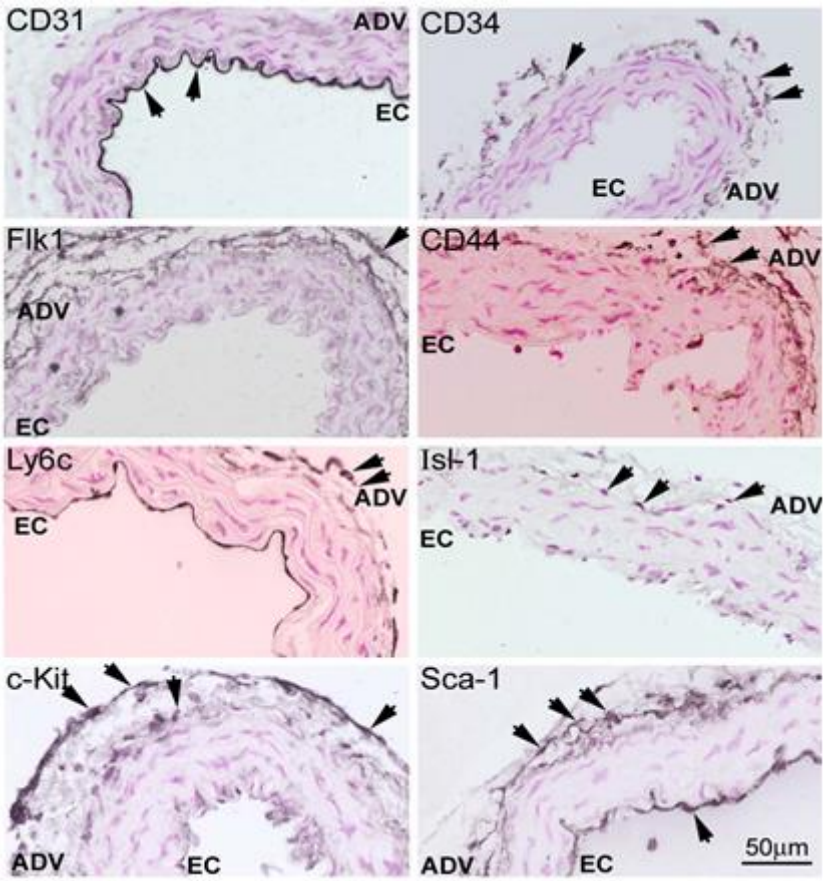

Figure 15. Immunohistological staining for different stem and progenitor cell markers on paraffin sections of freshly isolated mouse aorta.

3.3 Flow cytometric characterization of AoCs

Immunohistological staining results were further confirmed by flow cytometric analyses performed on total AoCs to study the expression of cardiac progenitor

markers such as Flk1, Isl-1(Moretti et al., 2006), c-Kit (Wu et al., 2006), and Sca-1 (Matsuura et al., 2004), and VW-SC markers such as CD34 (Zengin et al., 2006), and CD44 (Klein et al., 2011) as well as marker for monocyte progenitors such as Ly6c. The results are depicted in Figure 16 and give representative histograms of the FACS analysis. Total AoCs showed higher expression for Sca-1 (34.6%), Flk1 (16.3%) and Ly6c (12.5%). However, these cells showed moderate or lower expression for CD34 (4.2%), c-Kit (1.9%), CD44 (1.5%).

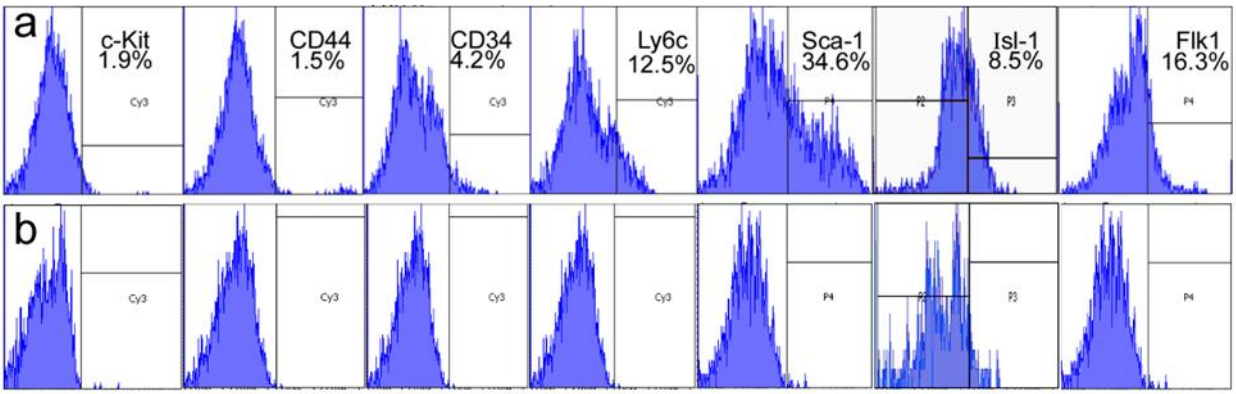

Figure 16. Flow cytometric characterization of AoCs. a) FACS analyses of total AoCs for c-Kit, CD44, CD34, Ly6c, Sca-1, Isl-1, Flk1. b) Controls (quantification is based on n=3).

3.4 Immunocytochemistry of Sca-1$^-$ and Sca-1$^+$ AoCs

Immunocytochemistry of Sca-1$^-$ and Sca-1$^+$ AoCs also revealed the presence of CD31$^+$, Brachyury$^+$, and Flk1$^+$ cells in both fractions, while only the Sca-1$^-$ fraction contained c-Kit$^+$ and Isl-1$^+$ cells (Figure 17a). Some of the Sca1$^-$ cells were also positive for both, Flk1 and Isl-1 (Figure 17b). These data show that the Sca1$^-$ fraction of AoCs preferentially harbours cells exhibiting well-acknowledged cardiogenic progenitor markers (Valente et al., 2014), (Beltrami et al., 2003), (Chong et al., 2011). Of note, the contribution of Sca1$^+$ and c-Kit$^+$ cells was already investigated in two

clinical trials (Bolli et al., 2011), (Makkar et al., 2012). The overall conclusion was that despite some moderate improvement of cardiac function both cell entities did not markedly contribute to CM generation (Valente et al., 2014).

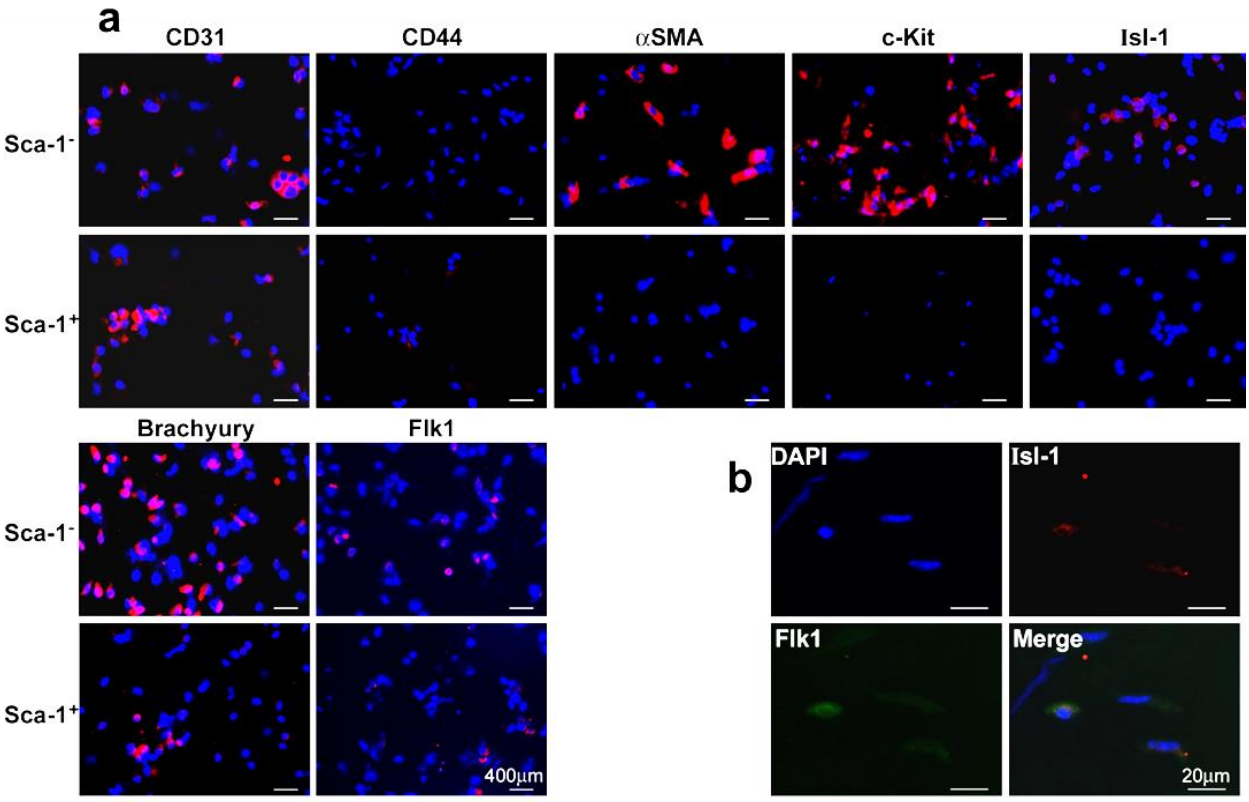

Figure 17. Immunocytochemistry of Sca-1$^-$ and Sca-1$^+$ fractions of AoCs prepared by cytospin. Sca-1$^-$ and Sca-1$^+$ AoCs in both fractions show the presence of CD31$^+$, Brachyury$^+$ and Flk1$^+$ cells, whereas only the Sca-1$^-$ fraction contained c-Kit$^+$ and Isl-1$^+$ cells **(a)**. Some of the Sca1$^-$ AoCs were double positive for Flk1 and Isl-1 **(b)**. Blue colour indicates nuclear staining with DAPI.

3.5 Immunocytochemistry and ultrastructural characterization of Ao-bCMs.

In order to further characterize the cardiomyocyte-like properties of Ao-bCMs, immunocytochemistry was performed for α-sarcomeric actinin (α-SRA), a major component of the sarcomeric Z-line. Imaging of the beating cells by combining video

recording (Movie 3), phase contrast and fluorescence microscopy revealed a clear cross-striated pattern of the α-SRA staining (Figure 18a) indicating a sarcomeric microfilament organization. Indeed, electron microscopic analysis showed typical Z-lines as well as A- and I-bands in $Sca1^-$ Ao-bCMs (Figure 18b, c). These structures were not observed in both freshly isolated $Sca1^+$ and $Sca1^-$ AoCs (Figure 18d) as well as in $Sca1^+$ AoCs on culture day 10 (data not shown). Overall, the Ao-bCMs displayed more mature sarcomeric structures than cardiomyocytes derived from iPS-cells (Kadari et al., 2015). Preliminary studies revealed that regardless of vessel size existence of cardiogenic VW-SCs can be assumed. To better characterize the cardiac phenotype and cellular origin of the Ao-bCMs, immunocytochemistry was performed for CD44, CD34, α-smooth muscle actin (αSMA), c-Kit, connexin 43, and α-SRA. A subset of $α\text{-}SRA^+$ Ao-bCMs exhibited staining for connexin 43, an important gap junctional protein in the myocardial syncytium. In addition, some of the $α\text{-}SRA^-$ cells were positive for connexin 43 (Figure 18e). These data indicate a potential gap junctional communication not only between Ao-bCMs, but also between Ao-bCMs and surrounding supportive cells. In this context, no particular accumulation of $αSMA^+$ and $CD44^+$ cells was found in Ao-bCM containing colonies (Figure 18f, g). In contrast, a strong accumulation of $c\text{-}Kit^+$ cells was observed in Ao-bCM containing clusters (Figure 18h), but c-Kit staining was not present in $α\text{-}SRA^+$ Ao-bCMs. However, a few Ao-bCMs with an immature, non-striated α-SRA staining pattern were also positive for CD34 (Figure 18i). This result indicates that $CD34^+$ AoCs might either give rise to Ao-bCMs or that CD34 is transiently expressed at a certain stage of Ao-bCM differentiation since fully differentiated mature Ao-bCMs

were negative for CD34 (Figure 18i). Furthermore, an immature non-striated α-SRA$^+$ AoCs were positive for Flk1 (Figure 18j).

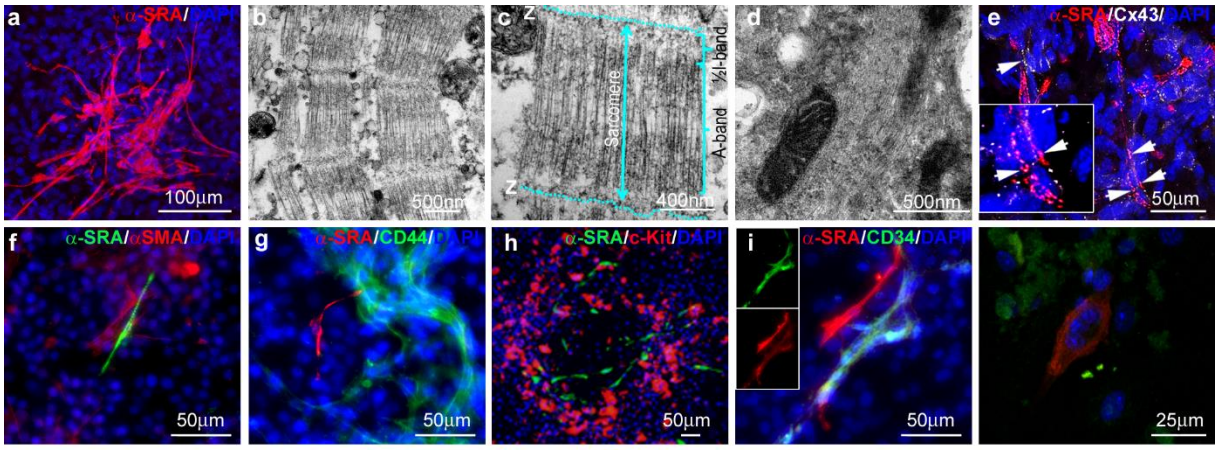

Figure 18. Immunocytochemical and ultrastructural analyses of Ao-bCMs. a) Immunocytochemical staining for α-sarcomeric actinin (α-SRA) of Ao-bCMs on culture day 10 (also see Movie 3). b, c) Sarcomeric structures with typical Z-lines, A- and I-bands in Ao-bCMs as imaged by electron microscopy. Absence of sarcomeric structures in Sca-1$^+$ AoCs (d). e) Connexin 43 staining (white) in a subset of α-SRA$^+$ (red) Ao-bCMs. f) Absence of co-staining for αSMA (red) in α-SRA$^+$ Ao-bCMs (green). g) Absence of co-staining for CD44 (green) in α-SRA$^+$ Ao-bCMs (red). h) Absence of co-staining for c-Kit (red) in α-SRA$^+$ Ao-bCMs (green) *vs.* co-staining for CD34 (green) in few immature α-SRA$^+$ cells (red) (i). j) Co-staining for Flk1 (red) in α-SRA$^+$ Ao-bCMs (green). Blue colour: nuclear staining with DAPI.

3.6 Real time gene expression analyses of Sca-1$^-$ and Sca-1$^+$ AoCs cultures

Next, the gene expression profile of factors typical of the developing heart including MEF2C, an early transcription factor of cardiac myogenesis (Lin et al., 1997), Isl-1 as a transcription factor, and a progenitor marker of the developing secondary heart field (Cai et al., 2003), and HCN4, which is required for the generation of pacemaker action potentials in the embryonic heart (Stieber et al., 2003) was studied. In addition, mature cardiomyocyte markers such as troponin I (Jumabay et al., 2010) and

cardiac-actin (ACTC1) (Sandstedt et al., 2014) were analysed. All three embryonic heart markers were detected in Sca-1$^-$ on day 0 at significantly higher levels than on day 10 (Figure 19a). In contrast, troponin I was significantly up-regulated only in Sca-1$^-$ AoCs on culture day 10 compared to culture day 0 (Figure 19a). Furthermore, an increased ACTC1 expression in Sca-1$^-$ AoCs was only observed on culture day 10 (Figure. 19a).

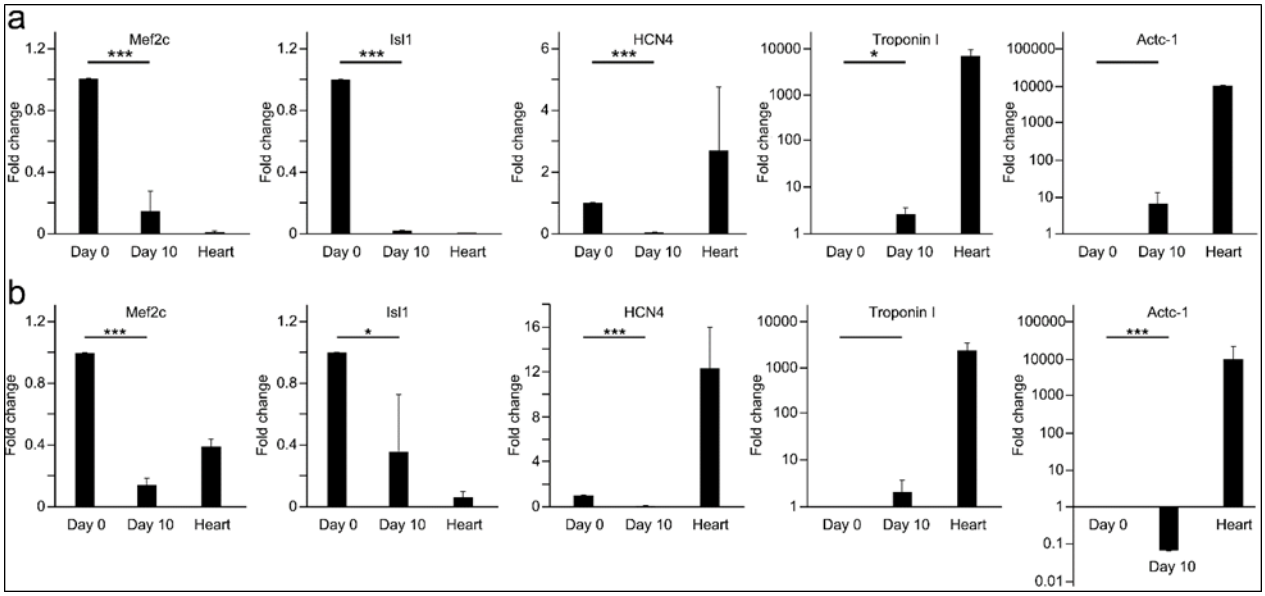

Figure 19. Relative gene expression analyses of early and mature cardiomyocyte markers in Sca-1$^-$ AoCs on culture day 0 *vs.* 10 (a) and Sca-1$^+$ AoCs on culture days 0 and 10 (b). Adult mouse heart tissue is used as a control. The gene expression levels in both, Sca-1$^-$ and Sca-1$^+$ AoCs, on day 0 are set to 1. The data are presented as means ± SD and are from three independent experiments.*p< 0.05, ** p<0.01, and *** p<0.001.

Similarly, all three embryonic heart markers such as MEF2C, Isl-1, and HCN4 were detected in Sca-1$^+$ on day 0 at significantly higher levels than on day 10 (Figure 19b). Moreover, troponin I was up-regulated in Sca-1$^-$ AoCs on culture day 10 compared to culture day 0 (Figure 19b). In contrast, significantly down regulated ACTC1 expression was observed in Sca-1$^+$ AoCs on culture day 10 compared to culture

day 0 (Figure 19b). Taken together, cardiac mature genes such as troponin I and ACTC1 (α-cardiac actinin) were highly upregulated in Sca-1$^-$ AoCs compared to Sca-1$^+$ AoCs on culture day 10.

3.7 Functional characterization of Ao-bCMs

To functionally characterize Ao-bCMs calcium oscillation was imaged, since Ca^{2+} is essentially needed to mediate electro-mechanical coupling and thereby the contractility of cardiomyocytes. Only Ao-bCMs exhibited fluorescence intensity sparks on culture day 10 as measured by the fluorescent Ca^{2+} indicator Fluo-4 AM (Figure 20a, Movie 5).

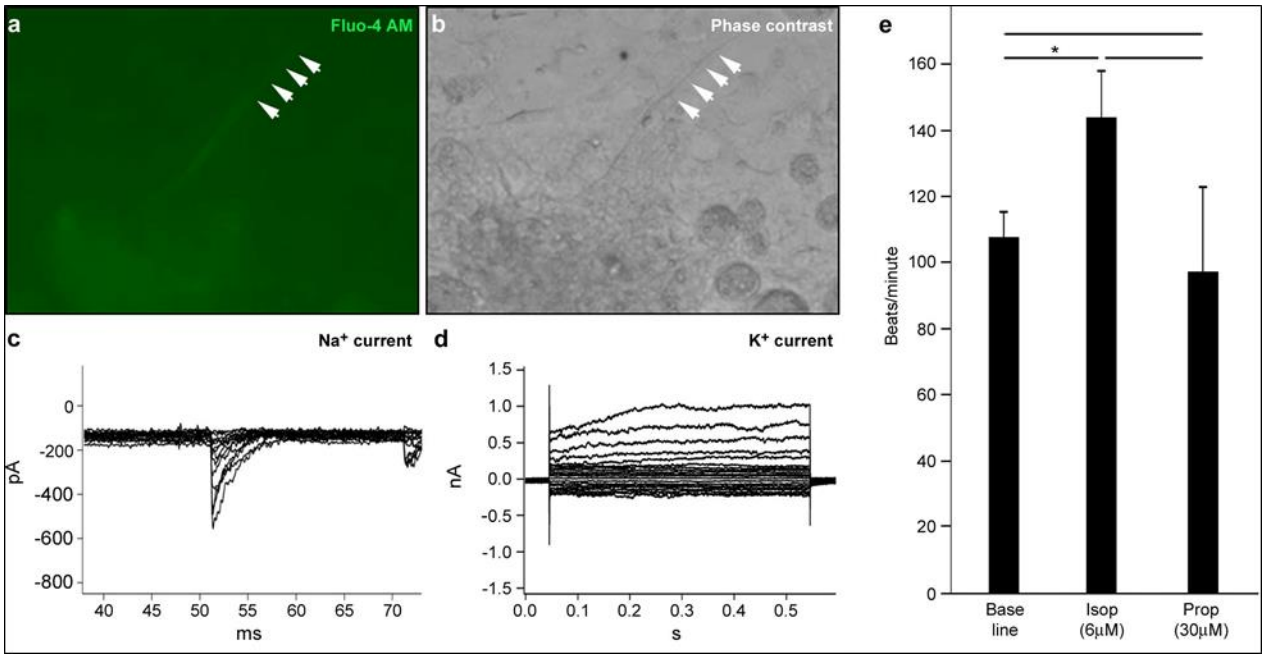

Figure 20. Functional characterization of Ao-bCMs. a, b) Fluorescence (a) and phase contrast images (b) (see also Movie 5 and 6). Beating cell showing calcium-dependent fluo-4 AM fluorescence (white arrows). c, d) Voltage-clamp recordings from -100 to +60 mV with typical voltage-activated Na^+ inward (c) and delayed rectifying K^+ outward currents (d) at a holding potential of -70 mV. e) Beating rate of Ao-bCMs under treatment with the β-adrenergic agonist, isoproterenol (Isop) (see also Movie 8), and the β-adrenergic antagonist propranolol (Prop) (see also Movie 9).

These sparks were limited to beating cells within the culture (Figure 20b, Movie 6). Next, electrophysiological properties of mouse Ao-bCMs were evaluated by measuring Na^+ and K^+ currents via patch clamp. Ao-bCMs showed characteristic voltage-dependent inward Na^+ and outward K^+ currents (Figure 20c, d). Furthermore, the receptiveness of Ao-bCMs for β-adrenergic drugs was examined. First, the baseline contraction rate was recorded in Ao-bCMs (Movie 7). Then, adrenergic pharmacological reagents were added to the culture. As expected, the application of the β-adrenergic agonist isoproterenol significantly increased the contractility rate of Ao-bCMs (Movie 8). Pre-incubation with propranolol, a non-selective β-adrenergic antagonist, reversed the acceleration induced by isoproterenol (Movie 9). Therefore, as in mature cardiomyocytes the beating properties of Ao-bCMs were modifiable by β-adrenergic pharmaceuticals indicating the presence of β-adrenergic receptors (Figure 20e). These results suggest that Ao-bCMs also have functional characteristics of cardiomyocytes.

3.8 Macrophage depletion in AoCs

Due to the strong accumulation of $F4/80^+$ macrophages in AoC colonies containing beating cells it was obvious to explore whether the aortic adventitia-derived macrophages play a role in generation of AoC-derived cardiomyocytes. Indeed, inflammatory pathways have recently been reported to induce endogenous reprogramming of somatic cells (Lee et al., 2012). Neonatal heart regeneration was shown to be accompanied by inflammation (Porrello et al., 2011) and dependent on macrophages (Aurora et al., 2014). Moreover, human and mouse vessel wall-derived stem cells were reported to differentiate into macrophages (Zengin et al., 2006), (Psaltis et al., 2014). Morphological imaging after macrophage depletion

demonstrated less confluence of AoCs in clodronate treated AoCS on day 11 when compared to those treated with PBS liposome-treated and untreated AoCs on day 11 (Figure 21). As mentioned above, immunocytochemical studies revealed strong accumulation of F4/80$^+$ cells within the Ao-bCM containing cell clusters (Figure 22a-d). After treatment AoC cultures with clodronate liposomes according to previously published protocol (Junt et al., 2007) for 11 days the majority of aortic adventitia-derived macrophages were depleted and only few F4/80$^+$ cells remained or were completely absent (Figure 22a). Interestingly, no beating cell clusters were observed after clodronate treatment. Immunofluorescence analyses confirmed that α-SRA$^+$ cells were only rarely present in clodronate-treated cultures. In contrast, F4/80$^+$ cells along with α-SRA$^+$ cells were always present in PBS (phosphate-buffered saline) liposome-treated and untreated cultures (Figure 22b, c). Moreover, the number of F4/80$^+$ cells was significantly higher in untreated and PBS liposome-treated cultures compared to clodronate-treated cultures (Figure 22d). These data indicate a crucial role of factors released by adventitia-derived macrophages for the differentiation of aortic VW-SCs into Ao-bCMs.

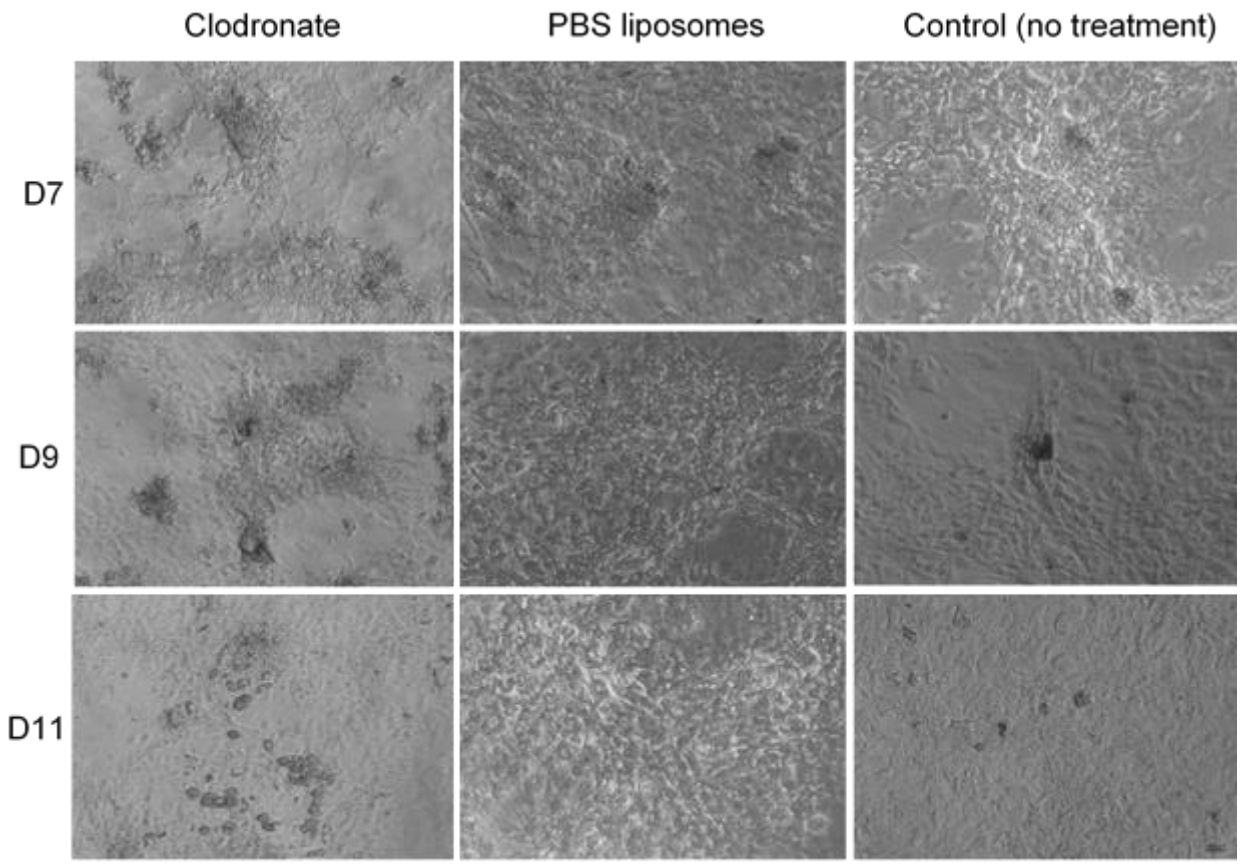

Figure 21. *In vitro* macrophage depletion in AoCs. Phase contrast images showing morphological features after treatment with clodronate liposomes, PBS liposomes, and untreated AoCs on days 7, 9, and 11. Less cell confluence of was observed in clodronate treated AoCS on day 11 when compared to those treated with PBS liposome-treated and untreated AoCs on day 11.

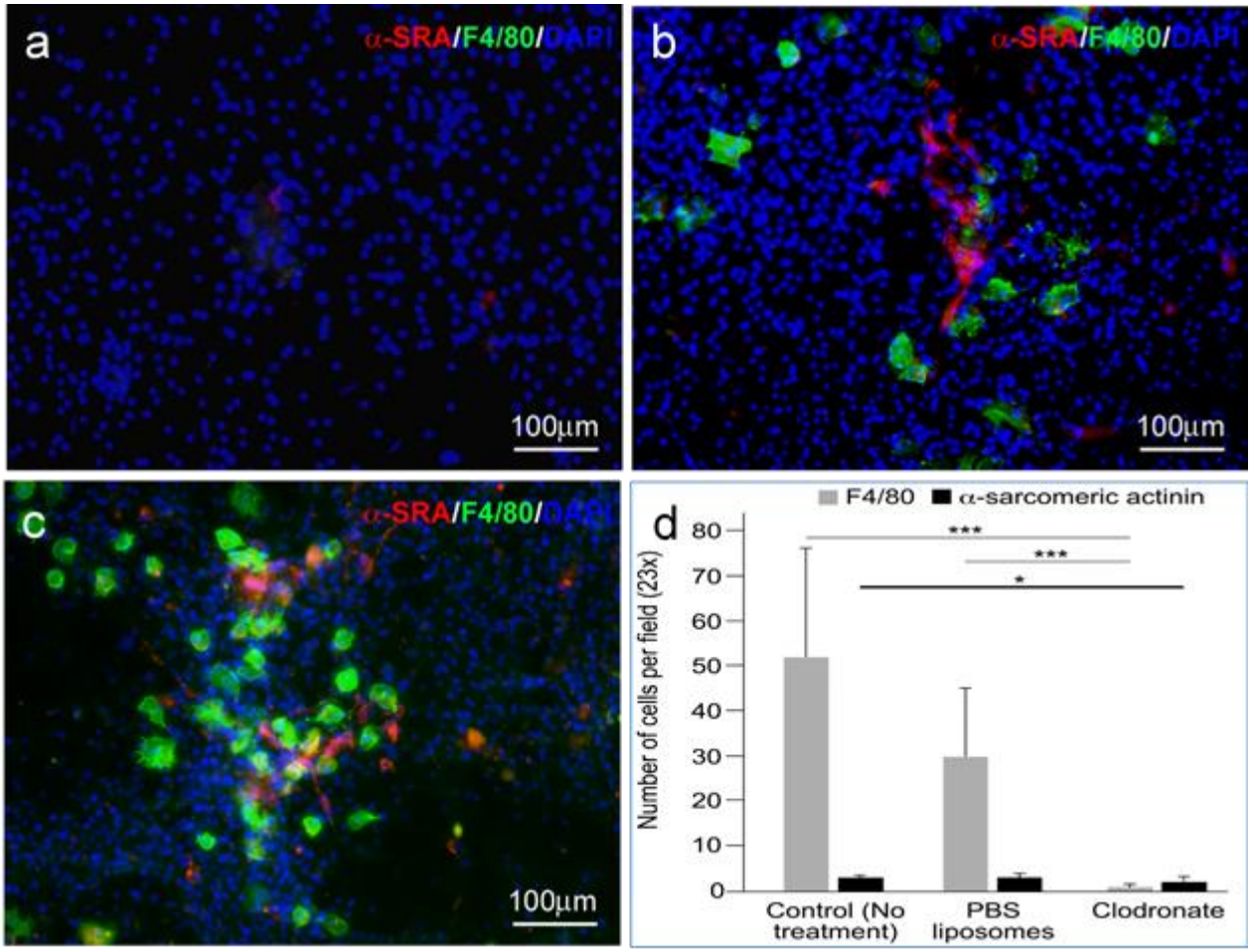

Figure 22. Immuncytochemical analyses on AoCs after macrophage depletion. Effective macrophage (F4/80, green) depletion and presence of only few α-SRA$^+$ cells (red) after treatment of AoCs with clodronate liposomes **(a)**. In contrast, accumulation of F4/80$^+$ (red) macrophages in colonies containing α-SRA$^+$ (green) cells in PBS liposome-treated **(b)**, and untreated AoCs **(c)**. Quantification of macrophages (F4/80$^+$) and α-SRA$^+$ cells per microscopic field at 23x magnification **(d)**. Data are presented as means ± SD and are from three independent experiments.*p< 0.05 and ** p<0.01 and *** p<0.001.

3.9 VEGF treatment of AoCs

Since it is well known that vascular endothelial growth factor (VEGF) is released by macrophages at a high level and VEGF in turn was reported to be involved in cardiac development (Lin et al., 2006), a potential role of VEGF in generation of Ao-bCMs was hypothesized. By promoting the proliferation and survival of ECs which in turn were shown to support cardiac differentiation, VEGF may act as an endogenous

paracrine factor in the culture of AoCs and support cardiac differentiation (Narmoneva et al., 2004). To test this hypothesis, the AoC cultures were treated either with the VEGFR-2 inhibitor E7080 or VEGF itself as a positive control. Similar to macrophage depletion, E7080 treatment blocked Ao-bCM generation. In contrast, VEGF treatment increased the size of Ao-bCM containing colonies and improved their synchronized beating (Movie 10) compared to untreated cultures (Movie.11). Moreover, the cell confluence in culture was increased after VEGF treatment on days 11 compared to those treated with anti-VEGFR-2 (E7080) and untreated AoCs (Figure 23). To evaluate whether this effect was related to the presence of Ecs, immunofluorescence staining was performed for CD31. The number of $CD31^+$ Ecs was significantly higher in VEGF-treated aortic wall-derived cells in comparison to those treated with E7080 or being left untreated (Figure 24a-d). Remarkably, in VEGF-treated cultures $CD31^+$ Ecs were strongly accumulated within Ao-bCM containing colonies as this also was confirmed by α-SRA staining. In addition, AoC cultures without VEGF treatment displayed a reduced size of beating colonies. These results indicate an essential involvement of VEGF not only in the generation of Ao-bCMs, but also in the improvement of their synchronized beating properties. This effect could be due to the enhanced number of Ecs in the culture, which in turn may improve Ao-bCM generation by paracrine effects. A direct effect of VEGF on CMs is also conceivable since they express VEGFR-2 (Chen et al., 2006).

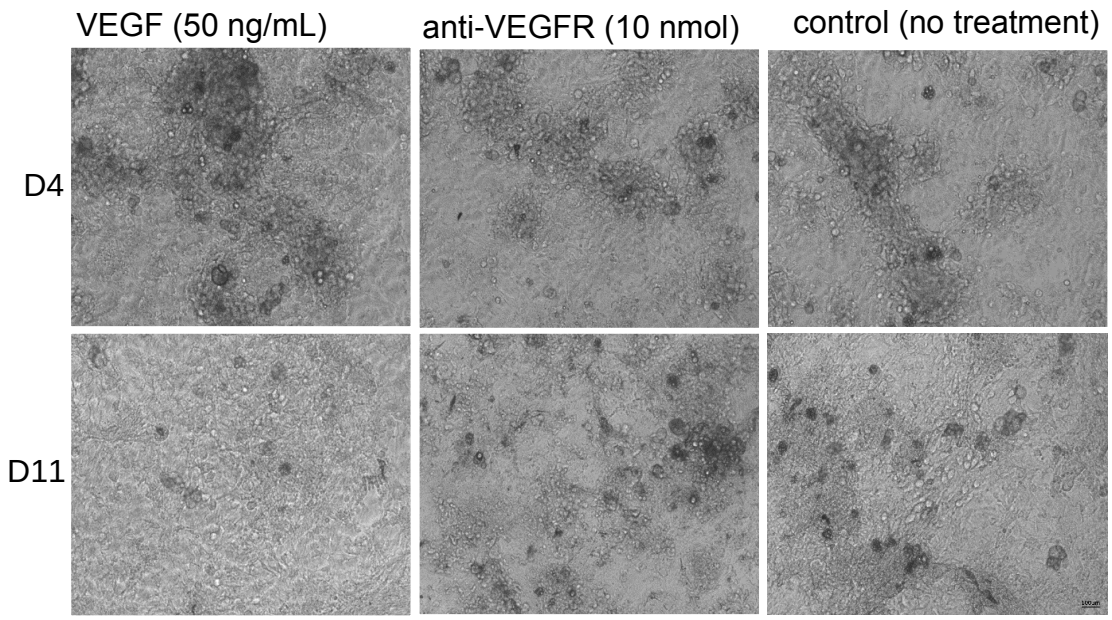

Figure 23. VEGF and anti-VEGFR treatment of AoCs. AoCs were cultured in normal cell culture conditions for days 4. After that AoCs were treated with VEGF (50 ng/mL) and anti-VEGFR-2 (E7080) (10 nmol) for days 11. Comparable cell confluence was observed before VEGF and anti-VEGFR-2 treatments on days 4. However, cell confluence was increased after VEGF treatment on days 11 compared to those treated with anti-VEGFR-2 (E7080) and untreated AoCs.

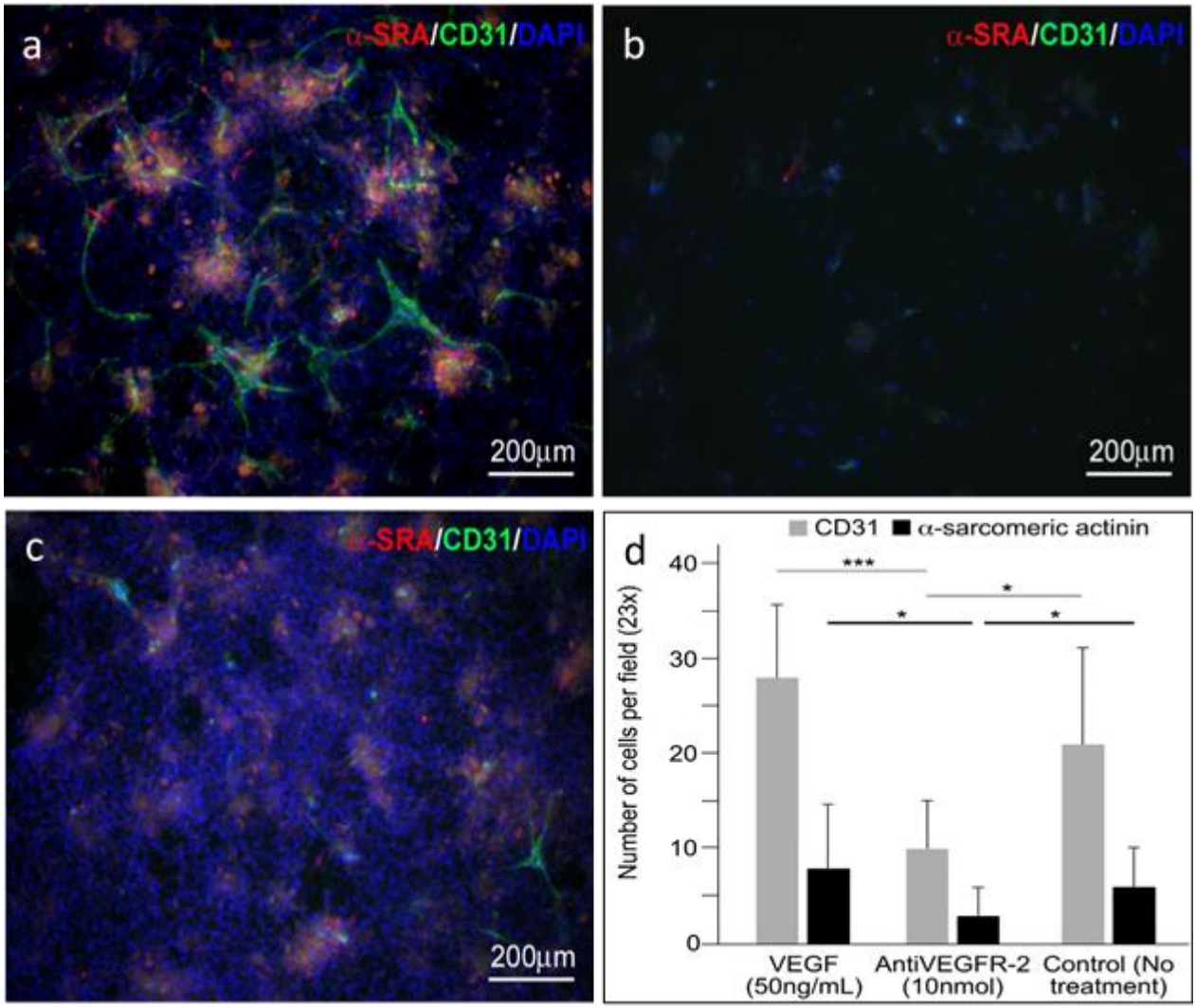

Figure 24. Immunocytochemical analyses after treatment of AoCs with VEGF and anti-VEGFR. The number of CD31$^+$ cells (green) in VEGF-treated cultures containing α-SRA$^+$ cells (red) (**a**, Movie 10), in comparison to those treated with anti-VEGFR-2 (E7080) (b) and untreated AoCs (Movie 11) (c). Quantification of ECs (CD31$^+$) and α-SRA$^+$ cells per microscopic field at 23x magnification (d). Data are presented as means ± SD and are from three independent experiments.*p< 0.05 and ** p<0.01 and *** p<0.001.

3.10 *In vivo* differentiation potential of AoCs in chick embryonic heart

To confirm their cardiac differentiation potential, *in vivo* AoCs were labelled with the green fluorescent dye CFDA (Goldstein et al., 2002) and subsequently injected into the hearts of chick embryos at developmental stage 16–17. This model has been

shown to be suitable to test the myocardial potential of target cells (Eisenberg et al., 2006), (Sedmera et al., 2000). Three days after implantation, green labelled AoCs were found adjacent to the chick heart tissue and could be distinguished from the host chick cells by their green fluorescence as well as a more pronounced DAPI staining (Figure 25a, b) (Fontaine-Pérus et al., 1997). In control sections from chick embryo heart injected with PBS only, no specific fluorescence signals were detected (Figure 26). Confirming the *in vitro* data, few Sca-1$^-$ cells became α-SRA$^+$ *in vivo* (Figure 25c-e). Some of the CFDA-labelled AoCs were double positive for Flk1 (VEGFR-2) and α-SRA (Figure 25f-j) as well as single AoCs were found to be positive for connexin 43 and α-SRA (Figure 25k-o) suggesting their differentiation into cardiomyocytes under *in vivo* conditions. These data imply that part of the Flk1$^+$ AoCs may have the capacity to differentiate into α-SRA$^+$ cells *in vivo* and might hence serve as a vessel wall-resident cardiomyocyte progenitor population.

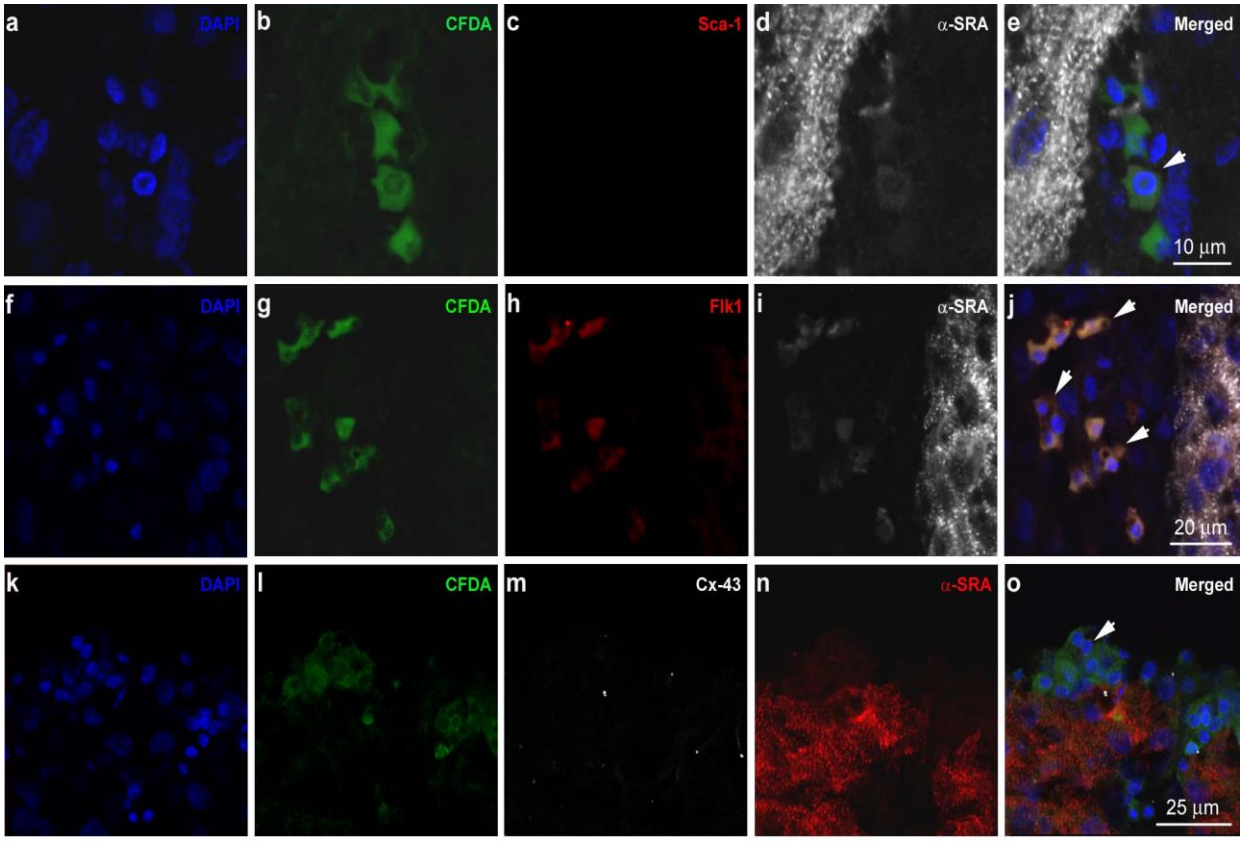

Figure 25*. In vivo* differentiation potential of AoCs in chick embryonic heart. AoCs were injected intracardially into stage 16–17 chick embryos. a-d) $DAPI^+$ (blue)/$CFDA^+$ (green)/$Sca\text{-}1^-$ (red) and $\alpha\text{-}SRA^+$ (white) cells. e) Merged image of panels a-d. f-i) Implanted $DAPI^+$ (blue)/$CFDA^+$ (green)/$Flk1^+$ (red)/$\alpha\text{-}SRA^+$ (white) cells. j) Merged image of panels f-i. k-n) Implanted $DAPI^+$ (blue)/$CFDA^+$ (green)/$Cx\text{-}43^+$ (white)/$\alpha\text{-}SRA^+$ cells (red). o) Merged image of panels k-n.

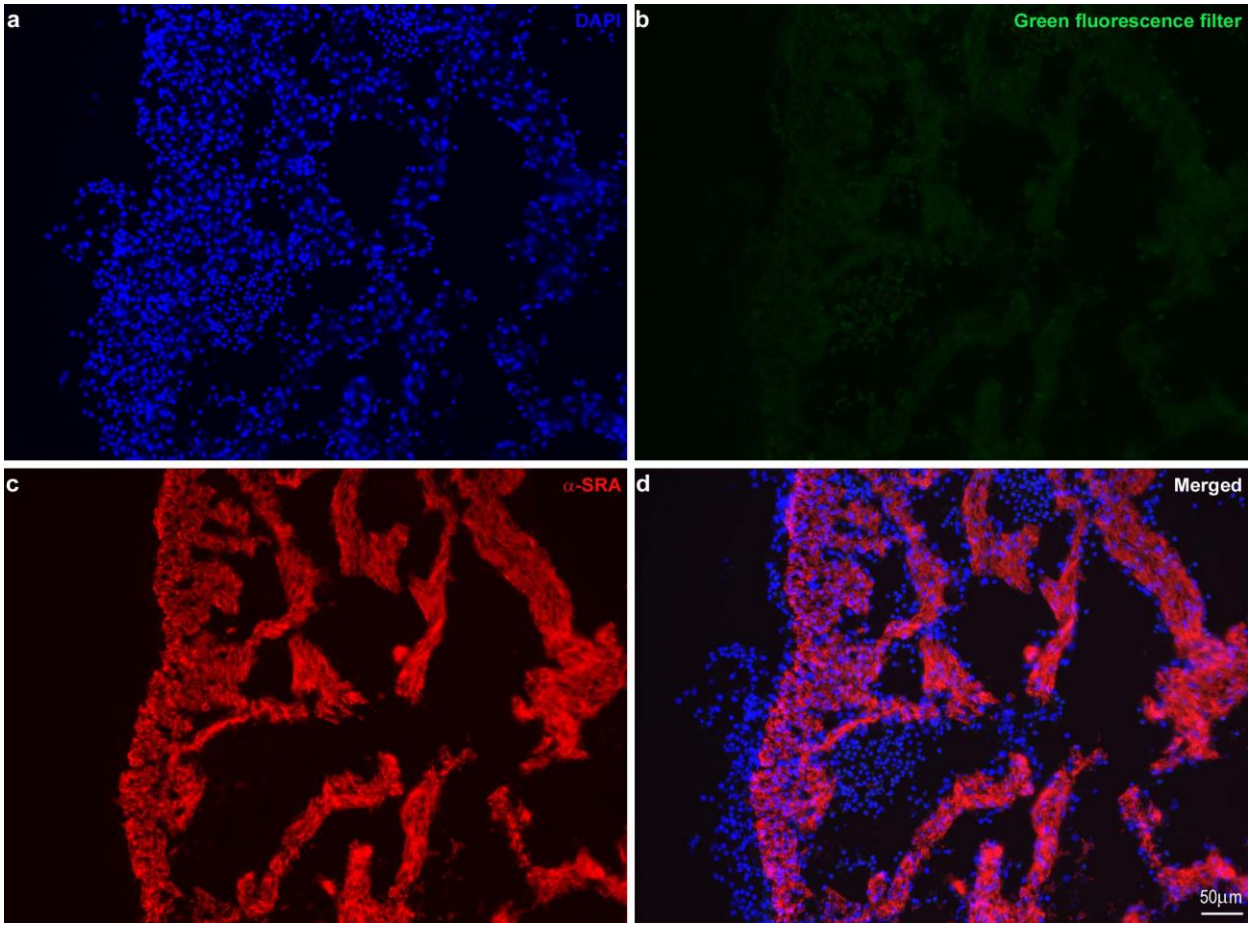

Figure 26. Intracardial injection of PBS into stage 16–17 chick embryos. Cryosections from this heart tissue was used as control to exclude unspecific background fluorescence. Compared to heart tissue after injection of CFDA-labelled cells (Fig. 4b, g, and l), no specific green fluorescence signals were detectable after injection of PBS. a-d) α-SRA staining (red) in the chick embryo myocardium. DAPI staining of the nuclei is shown in blue.

3.11 Genetic cell fate mapping of Ao-bCMs using Flk1 myr::mCherry transgenic mice

Hence, the results above suggest Flk1 as a marker of vascular adventitia-derived cardiac progenitors *in vitro* cell lineage tracing experiments were performed using cells isolated from the aortae of Flk1 myr::mCherry transgenic mice (Larina et al., 2009). Subsequently, the cells were cultured in mECGM. At day 3 in culture, most of the Flk1 myr::mCherry^{+} AoCs were found to also be positive for Isl1^{+} displaying a

nuclear staining as expected (Figure 27a). After 10 days in culture, Flk1 myr::mCherry^{+} AoCs gave rise to progeny in which some were CD34^{+} (Figure 27b), and at this time many Flk1 myr::mCherry^{+} AoCs showed a positive staining for α-SRA in a non-striated pattern suggesting the immature cardiac progeny phenotype of these cells. Interestingly, mature cardiomyocytes expressing α-SRA in a more striated pattern exhibited a weak or lacking Flk1 myr::mCherry signal suggesting down-regulation of Flk1 expression in maturing cardiomyocytes (Figure 27c). Flk1 myr::mCherry^{+} AoCs mostly co-expressed CD31 which is a marker of mature endothelial cells (Figure 27d). Whereas a subset of Flk1 myr::mCherry^{+} cells was also shown to be positive for F4/80 (green in Figure 27e). Moreover, performing immunostaining for Fkl1 on Flk1 myr::mCherry^{+} aortic wall-derived cells revealed that almost all Flk1 myr::mCherry labelled^{+} AoCs were also marked by Flk1 antibody (Figure 27f) conforming the Fkl1 expression in these cells. These results from cell tracing studies suggest that vessel wall-derived Flk1^{+} cells contribute to generation of both Ao-bCMs and their supportive cells such as endothelial cells and macrophages.

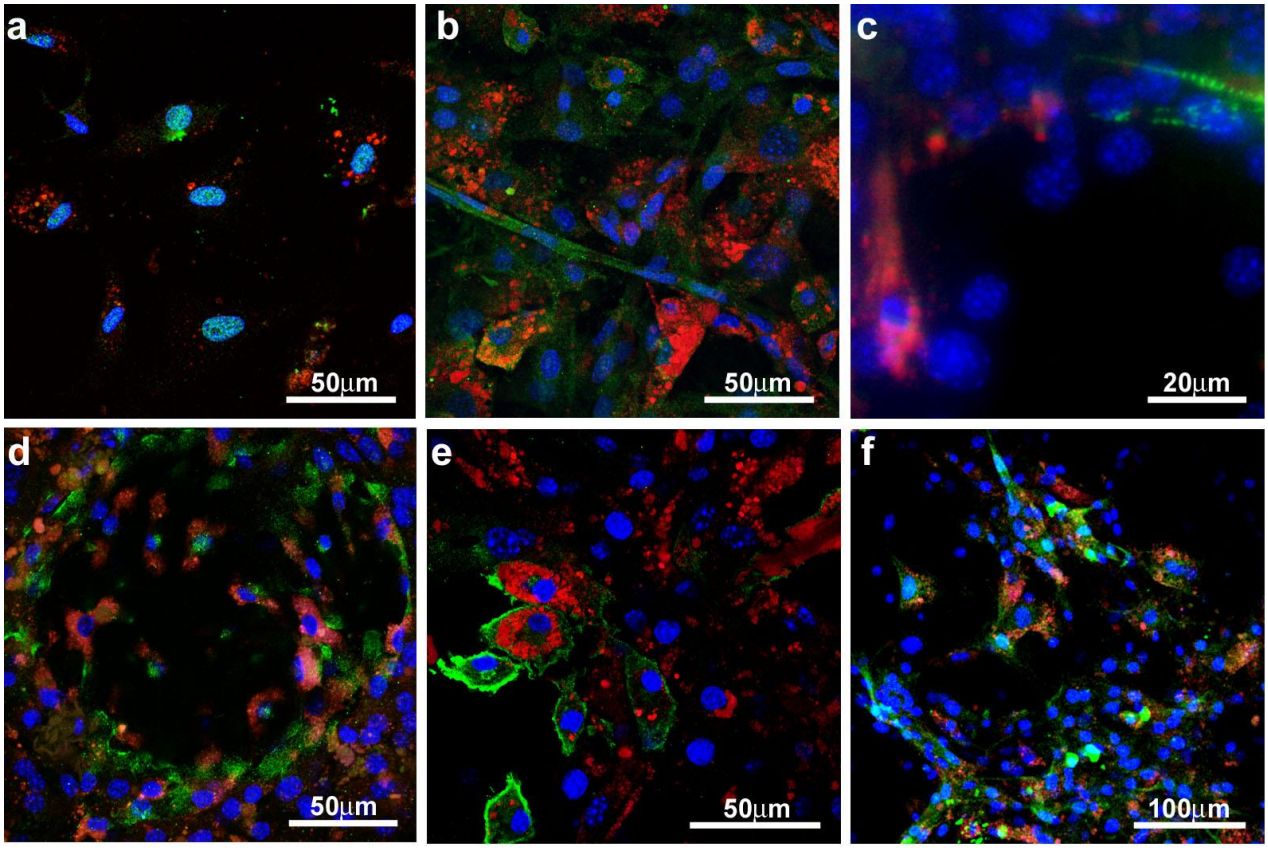

Figure 27. Genetic fate mapping of Ao-bCMs using Flk1 myr::mCherry labelled mice. Immunocytochemical staining in Flk1 myr::mCherry^{+} aortic cell cultures. Flk1 myr::mCherry^{+} AoCs (red) are mostly co-expressed with Isl1^{+} (green) (a), subset of CD34^{+} cells (green) (b) and immature α-SRA^{+} cells (green) (c), as well as most of the CD31^{+} cells (green) (d), whereas subset of Flk1 myr::mCherry^{+} cells (red) exhibits co-expression of F4/80^{+} (green) (e). Furthermore, confirmed the overlapping expression pattern between Flk1 myr::mCherry labelled^{+} cells and Flk1^{+} immune staining AoCs (f).

4 Discussion

Vascular wall has been shown to harbour various stem and progenitor cells termed VW-SCs that can give rise to endothelial, smooth muscle cells as well as macrophages (Ergün et al., 2011), (Zengin et al., 2006), (Psaltis et al., 2012), (Psaltis et al., 2014). Furthermore, recently it was shown that vascular adventitia-derived PDGFRß$^{+}$ (platelet-derived growth factor receptor ß) cells have the capacity to differentiate into follicular dendritic cells and to support follicle organization (Krautler et al., 2012). Briefly, these data suggest a broad spectrum of differentiation capacity of vascular adventitia-derived stem and progenitor cells and/or a broad spectrum of adventitial subpopulation differentiation into various mature cell types.

In this context, the aim of the current doctoral thesis was to analyse whether and which type(s) of VW-SCs might have the capacity to differentiate into other cell lineages such as cardiomyocytes. The present results show that it is possible to isolate cardiac progenitors from vessel wall that can give rise to Ao-bCMs. First, the cell culture conditions to obtain Ao-bCMs were optimized in this study. Second, the AoCs were sorted using stem cell surface marker Sca-1 and it could be shown that Ao-bCMs are derived from Sca-1^{-} AoCs fraction. Third, Ao-bCMs were characterized *in vitro* and functionally, e. g. by analysing their responsiveness to β-adrenergic drugs and capacity for calcium oscillation. Fourth, mechanisms that regulate differentiation of Ao-bCMs were studied in detail, e. g. the role of VEGF-VEGFR-2-system and local generated inflammatory microenvironment. Fifth, the cardiac differentiation potential of Ao-bCMc was tested *in vivo* using the transplantation of fluorescently labelled AoCs into the developing chick embryo heart**.**

4.1 Adventitial stem cell niche in mouse aorta and optimization of cell culture conditions for AoCs to generate beating cells

As mentioned in the introduction, adult vascular wall adventitia harbours various stem or progenitor populations, e. g. for vascular cells such as endothelial and smooth muscle cells/pericytes as well as non-vascular cells such as macrophages or hematopoietic stem cells (Ergün et al., 2011), (Zengin et al., 2006), (Psaltis et al., 2012) (Psaltis et al., 2014). In the current study, the first aim was to identify the exact localization of the vascular wall stem cell niche and analyse the potential presence of stem and progenitor cells with cardiogenic potential. Hence, immunostainings for CD34, CD31, CD44, Flk1, Sca-1, c-Kit, and Isl-1 were performed on adult mouse aortic sections. In this study, $CD31^+$ staining was localized only in mature endothelial cells of the intima, whereas stem or progenitor cells such as $CD34^+$, $CD44^+$, $Flk1^+$, and $Sca\text{-}1^+$ were localized in the vascular adventitia (Figure 15)., Previous studies had already shown such a localization of $CD34^+$, $CD44^+$, $Sca\text{-}1^+$, and $Flk1^+$ cells within the adult vascular adventitia (Klein et al., 2013), (Passman et al., 2008), (Zengin et al., 2006), (Psaltis et al., 2014). Interestingly, in the current study, expression of cardiac progenitor markers such as $c\text{-}Kit^+$ and $Isl\text{-}1^+$ were also detected in cells of the adult vascular adventitia, which has not been demonstrated before (Beltrami et al., 2003), (Cai et al., 2003). In earlier studies, CD31 expression was detected on $Sca\text{-}1^+$ cells from adult mouse myocardium (Oh et al., 2003). Moreover, after transplantation, human adult $CD34^+$ peripheral blood cells had ability to turn into cardiomyocytes in the injured tissue areas of the mouse heart (Yeh et al., 2003). In another study, it has been reported the detection of $c\text{-}Kit^+$ and $CD44^+$ co-expressing cells in the human heart auricle primary cultures (Fang et al., 2009). In addition,

earlier studies have demonstrated the expression of cardiac progenitor markers such as $Flk1^+$, $Isl\text{-}1^+$ in the embryonic stem cells (Moretti et al., 2006). Furthermore, the presence of $c\text{-}Kit^+$ cells in the mammalian heart (Wu et al., 2006), and $Sca\text{-}1^+$ in the adult murine heart was reported (Matsuura et al., 2004). However, in the current study subset of $CD34^+Flk1^+isl\text{-}1^+Sca1^-$ VW-SCs might serve as cardiac progenitors. Largely, adult vascular wall adventitia acts as niche for various vascular stem or progenitors, which have cardiogenic potential. One possible explanation for the presence of abundant progenitor cells in the adventitia is that these cells are preserved during development from the embryonic to the adult period (Ergün et al., 2011), (Zhang and Xu, 2014). In embryonic blood vessels, cells with cardiac potential have been already reported and termed mesoangioblast progenitors (De Angelis et al., 1999), (Minasi et al., 2002). These cells can give rise to vascular cells as well as other mesodermal lineages including skeletal and cardiac muscle (Motoike et al., 2003). Mesoangioblasts express CD34, Flk1, Sca-1, Thy-1, and c-Kit (Sampaolesi et al., 2003). Cardiogenic VW-SCs identified in this study similary express CD34 and Flk1 but differ in their expression regarding Sca-1. Furthermore, they have a broader differentiation potential, as they can give rise to macrophages. Therefore, additional studies will be required to clarify similarities and differences between mesoangioblasts and VW-SCs and to explore their relationship as well as developmental origin. Taken together, adult vessel wall adventitia harbours various stem or progenitor populations, which have cardiac genic potential.

In order to induce cardiomyocyte-like cells, AoCs were cultured in DMEM, RPMI, and mECGM. However, beating cells were only found in mECGM after 10 days of culture. The cultivation in mECGM alone resulted in few spontaneously beating cell clusters

starting at around 8-10 days of culture. Besides, AoCs cultures were treated with different combinations of growth factors such as BMP4, Chir99021, Vitamin C, and IWR1 that have been used for cardiac differentiation of human iPS cells (Kadari et al., 2015). However, beating cells were not observed in cultures along with combinations of these small molecules and growth factors. The reason could be that AoCs may not respond to Vitamin C, early BMP4, WNT activation (Chir) and late inhibition (IWR1) driving cardiac induction and specification during embryonic development. Hence, mECGM was chosen as cell culture medium in this study protocol for Ao-bCMs generation. The second relevant point is that optimal cell numbers are also essential in order to generate spontaneously beating cells from aortic cultures. The optimal induction was achieved by culturing $2x10^6$ AoCs in a single well of 48 well plate. This might be due to optimal cell-cell interactions and microenvironment that enables the formation of colonies containing cardiac progenitors which then differentiate into mature beating cells. It has been reported that endothelial cells promote cardiac myocyte survival and spatial reorganization (Narmoneva et al., 2004). On the other hand, age is another important factor for the generation of cardiomyocytes from adult stem cells (Jumabay et al., 2010). In recent study, the number of stem cells was found to be changed based on age factor. It has been reported that the c-Kit^+ population was declined with an increase in age (Sanada et al., 2014). In this study, initially young mice (2 months of age) were used yielding in Ao-bCMs, but Ao-bCMs could be also generated in cultures derived from mice aged between 3 to 10 months. Similarly, earlier studies also showed generation of cardiomyocyte-like cells in cultures of adipocytes from mice aged more than 12

months (Jumabay et al., 2010). Hence, generation of Ao-bCMs was not dependent on mice age.

Ao-bCMs could be generated on plastic surfaces as well as fibronectin coated glass cover slips, but not on gelatin and matrigel coated glass cover slips. In line with these results, a recent study reports that fibronectin is one of the major ECM proteins that promotes the adhesion of cardiomyocytes (Wu et al., 2010).

4.2 Sca-1$^-$ population of AoCs gives rise to spontaneously beating cells

The purification of a cardiac stem cell population is challenging, especially due to lack of specific surface markers (Takamiya et al., 2011). After initial discovery of the c-Kit$^+$ cardiac stem cell (Beltrami et al., 2003), other cardiac progenitor cell (CPC) populations have been identified. In rodents, different CPCs have been isolated on the basis of the expression of stem cell antigen 1 (Sca-1) (Matsuura et al., 2004). Afterwards, *in vivo* cell-tracing studies using the Flk1-lacZ reporter mouse indicate that some cardiomyocytes arises from a population that expresses the VEGFR-2 (Flk1) (Ema et al., 2006). Another study demonstrated transdifferentiation of human peripheral blood CD34$^+$ cells into cardiomyocytes, endothelial cells, and smooth muscle cells in the injured mouse heart (Yeh et al., 2003). Based on all these published data, we applied microbeads for various surface markers such as c-Kit, Sca-1, CD34, and AoCs were pre-sorted using MACS technology. However, Ao-bCMs could be generated only by culturing the Sca-1$^-$ fraction of AoCs. This result was unexpected because several previous publications suggested that Sca-1$^+$ cells from different sources function as cardiac progenitors (Oh et al., 2003), (Tateishi et

al., 2007). Only recently, however, this assumption was challenged since in-depth analyses that also encompassed genetic approaches did not result in a considerable contribution of Sca-1^{+} cells to cardiomyocyte (CM) generation (Valente et al., 2014). Considering the fact that even Sca-1^{-} AoCs are heterogeneous, and thus, cell-cell interactions in this cell mixture may be required for the generation of Ao-bCMs, further immunophenotypic studies on Sca-1^{-} AoCs were performed. These analyses revealed that the Sca-1^{-} fraction is composed of c-Kit^{+}, Isl-1^{+}, and Flk1^{+} cells, which may be involved in generation of Ao-bCMs. Furthermore, higher expression levels were observed for cardiac progenitor markers such as Flk1 (16.3%), Isl-1(8.5%) compared to the Flk1^{+} fraction. It was shown that cells expressing Isl-1, Flk1 (Moretti et al., 2006), and c-Kit (Wu et al., 2006) serve as cardiovascular progenitors (CVPCs) and have the potential to differentiate into cardiomyocytes. The immunocytochemical studies of AoCs revealed the presence of Flk-1^{+}, c-Kit^{+}, and Isl-1^{+} cells within colonies formed by AoCs after 7-11 days of culture, suggesting that some of these cell types might serve as progenitors of Ao-bCMCs, while others might support their generation in culture.

4.3 Ao-bCMs exhibit morphological and functional features of cardiomyocytes

4.3.1 Immunopheotypic characteristics

In order to know which cardiac progenitors are giving rise to Ao-bCMs, double immunostainings on AoCs and Ao-bCMs were performed. Among the markers used for cardiac progenitor cells Isl-1 is of particular relevance. It has been clearly shown that besides the nervous system and gastrointestinal development Isl-1 plays a critical role in early cardiac development (Cai et al., 2003). The impact of this transcription factor for cardiac development is underlined by the fact that *Isl-1*$^{-/-}$ mice

die at approximately E10.5, because of heart formation defects (Li et al., 2014). Isl-1, a LIM homeodomain transcription factor, was originally described as an Insulin gene enhancer binding protein (Karlsson et al., 1990). Regarding the cardiac development it has been shown that murine Isl-1$^+$ cells maintain their cardiovascular differentiation potential under culture conditions *in vitro* (Laugwitz et al., 2005) suggesting them as real cardiac progenitor cells. In a recent study, using nLacZ$^+$ as a reporter gene for Isl-1 expression Isl-1$^+$ cells were also found in the proximal part of large vessels (near to the heart) including proximal aorta beside some areas of the mouse heart tissue, while the major part of myocardium was found to be free of the Isl-1 expression (Weinberger et al., 2012). Confirming these findings, the present data clearly show few Isl-1$^+$ cells within the adventitia of adult mouse aorta (Figure 15), and a part of AoCs was found to be double positive for both, Flk1 and Isl-1 (Figure 17b), suggesting the existence of cardiac progenitors in the adult vessel wall and among the AoCs. In addition, the expression of further stem and progenitor markers such as c-Kit, CD34, and Flk1 as well as endothelial cell marker CD31 and smooth muscle cell marker αSMA was studied. Both CD34 and Flk1 were found to be expressed in immature Ao-bCMs where it was found to be co-localised with α-SRA$^+$ (Figure 18i, j). These data are in line with previously published results that showed the potential of CD34$^+$ cells to turn into cardiomyocytes in the injured mouse heart (Yeh et al., 2003) and multipotent Flk1$^+$ cardiovascular progenitor cells that give rise to cardiomyocytes (Kattman et al., 2006). In contrast to some results published in the literature, (Oh et al., 2003), (Beltrami et al., 2003), (Potta et al., 2010) neither early nor mature Ao-bCMs were found to express CD31, c-Kit or αSMA. Taken together, the data

presented in this work suggest that a subset of $CD34^{+}Flk1^{+}Isl\text{-}1^{+}Sca1^{-}$ VW-SCs might serve as cardiac progenitors present in adventitia of the adult aorta.

4.3.2 Structural characteristics of Ao-bCMs

In order to study detailed structural features, single and double immunostainings as well as electron microscopic analyses were performed on Ao-bCMs. During transmission electron microscopy, the sample preparation process can have an impact on the quality of the resulting sample. For cell isolation, most cells were grown adherent and had to be enzymatically or mechanically detached (Schrand et al., 2010). In this study, both, enzymatically and mechanically detached Ao-bCMs were used. However, intact cell membranes and organelle structures were better preserved using mechanical detachment via cell scrapers than by enzymatical detachment by adding trypsin EDTA. TEM-analyses showed well-organized sarcomeric structures in Ao-bCMs with distinct A and I bands as well as Z-lines. In higher magnification also structural components of the sarcoplasmic reticulum, which represents the intracytoplasmic site of calcium storage were identified. In immunocytochemistry, a subset of $\alpha\text{-SRA}^{+}$ Ao-bCMs stained positive for connexin43, a major component of gap junctions in the ventricle, which are essentially required for electrica and metabolic coupling between adjacent cardiomyocyte (Severs et al., 2008). These results strongly indicate that intercellular communication through gap junctions and mature sarcomeric organization is present in Ao-bCMs. These Ao-bCMs were generated from AoCs cultures. As mentioned in results, AoCs were cultured in mECGM for 8-10 days and then surprisingly, spontaneously beating cells were appeared in the culture, which were termed as Ao-bCMs (aortic wall-derived

beating cardiomyocytes These Ao-bCMs displayed more mature sarcomeric structures than cardiomyocytes derived from iPS-cells (Kadari et al., 2015).

4.3.3 Expression of cardiac genes is regulated during the differentiation of AoCs into Ao-bCMs

Cardiac transcription factors as well as early-stage cardiac genes such as Mef2c, Isl-1 and HCN4 (Scavone et al., 2013) are highly expressed on day 0 (AoCs), suggesting that AoCs could be maintained in a primitive cardiac progenitor stage prior to differentiation, whereas cardiac structural genes such as Troponin I and ACTC1 (Amid et al., 2012) were expressed on day 10 (Ao-bCMs), suggesting that mature cardiac genes are upregulated during the differentiation of AoCs into Ao-bCMs. These data again underline the presence of cardiogenic progenitors among AoCs with the capacity to differentiate into cardiomyocytes *in vitro* without any external genetic manipulation.

4.3.4 Functional characteristics of Ao-bCMs

One of the specific properties of cardiomyocytes is to display electrical activity and generate action potentials (Grant, 2009). The patch clamp method was used to measure ionic currents. Ao-bCMs showed sodium *(*Na) and potassium (K*)* currents indicating the presence of the corresponding ion channels. Sodium and potassium channels are essential in the correct initiation and propagation of the cardiac action potential (Sharma et al., 2013). A difficult task during this thesis was the detection of Ca^{2+} transients using Fluo-4 Sparks of $Ca2^{+}$ florescence were not observed in Ao-bCMs cultured in mECGM medium. This could be due to presence of lower calcium concentration levels in mECGM. However, a previous publication reported a calcium-induced calcium release (CICR) whereby calcium is able to activate or increase

calcium release from intracellular Ca^{2+} stores (sarcoplasmic reticulum) in cardiomyocytes (Fabiato, 1983). Adding another medium (DMEM, 10% FBS) as an additional source of calcium to the mECGM medium resulted in intensive calcium sparks from Ao-bCMs. Furthermore, the response of Ao-bCMs cells to β-adrenergic stimulants such as isoproterenol (agonist) and propranolol (antagonist) were studied which are known to influence heart beating rate (Mougeot et al., 1981). The beating rate of Ao-bCMs was significantly increased by stimulating them with Isoproterenol (6µM) and reversely this effect could be blocked by adding propranolol (30µM). It has been shown that mouse ES cell derived cardiomyocytes respond to stimulation with the adrenergic agonist isoprenaline with an increase in beating rate. This positive chronotropic effect was abolished by adrenergic antagonists suggesting that ES derived cardiomyocytes expressed β-adrenergic receptors (Wobus et al., 1991). In line with these data, the present results suggest that Ao-bCMs are equipped with β-adrenergic receptors. In summary, also at the functional level the Ao-bCMs display features characteristic for mature cardiomyocytes.

4.4 Mechanisms that regulate the *in vitro* generation of Ao-bCMs

4.4.1The role of local generated macrophages in AoCs cultures

Recent experimental evidence suggests that macrophages may be critically involved in cardiac repair and regeneration (Frangogiannis, 2015). Furthermore, it was also shown that depletion of macrophages denied regeneration of the neonatal heart, suggesting that neonatal cardiac macrophages exhibit a unique regenerative phenotype and secrete soluble mediators that contribute to formation of new myocardium (Aurora et al., 2014). As demonstrated in the results section of this work,

AoCs-derived colonies containing beating cells displayed increased accumulation of F4/80$^+$ macrophages suggesting that these might have an effect on the generation of Ao-bCMs. Therefore, the role of vessel wall adventitia-derived macrophages in the cardiac differentiation of AoCs was tested *in vitro*. To this end, a well-established technique to deplete macrophages *in vitro* was used which is based on their ability to ingest and digest clodronate liposomes (Buiting and Van Rooijen, 1994). Phagocytotic activity shuttles clodronate into the cytoplasm inducing apoptosis (Van Rooijen, 1989). In this study, F4/80$^+$ cells were efficiently depleted by treatment with clodronate liposomes and this interestingly stopped the generation of spontaneously beating cells. These results demonstrate that Ao-bCM generation seems to be dependent on an inflammatory microenvironment, e. g. the presence of macrophages.

4.4.2 Vascular endothelial growth factor (VEGF) and anti-VEGFR-2 treatment in AoCs cultures

Furthermore, the role of VEGF and VEGFR-2 in generation of Ao-bCMs was studied using the VEGFR-2 inhibitor compound E7080. A recent study demonstrated that VEGF is required for effective cardiomyocyte differentiation from human iPS cells (Ye et al., 2013). Moreover, it has been described that VEGF is an essential growth factor for vascular endothelial cells (Ferrara, 1999). Others highlighted the significance of interactions between cardiomyocytes and endothelium for normal cardiac function (Narmoneva et al., 2004). Due to this background, the role of the VEGF system was analyzed by adding VEGF to the AoCs cultures. This treatment resulted in a significantly increased number of synchronized beating colonies as well as of endothelial cells. In contrast, the inhibition of VEGFR-2 signaling by simultaneous

treatment with E7080 prevented Ao-bCM generation and decreased the number of endothelial cells. Indeed, E7080 has been shown to be a multi-targeted kinase inhibitor whose targets also include vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2) and shown to inhibit tumor angiogenesis by targeting endothelial cells (Glen et al., 2011). The results presented here, indicate that the presence of endothelial cells in aortic cell culture seems to be necessary for generation of beating cells. Moreover, in this study, double immunostainings further showed co-expression of Flk1 (VEGFR-2) on Ao-bCMs that also underlines an essential role of VEGF in generation of Ao-bCMs. Besides, earlier investigators have reported that VEGF was included in cardiac differentiation protocol to promote the expansion and maturation of the KDR^+ population (Yang et al., 2008). Taken together, the present data show that VEGF and its receptor system are needed for an effective generation of Ao-bCMs *in vitro*.

4.5 AoCs also displayed a cardiac differentiation potential *in vivo*

Next, the cardiac differentiation capacity of AoCs was studied under *in vivo* conditions using their implantation into the developing chick embryo heart. The key advantages of using chick embryo model for the present study are easy handling, standard incubation timings, and stage embryos accurately for experimental standardization, high accessibility for a variety of surgical implantation procedures and the lack of a developed immune system. Furthermore, stem cells injected into embryonic tissues (chick embryo) are exposed to a range of developmental signals that are able to direct their differentiation. Thus this model is more likely to enable the differentiation ability of implanted stem cells than when injected into adult organs (Rashidi and Sottile, 2009). Using such a strategy, mouse ES derived $Flk1^+$ cells

were transplanted into the chick model to demonstrate that Flk1$^+$ progenitors can exhibit a vascular phenotype (Yamashita et al., 2000). Similarly, another study has also reported that donor bone marrow cells exhibited a myocardial phenotype after they were incorporated into the developing chick embryo heart (Eisenberg et al., 2006). In the present study, AoCs were labeled with the green carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) fluorescent dye and then injected into heart of chick embryos. Earlier studies have reported that CFDA, a membrane permeant dye that covalently links to free amines of cytoplasmic proteins, effectively marks cells such as hepatocytes, lymphocytes, CD34$^+$ cells, neural stem cells, fetal CNS cells, and human intervertebral disc cells (Gruber et al., 2000), (Weijer et al., 2002), (Groszer et al., 2001). The results presented in this study show that after 3 days post transplantation, CFDA labelled AoCs were found adjacent to the chick heart tissue, and some of these cells were double positive for Flk1 and α-SRA markers. Moreover, another study has reported that Flk1$^+$ cells show a better engraftment after injecting into the mouse with an acute MI (Mauritz et al., 2011). A more recent study reported about transplantation of ES-derived Flk1$^+$ cells into cardiomyopathic hearts that effectively differentiated into cardiomyocytes and showed the potential to communicate with host cardiomyocytes and contribute to the contractile function of the heart (Baba et al., 2007). Taken together, the present results suggest that a subset of the Flk1$^+$CD34$^+$Isl-1$^+$ AoCs may have the capacity to differentiate into α-SRA$^+$ cells *in vivo* and might hence serve as a cardiomyocyte progenitor population.

4.6 Genetic fate mapping of Ao-bCMs

In order to follow the differentiation path of AoCs into Ao-bCMs, *in vitro* aortic cells prepared from Flk1 myr::mCherry transgenic mice were used. As mentioned in the

results, co-expression of mCherry and Isl1^{+} was observed in day3 AoCs cultures. Expression of Flk1^{+} (Yang et al., 2008) and Isl1^{+} (Moretti et al., 2006) has been suggested to be characteristic for cardiovascular precursor cells. Besides, a subset of Flk1 myr::mCherry^{+} cells showed expression of CD34 in AoCs cultures on day 10. It has been reported that CD34 and Flk1 co-expression is characteristic for vascular wall progenitors (Zengin et al., 2006). Furthermore, Flk1 myr::mCherry^{+} cells showed expression of α-SRA indicating immature Ao-bCMs. As expected, Flk1 myr::mCherry expression was absent in mature Ao-bCMs showing the typical striated staining pattern for α-SRA. Supporting the results presented above the *in vitro* cell lineage fate mapping experiment suggests that vascular adventitia-resident Flk1^{+} cells serve as progenitors differentiating into Ao-bCMs under specific culture conditions. Previous *in vivo* fate mapping studies also suggested that Flk1^{+} cells are progenitors for cardiac muscles along with hematopoietic and vascular endothelial cells in the developing mouse fetus (Motoike et al., 2003). Furthermore, in the current study, the majority of Flk1 myr::mCherry^{+} cells differentiated into CD31+ endothelial cells. It has been reported that Isl1, Flk1 and CD31 expression is needed for the differentiation of cardiac precursors into endothelial cells (Moretti et al., 2006). Notably, a subset of Flk1 myr::mCherry^{+} cells showed F4/80 expression indicating their potential to differentiate into macrophages. Since F4/80^{+} macrophages are essentially needed for the generation of Ao-bCM, these data suggest that endothelial cells as well as Ao-bCMs and F4/80^{+} macrophages are of the same vascular origin, originating from Flk1^{+} vascular wall stem cells in the adventitia. Moreover, it has been reported that F4/80^{+} macrophages are closely associated with Flk1 myr::mCherry^{+} vasculature in order to engulf EC debris (Poché et al., 2009). These *in vitro* cell

lineage fate mapping results suggest that Flk1^{+} cells deliver not only Ao-bCMs, but also endothelial and hematopoietic cells which are crucially supportive for Ao-bCM generation.

5 Conclusion

Taken together, the identification and characterization of Ao-bCMs suggests VW-SCs as a novel endogenous source for cardiomyocytes. The presented *in vitro* and *in vivo* data indicate that a subset of $CD34^{+}Flk1^{+}Isl\text{-}1^{+}Sca1^{-}$ VW-SCs might serve as cardiac progenitors. *In vitro* cell lineage tracing experiments confirmed the origin of Ao-bCMs from $Flk1^{+}$ cells using Flk1 myr::mCherry transgenic mouse models. These findings might pave the way for endogenous therapeutic strategies for myocardial regeneration after myocardial infarction. The current data suggest that VW-SCs resident in the vascular adventitia can deliver both, Ao-bCMs and an inflammatory microenvironment, which in turn is essentially involved in the generation of Ao-bCMs. Notably, VEGF and VEGFR signaling are needed for the robust generation of Ao-bCMs. In conclusion, VW-SCs represent an easily accessible, abundant and highly attractive endogenous source for cardiac progenitors. Since these cells are also present in the wall of coronary vessels, they might serve as candidate cells for endogenous myocardial regeneration. Future studies will show how and to which extent they really can be manipulated therapeutically.

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7 Abbreviations

% = percent

°C = degree Celsius

µg = microgram

µL = microliter

µM = micromolar

BM-EPCs = bone marrow-derived endothelial progenitor cells

BMP = bone morphogenic protein

C-EPCs = circulating endothelial progenitor cells

ECs = endothelial cells

EDTA = ethylenediaminetetraacetic acid

EPCs = endothelial progenitor cells

FACS = fluorescence activated cell sorting

FBS = fetal Bovine Serum

Flk1 = fetal liver kinase 1

hITA = human internal thoracic artery

HPCs = hematopoietic progenitor cells

HSCs = hematopoietic stem cells

IEL = inner elastic membrane

iPS = induced pluripotent stem cells

MACS = magnetic activated cell sorting

mg = milligram

MHCs = major histocompatibility molecules

ml = milliliter

MI = myocardial infarction

MSCs = mesenchymal stem cells

ng = nanogram

OEL = outer elastic membrane

PBS = phosphate buffered saline

PFA = paraformaldehyde

αSMA = alpha smooth muscle actin

SMC = smooth muscle cells

VEGF = vascular endothelial growth factor

VEGFR = vascular endothelial growth factor receptor

VW-EPCs = vascular wall-resident endothelial progenitor cells

VW-MSCs = vascular wall-resident mesenchymal stem cells

VW-SCs = vascular wall-resident stem cell

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9 Affidavit

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Erklärungen nach §4 Abs. 3 Satz 3, 5, 8 der Promotionsordnung der Fakultät für Biologie der Julius-Maximilians-Universität Würzburg.

I hereby declare that my thesis entitled "**Generation of cardiomyocytes from vessel wall-resident stem cells**" is the result of my own work.

I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

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Unterschrift

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